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REGULATION OF CARTILAGE ACID HYDROLASES AND THEIR RELATIONSHIP TO SKELETAL GROWTH

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REGULATION OF CARTILAGE ACID HYDROLASES AND THEIR RELATIONSHIP TO SKELETAL GROWTH

by

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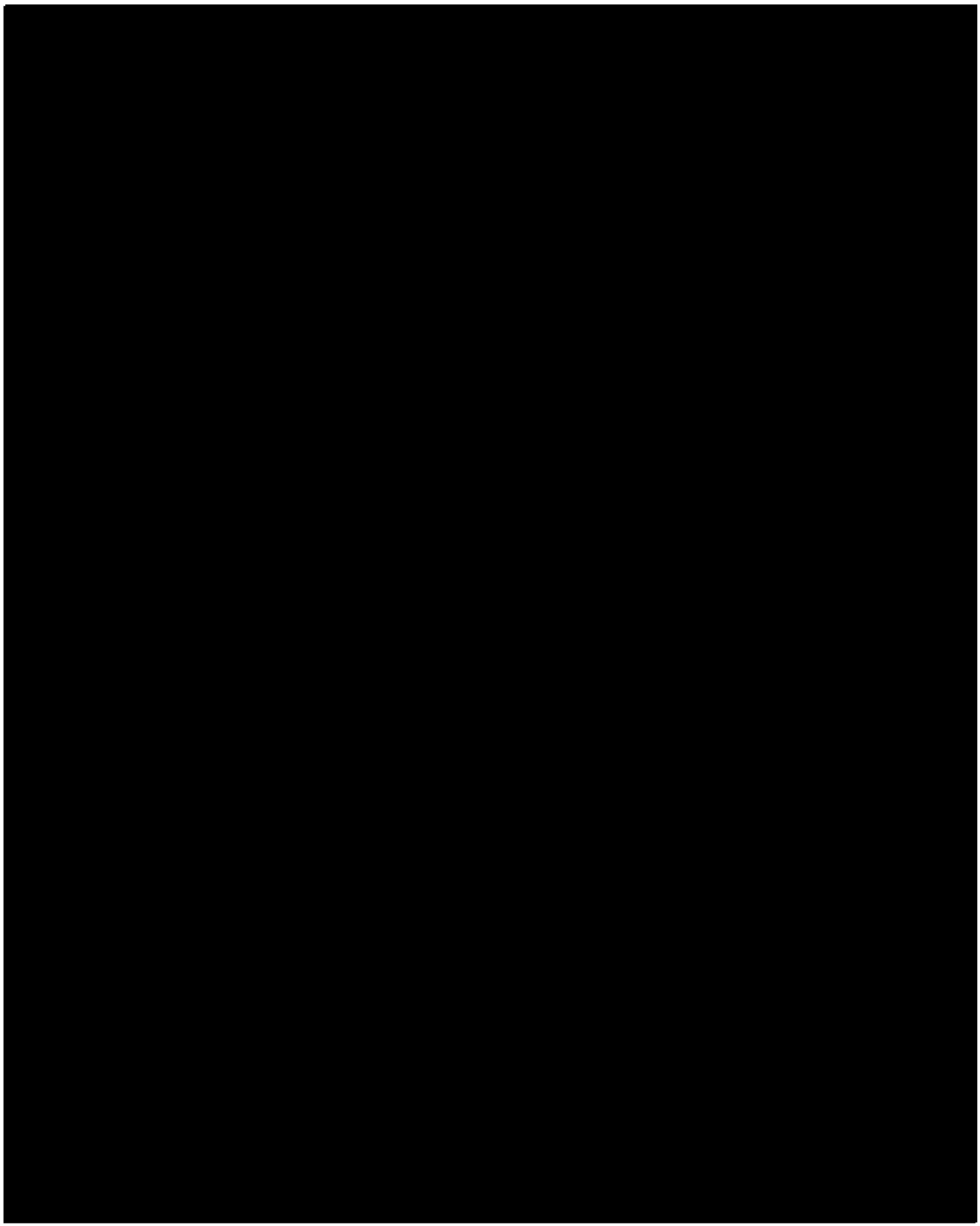
ABSTRACT

The *in vivo* regulation of several acid hydrolases by growth hormone was investigated in an attempt to lend insight into the mechanism of growth hormone action. In addition, the relationship of these enzymes to age-dependent changes in skeletal growth rate was examined. Initial studies showed that hypophysectomy reduced the activity of an unknown cartilage protease, which was assayed by digestion of gelatin-membrane substrates at pH 4.0. Treatment of hypophysectomized rats with growth hormone enhanced this activity to about normal levels. The unidentified protease activity was also shown to be higher in younger, more rapidly growing rats, than in older, less rapidly growing rats. These observations suggested that one or more lysosomal, acid proteases, called cathepsins, may be related to skeletal growth. Therefore, the relationship between three known lysosomal enzymes, namely cathepsin D, cathepsin B and acid phosphatase, and cartilage growth rate was examined. Cartilage cathepsin D and acid phosphatase activities of hypophysectomized rats were reduced relative to normal controls. Treatment of hypophysectomized rats with growth hormone enhanced these two enzyme activities towards normal levels. Pepstatin titration experiments suggested that the elevated cartilage cathepsin D activity corresponded to an increased enzyme concentration. Cartilage cathepsin B activity was refractory to hypophysectomy and growth hormone-treatment. The cartilage activity of all three enzymes was much greater in younger, more rapidly growing normal animals, than in older, less rapidly growing normal animals. Apparently, this was a result of decreased cell number and enzyme concentration. The growth

rate and cartilage acid hydrolase activities of hypophysectomized animals varied minimally with increasing rat age. The enzyme modulations cited could be, at least in part, tissue specific as liver acid hydrolase activities did not parallel growth hormone and age-dependent growth rate. Overall, these results suggest that cartilage cathepsin D and acid phosphatase activities are coordinately controlled and related to skeletal growth rate. Taken together with other studies relating acid hydrolases to tissue growth, these observations stimulate speculation that the mechanism of growth hormone action and, derivatively, skeletal growth in general may occur via acid hydrolase-mediated processes.

CURRICULUM VITAE

John R. Hubbard



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Chapter I

INTRODUCTION

A. Overview - The fundamental biochemical mechanism of growth hormone (GH) action is only partially understood. Skeletal tissue is the cardinal target tissue of GH; it was therefore chosen as the primary tissue to study GH action in this investigation. The elucidation of the biochemical events regulated by GH may lend insight into the mechanism of GH action and, derivatively, skeletal growth. In addition, the examination of correlations between age-dependent changes in growth rate and age-dependent metabolic alterations, could also improve our understanding of the molecular mechanism(s) of growth.

B. Skeletal Growth - The biochemical mechanisms of skeletal growth are essentially unknown. However, certain observations may form a basis upon which research can be initiated. Mammalian bone is initially formed as cartilagenous tissue, or in a few cases as condensed mesenchyme, which is later ossified into bone (1a,b). Because bone is not able to grow, the growth rate of its precursor cartilage significantly controls the overall growth of skeletal tissue.

Cartilage is primarily composed of cartilage cells (chondroblasts and mature chondrocytes) which lie in small spaces called lacunae (1a,b). They are surrounded by a dense intercellular matrix which is primarily composed of a coordinated complex of collagen and sulfated chondromucoproteins. Therefore, the process of cartilage growth involves both the proliferation of cartilage cells and the synthesis of matrix material. Several biochemical parameters can be used to determine the anabolic

activity of cartilage. These methods primarily include (1) uptake of [^{3}H]-thymidine into DNA, (2) uptake of [^{35}S]-sulfur into sulfated mucoproteins, (3) uptake of [^{3}H]-amino acids into protein, (4) hydroxylation of proline to hydroxyproline of collagen, and (5) uptake of [^{3}H] uridine into RNA.

As chondrocytes divide, small clusters of chondroblasts form initially (1a, b). These chondroblasts secrete matrix material which also adds to the growth of the cartilage and helps to separate the clustered cells. This interstitial growth is probably facilitated by increased tissue plasticity in young animal tissue (1a). The control of cartilage growth involves many factors including nutritional status, vitamins, and certain hormones (particularly growth hormone, somatomedin, and thyroxine) (1a, b). Cessation of skeletal growth generally occurs either as a consequence of nearly total transformation of cartilage into bone or of decreased sensitivity of cartilage to regulatory factors (particularly apparent in the aging process) (1a, b).

C. Growth Hormone

1. Overview - Growth hormone (GH) is a polypeptide hormone which is composed of a single polypeptide chain of approximately 190 amino acid residues (2). In 1945, Li, Evans and Simpson (3) obtained the first highly purified preparation of GH. GH has a molecular weight of about 21,500 and contains 2 disulfide bridges. The amino acid sequence of GH is known for several species (4a, b, c). Although there is a high degree of sequence homology between species, only human and primate GH can stimulate human growth. Of particular interest to this study, is the observation that bovine GH stimulates rat growth and many metabolic processes (5). Interestingly, the primary amino acid sequence of GH dis-

plays a high degree of homology to prolactin and placental lactogen (6). Thus GH of many species exerts mild lactogenic properties.

GH is synthesized (about 500 ug per day in humans) and is secreted by the anterior pituitary. It circulates in the blood at about 10^{-10} M (0 to 5 ng per ml in human plasma). Pituitary secretion of GH is regulated by the hypothalamus. In acromegaly, a disorder characterized by high GH levels, plasma GH averages between 15 and 80 ng/ml (7). Interestingly, circulating levels of GH can fluctuate by an order of magnitude within minutes. Stress, exercise, hypoglycemia, amino acids (particularly arginine), high protein intake, ACTH, catecholamines and malnutrition enhance GH concentration in the blood. Glucocorticoids and glucose are the primary factors that decrease GH levels.

2. Growth hormone regulation of metabolic processes and skeletal growth - GH appears to affect essentially all body tissues (8a, b). In muscle tissue, GH stimulates the transport of amino acids and the synthesis of proteins, RNA and DNA (8a, b). It also inhibits glucose transport (antagonizing the effect of insulin) and glycolysis. In adipose tissue, GH is slightly lipolytic, thus stimulating release of glycerol and free fatty acids within 30 to 60 min after administration in vivo. In liver tissue, GH enhances fatty acid oxidation and glycogen synthesis (apparently via gluconeogenesis). GH is also known to affect mineral metabolism. Intestinal absorption of calcium and retention of phosphate, magnesium, chloride and potassium also appear to be partially regulated by GH (9).

Despite the manifold affects of GH on overall body metabolism, the physiological importance of GH in general metabolic regulation is unknown. This is well illustrated in certain endocrine pathologies. For

example, adult subjects with a specific deficiency in GH have no major metabolic problems. In addition, hypopituitary patients need only to be treated with thyroxine, corticosterone, and gonadal steroids to maintain normal metabolism. Thus although GH appears experimentally to influence many metabolic processes, the significance of its influence in vivo remains obscure.

The major role of GH appears to be the stimulation of skeletal growth and metabolism (8a, b). The dependency of skeletal growth on GH is illustrated by the development of dwarfism when GH is absent or in very low concentration during childhood. Although thyroid hormones and thyroid stimulating hormone appear to enhance certain cartilage metabolic activities, cartilage growth seems to be primarily stimulated by GH. ACTH, gonadotrophins, and gonadal steroids do not affect cartilage growth rate to a significant degree (10).

The effect of GH on skeletal growth occurs on both cartilage growth and mineralization. In vivo GH stimulates cell mitosis, matrix sulfation, and the synthesis of DNA, RNA, general proteins, collagen, and chondromucoproteins (8a, b). In addition, GH enhances blood mineral levels (as described previously) thus influencing cartilage mineralization. Therefore, cartilage tissue may represent the tissue of choice to study the anabolic activities and mechanism of action of GH. However, comparitively few studies with GH have utilized cartilage as the experimental tissue. The reasons for the more prevalent use of other tissues, particularly liver, rather than cartilage are probably due to a) the comparatively high degree of difficulty to obtain large quantities of cartilage, b) the relatively sluggish metabolic rate of cartilage and c) the comparatively tenacious make-up of cartilage tissue. However, as carti-

lage may be the primary target tissue of GH action, we chose to use cartilage as our major tissue of study. Liver tissue was also examined since 1) a large body of data are available on this tissue, and 2) it offers the basis to compare perturbations in cartilage metabolism with a second tissue.

3. Mechanism of growth hormone action and relationship to somatomedin - The biochemical mechanism of GH stimulation of cartilage growth is not well understood. A complicating factor in the elucidation of its mechanism results from the inability of GH to regulate directly cartilage metabolism *in vitro* (11). In 1957, Salmon and Daughaday (12) presented the first evidence that GH may not directly regulate cartilage growth *in vivo* but rather may exert its effects *via* a GH-dependent substance(s) initially termed "sulfation factor". A good deal of evidence, albeit indirect, has since supported their hypothesis and it is now generally believed that a family of GH-dependent peptides, called "somatomedins" (SM), may mediate many of the effects of GH *in vivo*, particularly on cartilage growth and development (13a, b). At least 5 different somatomedin molecules have been identified. These include SM-A, SM-C, insulin-like growth factor I (IGF-I), IGF-II, and multiple stimulating activity (14). Interestingly, the IGF peptides have a high sequence homology to insulin (15). The serum levels of the SM's are elevated in acromegaly and decreased in hypopituitary subjects (16). GH treatment can restore SM activities to normal levels. However, the mechanism of SM generation is unknown. Many investigators suggest that SM synthesis is stimulated by GH, particularly in liver tissue (17). However, other studies indicate that certain SM-like peptides could be generated from proteolysis of GH. For example, we have demonstrated that a 5,000 MW

tryptic fragment of bovine growth hormone (bGH, 96-133) has several SM-like bioactivities (18, 19). It should be noted that there has actually been little or no direct evidence that SM mediates GH action *in vivo* or that SM even stimulates growth *in vivo*. The lack of evidence, however, partially resides in a) the scarcity of purified SM's which precludes sufficient quantity for study and b) rapid SM turnover *in vivo* in hypopituitary subjects. This latter point is possibly due to the lack of a GH-dependent SM carrier protein. Preliminary serum fractionation studies by the author support the existence of a SM carrier protein in human and rat serum (data not shown). Thus, experiments concerning the mechanism of GH action *in vivo* must be carefully interpreted. It should also be noted that at present, studies on cartilage growth *in vivo* generally utilize GH administration *in lieu* of SM treatment, while *in vitro* studies often use SM or serum from GH-treated animals *in lieu* of GH alone.

Thus, due to both the problems of working with cartilage tissue and the undefined roles of GH, SM and perhaps other GH-dependent factors, progress in elucidating the biochemical mechanism of GH-stimulated skeletal growth has been particularly difficult. Several observations however have begun to form a foundation upon which the mechanism may one day be resolved. For example, there is some evidence that cAMP levels are decreased by GH-dependent SM (20). In many other systems characterized by rapidly growing tissue, e.g. malignant growth, low levels of cAMP have also been observed (21a, b). Changes in cAMP levels are of considerable importance since they can directly affect the activity of several enzymes (22a, b) and can regulate DNA and RNA synthetic processes (23). In addition, cAMP changes appear to occur in the cell membrane immediately after hormone receptor interaction (14a, b). Thus alteration

of cAMP could be one of the initial steps in the mechanism of GH (or SM) action.

In other studies, the GH-dependence of particular enzymes whose activities may be involved in the mechanism of tissue growth have been examined. Enzymes which may be involved in the growth process would generally be expected to 1) be altered by hypophysectomy or other means that modulate GH levels, 2) revert towards normal levels by replacement of physiologically relevant doses of GH after hypophysectomy, 3) exhibit a similar relationship to growth rate during manipulation of growth by other means, and 4) have a logical causal relationship between their activity and the rate of growth.

The relationship of hypophysectomy and GH treatment to enzyme activity has been extensively studied. In liver tissue, numerous enzymes have been investigated but few have been shown to be GH-dependent (25a, b, c, d, e). Of particular interest, however, is the enhancement of hepatic DNA polymerase and ornithine decarboxylase activities by GH (26, 27a, b). These enzymes may act as mediators of GH action via stimulation of DNA and protein synthesis, respectively. DNA polymerase is an enzyme directly responsible for the replication of DNA, while ornithine decarboxylase is related to polyamine synthesis which has been implicated in RNA synthesis and stabilization (27a, b, 28). In addition, GH decreases hepatic glutamic pyruvic transaminase which may act to retard amino acid catabolism (29). In regard to lysosomal enzymes, Mathies, Palm and Gaebler (30) reported that hypophysectomy reduced liver cathepsin D activity but that this lower activity was unaltered by GH treatment. In another study, large doses of GH (10 mg per day for 14 days) were not able to alter hypophysectomized (Hx) rat liver acid phosphatase activity (31).

In muscle tissue, glutamic oxalacetic transaminase activity was elevated in Hx animals and reduced by administration of GH (32). This enzyme may also serve to reduce amino acid catabolism. Interestingly, Cohen and Hekleus reported low transaminase activity in rapidly growing transformed, embryonic, and regenerating tissues (33). Renal glutamic oxalactic transaminase was also decreased by GH (34). Hypophysectomy decreased kidney cathepsin D activity but only large doses of GH, i.e., 1 mg per day for 20 days, enhanced the activity to normal levels.

The relationship of GH to enzyme activities has been examined in several tissues. However, very few studies have utilized skeletal tissue (for reasons discussed in section C.2), which may be the primary target tissue of GH (or SM). Buchwald and Hudson (35) showed that hypophysectomy decreased bone alkaline phosphatase activity. Later studies supported their observation and showed that this effect could be reversed by GH treatment (36a, b). In light of other information implicating a relation of alkaline phosphatase activity and bone mineralization (37a, b), these observations suggest that GH-dependent skeletal calcification may occur, in part, via GH-dependent alkaline phosphatase activity. A very high dose of bGH (3.0 mg per mouse) enhanced acid phosphatase activity of cartilage (38). The significance of this observation is questionable, however, due to the supraphysiologic level of GH used. In 1977, Kasaina, Torkenko, and Ukhimn (39) reported that 3 h after treatment of rabbits with GH (4 units per kg weight) several cartilage and bone lysosomal glycosidase activities were decreased.

Thus, the relationship of GH to several tissue enzymes has provided some insight into the mechanism of GH action. However, distinguishing whether GH-dependent enzymes are obligatory for growth or merely

influenced by the growth process will require a great deal more investigation. Nevertheless, elucidation of GH-dependent enzymes (particularly in cartilage) may prove quite valuable in the elucidation of the biochemical mechanisms of GH (or SM) action.

D. Lysosomes and Their Hydrolytic Enzymes

1. Overview - Lysosomes (lytic bodies) were first discovered by de Duve and others (40a, b) between 1955 and 1959 using differential centrifugation of cellular components, followed by morphologic and chemical analysis. Lysosomes are intracellular organelles which have been found in essentially all animal tissues. They are about 0.25 to 0.8 um in diameter and contain several classes of hydrolytic enzymes. These enzymes are apparently synthesized by the endoplasmic reticulum and encompassed (in the golgi-gerl region) by a single lipoprotein membrane. This membrane is characterized by a high degree of intracellular stability, impermeability to the enzymes it encompasses, and insensitivity towards hydrolytic enzyme activities (41). In vitro, however, lysosomal membranes can be disrupted by exposure to hypotonic solutions and to certain detergents such as Triton X-100 (41).

Lysosomes contain several classes of enzymes including cathepsins, glycosidases, phosphatases, ribonucleases, and deoxyribonucleases. These enzymes hydrolyze substrates such as proteins and peptides, polysaccharides, phosphate esters including those of mononucleotides and phosphoproteins, ribonucleic acids, and deoxyribonucleic acids. Characteristics of the lysosomal enzymes examined in this study (cathepsin D, cathepsin B and acid phosphatase) will be discussed in section D.2.

The physiologic role of lysosomes is not totally clear but may be multifacited. Certainly considerable evidence suggests that lyso-

somal enzymes are used to degrade phagocytized macromolecules, thus supplying the cell with precursors for new molecular synthesis. Lysosomal hydrolases may also digest intercellular components by either discharging the lysosomal enzymes at the cell surface, or by releasing the entire lysosomal organelle. Lysosomal cathepsins may also function in the partial hydrolysis of certain proteins, perhaps yielding an active product. For example, several hormones are synthesized in large precursor forms which must be degraded to active structures (42a, b). Thus, lysosomal hydrolases could serve regulatory functions via their ability to chemically modify macromolecules. Since lysosomes are quite mobile (43), their action could occur not only in the cytoplasm but at the cell surface or at, or within, the nucleus as well. Thus, the regulation of lysosomal hydrolases could provide a means of controlling many diverse cellular events.

2. Characteristics of certain lysosomal enzymes

a. Cathepsin D - Cathepsin D (International enzyme commission number IEC 3.4.23.5) is a carboxyl endoprotease with a molecular weight between 40,000 and 60,000. It is found primarily in cellular lysosomes. Cartilage and uterine cathepsin D have molecular weights of about 43,000 while liver cathepsin D is about 51,000 m.w. Depending on the tissue of origin, cathepsin D has between 1 and 12 isozymes (44). Cartilage cathepsin D has only one or two forms while uterine cathepsin D has at least 12 isozymes (45a, b). Chemically, cathepsin D is characterized by 1) a high glycine content, 2) 8 half cysteines per molecule, 3) several glucosamine residues and 4) about twice the number of acidic amino acids (many of which are amides) as basic amino acids.

Cathepsin D, like pepsin, characteristically hydrolyzes

peptide bonds on the carboxyl side of aromatic amino acids (46a, b). In addition it preferentially cleaves bonds that have hydrophobic residues on either side of the bond to be cleaved. While cathepsin D generally acts as an endopeptidase, it can also act as an exopeptidase at the amino terminus of certain proteins. The pH optimum of cathepsin D appears to depend on the substrate used. Thus, while hemoglobin is optimally hydrolyzed between pH 3.0-3.8, cartilage proteoglycans are optimally cleaved at pH 4.0-5.0 (47a, b). Cathepsin D appears to hydrolyze cartilage chondromucoproteins only in areas of relatively low carbohydrate concentrations.

Cathepsin D activity is neither affected by divalent cations such as calcium, manganese, and magnesium, nor by chelating agents such as EDTA (48). However, as with other carboxyl proteases, cathepsin D is inactivated by pepstatin. Pepstatin was first isolated by Umezawa (49) from filtrates of streptomyces and used as an inhibitor of pepsin. Pepstatin binds to cathepsin D with high affinity (Kass = 6×10^5 M at pH 3.5, to 2×10^9 M at pH 6.4) stoichiometrically at the active site. Thus, cathepsin D concentration can be determined by titration with pepstatin (50). Immunoglobulins directed against cathepsin D have also been useful in inhibiting its activity (51a, b).

b. Cathepsin B - Cathepsin B (IEC number 3.4.22.1) is also an important lysosomal protease. Cathepsin B (or B1) is a thiol protease which has a molecular weight of 25,000 (52). This enzyme appears to have a cysteine and histidine residue at its active site. It cleaves many proteins with a specificity resembling papain (53). Cathepsin B cleaves hemoglobin optimally at pH 4.0-4.5 but has an optimum for most substrates between pH 5.0 and 6.5. Like other thiol proteases, cathepsin

B is inhibited by leupeptin (54).

Cathepsin B is probably universally found in mammalian species and in most tissues including cartilage. The physiological function of cathepsin B is still poorly understood but it appears to play a major role in lysosomal catabolism of proteins (55).

c. Acid phosphatase - Acid phosphatase or orthophosphoric monoester phosphohydrolase (IEC number 3.1.3.2) is a lysosomal enzyme which hydrolyzes phosphoric monoesters at acidic pH. Acid phosphatase is often used as a lysosomal marker and is actually a part of the biochemical definition of lysosomes (56). Acid phosphatase may have several chemical forms, and most of the lysosomal activity has a M.W. of 100,000-111,000 (56). The physiological function of acid phosphatase is still obscure. However, there is some evidence that it is involved in cartilage remodeling (57).

3. Relationship of lysosomal enzymes to tissue growth - An intriguing relationship between tissue growth and lysosomal enzymes (particularly proteases) is becoming increasingly apparent. Ostensibly the most efficient mechanism to explain tissue growth could involve the enhancement of anabolic processes and the reduction of catabolic processes of the tissue. Therefore, it is not surprising that some investigators have reported a decrease in protein degradation during growth *in vivo* (58a, b) and *in vitro* (59a, b). However, in many other studies, high lysosomal activities have been correlated with rapid tissue growth.

Comparative studies have shown that tumors are generally characterized by high proteolytic and other lysosomal activities (60a, b, c). For example, increased levels of cathepsin D were apparent in tumors of human breast and colon (61, 62). Acid phosphatase and several glyco-

sidases were also elevated in breast tumors but not colon (61). Cathepsins, acid phosphatase and other lysosomal enzymes are also elevated in many transformed cell lines, such as in murine saroma virus transformed fibroblasts (MSV-3T3) and rous sarcoma virus transformed fibroblasts (RSV-3T3) compared to normal embryonal fibroblasts (64a, b, c). Interestingly, certain protease inhibitors have been able to reverse some changes in transformed cells towards normal characteristics (65a, b). Similar findings prompted Stubblefield and Brown to propose in 1977 a protease theory of cancer (66).

Other systems associated with rapidly growing tissue, such as regenerating liver, also commonly exhibit high lysosomal enzyme activities. Interestingly, Miyamoto et al. (67) in 1973, showed that intraperitoneal injections of leupeptin and pepstatin slowed or inhibited DNA and RNA synthesis as well as cell mitosis in regenerating rat livers. In addition, cell DNA synthesis and mitosis have been stimulated by certain proteases and lysosomal lysates (68a, b, c).

Although proteases and other lysosomal enzymes are clearly associated with abnormally rapid tissue growth, their relationship to normal tissue growth is unclear. In addition, the role of these enzymes in tissue growth is not well understood. Some investigators suggest that during tumor growth these hydrolases break down tissue surrounding the tumor and allow space for expanded tumor growth and invasion (69). Many other investigators believe that these hydrolases may have a direct effect on promoting cellular mitosis by 1) disruption of cell contact inhibition, 2) activation of nuclear regulatory molecules, and 3) alteration of cAMP levels (70a, b). Taken together, these observations underscore the idea that certain lysosomal hydrolases are correlated with, and perhaps obligatory for, growth of certain tissues.

Cartilage lysosomal enzymes have been investigated primarily with regard to disease states such as osteoarthritis (71a, b, c). Curiously however, osteoarthritis is characterized by both high lysosomal enzyme activities and enhanced cell proliferation (71a, b, c). This proliferation is obtusely interpreted as tissue growth to replace lost tissue, but is also consistent with a direct causal relationship between lysosomal enzymes and chondrocyte proliferation. In addition, certain proteases and lysosomal lysates have been shown to stimulate cartilage cell proliferation. The relationship of endogenous cartilage hydrolytic enzymes to growth rate is essentially unknown, but could reveal insights into the mechanism of skeletal growth.

4. Relationship of lysosomal enzymes to age - The relationship of lysosomal enzymes to aging has been studied for many years primarily because these enzymes could be involved in age-dependent tissue degradation. However, the exact nature of this relationship still remains somewhat obscure. Although many studies have utilized liver tissue in aging studies, consistent observations have not been obtained (72a, b, c). Possibly the complex regulation and cell population of liver tissue may have added to the inconsistancy in results. In rat and rabbit cardiac tissue, differential changes in lysosomal enzymes with age were reported (73). Thus, in both species, while cathepsin D activity increased with age, acid phosphatase activity was unaltered and glucosaminidase activity was either lower or unchanged in cardiac tissue.

In cartilage tissue, changes in lysosomal enzymes with age are also rather obscure. In canine hip cartilage, cathepsin D and acid phosphatase activities per weight of tissue decreased with age (between new born and 12 months of age) (74). However, alteration of DNA content

suggested that this change was primarily due to age-dependent decreases in cell number. Silberberg and Lester (75) studied age-dependent changes in guinea-pig articular cartilage. Their results indicated that lysosomal cathepsin activity and glycosidase activities generally decreased from birth to midlife and then began to increase in later years. Histochemical localization of rat costal cartilage acid phosphatase and glycosidases indicated an increase in activity between newborn and 1-2 week old rats, followed by a decrease to adult levels by 8 weeks of age (76).

5. Hormonal regulation of lysosomal enzymes - There is increasing evidence that hormones regulate lysosomal lability and enzyme activities. Szego (77) has put forth a model for lysosomal mediation of hormone action, in which lysosomes could 1) act in nucleocytoplasmic communication, 2) function in the process of hormone receptor modification and degradation, 3) cause cell membrane alteration and 4) alter nuclear regulatory constituents. The relationship and role of lysosomes to hormone action is however still unclear.

Several hormones have been shown to alter lysosomal enzyme activities. For example, DeMartino and Goldberg (78) reported that both anabolic and catabolic levels of triiodothyronine (T_3) stimulated a 2-3 fold increase in liver cathepsin D, cathepsin B, acid phosphatase and several other enzymes. Pepstatin titration experiments indicated that the increase in cathepsin D activity was due to an increased number of enzyme molecules. The effect of T_3 differed between tissues as T_3 enhanced muscle tissue lysosomal enzymes about 50% and had no detectable effect on heart and kidney tissue. Woessner (79) showed that estrogen decreased uterine cathepsin D and acid phosphatase activities. After hypophysectomy muscle cathepsin D activity decreased but even high doses of GH (250 ug per day for 15 days) did not reverse the effect (80).

Hormones also affect lysosomal membrane lability. For example estrogen and testosterone appear to increase lysosome lability in target tissues (81a, b). On the other hand, cortisone appears to stabilize lysosomes in target tissues as well as increase lysosomal enzyme activities (81a).

In skeletal tissue, parathyroid hormone enhanced synthesis of bone and release of lysosomal enzymes including cathepsin D and acid phosphatase (82). This observation may suggest a role of these enzymes in bone demineralization. Steinetz and Manning (83) showed that a high dose of GH (3.5 mg per mouse) increased cartilage acid phosphatase activity. However the physiological significance of the observation remains obscure due to the high concentration of hormone used.

Thus, available preliminary evidence suggests that lysosome stability and enzyme activities may be related to, and possibly required for, hormone action.

E. Summary - Previous studies suggest that certain lysosomal enzymes may be related to, and perhaps mediators of, tissue growth and hormone action. However, the precise nature of these relationships and the roles they play in the biochemical mechanism of tissue growth are still poorly understood.

Skeletal growth, as with growth of other tissues, may also be related to lysosomal enzyme activities. However, at present, the relationship between endogenous lysosomal enzyme activities and skeletal growth is unknown. As growth hormone is a major regulator of skeletal growth in vivo, determination of its effect on cartilage lysosomal enzymes may clarify the relationships of lysosomal enzymes to skeletal growth. Similarly age-dependent differences in growth rate may also prove to be a useful

model to probe the relationships between skeletal growth and cartilage lysosomal enzyme activities.

F. Research Aims - The primary purpose of this research was to investigate the regulation of certain cartilage acid hydrolases by growth hormone in an attempt to lend insight into the mechanism of growth hormone action. Secondarily, the relationship of these enzymes to age-dependent changes in skeletal growth rate was examined to determine if growth hormone-dependent metabolic alterations could represent general growth-dependent processes.

Chapter II

MATERIALS

The materials used in this investigation were obtained from the following sources:

Materials	Source
Bovine growth hormone (NIH-GH-B-18) 0.81 I.U./mg	National Institutes of Health, and the National Pituitary Agency, University of Maryland
Bovine serum albumin	Sigma Chemical Co., Missouri
Calf thymus DNA	Sigma
Cathepsin B	A. Barrett, Strangeways Laboratories, Cambridge, England
Cathepsin D	A. Barrett
Dimethyl sulphoxide	Sigma
Diphenylamine	Eastman Kodak Co., N.Y.
Dithiothreitol	Sigma
Disodium (ethylenedinitrilo) tetraacetate (EDTA)	J.T. Baker Chemical Co., N.J.
Folin-Cioculteau Phenol reagent	Fisher Scientific Co., N.J.
Gelatin-substrate membranes	N. Owers, Department of Anatomy, MCV/VCU
Hemoglobin	Sigma
Leupeptin	A. Barrett
N,α-benzoyl-D,L-arginine-L-naphthylamide (BANA)	Sigma
Pepstatin	A. Barrett
p-nitrophenol	Sigma

p-nitrophenyl phosphate	Sigma
Polytron homogenizer	Brinkman Instruments Inc., N.Y.
Sprague-Dawley male hypophysectomized rats	Zivic-Miller Labs, Allison Park, PA
Sprague-Dawley male normal rats	Zivic-Miller Labs, Allison Park, PA
Tissue-Tek O.C.T. compound	Scientific Products
Trichloroacetic acid (TCA)	J.T. Baker Chemical Co.
3,3',5'-triodo-L-thyronine	Sigma
Triton X-100	Sigma
Tyrosine	Calbiochem, Cal.

Chapter III

METHODS

A. Nonspecific Protease Assay - Gelatin Membrane Substrate

1. Laboratory animals and hormonal treatment - Normal and 21 day post-operative hypophysectomized (Hx) male Sprague-Dawley rats were used in this study. A solution of bGH (1.5 to 500 ug total dose) in alkaline saline (3 drops 0.1 N NaOH in 8 ml 0.9% NaCl) or alkaline saline alone was injected subcutaneously, one dose per day for four days. Rats were sacrificed on the 5th day along with normal rats comparable in age (N age, normal age control group) or weight (N wt, normal weight control group) to Hx animals.

2. Detection of cartilage protease activity - Cartilage protease activity was assessed by a slightly modified method of Owers (84a, b, c). This assay was based on the hydrolysis of gelatin film membranes which were fixed with glutaraldehyde onto glass microscope slides.

Although quantitation of this protease assay was arduous, it afforded the advantages of high sensitivity and low specificity. A similar procedure has been used by others to study the parathyroid hormone-dependence of a bone proteolytic activity (82). In the standard assay, 3 rat costal cartilages (from ribs 3-5) were excised from each rat. Embedding medium was applied to cartilage triplicates from 7-9 rats, representing all treatment groups, and solidified at -20°. Sixteen micron thick cross-sectional slices (starting 1 cm from the sternum) were prepared at -20° using a cryostat. Each slice was placed on a gelatin

membrane and overlaid with 0.03 M veronal-acetate buffer, pH 4.0. The samples were immediately incubated in a humid chamber at 37° for the times indicated. After incubation the slides were washed with buffer and dried prior to quantitation.

To visualize gelatin digestion, the membranes were either mixed with India ink prior to fixation (84a) or stained with trypan blue after digestion. These methods gave essentially identical results, when expressed on a percentile basis. Data from both methods were therefore combined. Membranes to be stained with trypan blue were cooled 5 minutes at 4° after the reaction with cartilage. They were then washed sequentially at 4° for about 2 minutes to 10 minutes in 0.03 M veronal-acetate, pH 8.0, 1 min in 95% ethanol, 1 minute in 50% ethanol, 10 seconds in 0.03 M veronal-acetate, pH 5.5, 10 seconds in 1.0% trypan blue in 0.03 M veronal-acetate, pH 5.6, 10 seconds in deionized water and 10 seconds in a second water wash. The membranes were dried at room temperature and washed twice with deionized water at room temperature for 5 minutes each. Membranes were dried before quantitation.

3. Quantitation of protease activity - Protease activity resulted in decreased stain under the cartilage slice. To quantitate this activity, a photometric method was developed by the author. After incubation, the membranes were washed, stained, and photographed. The photographs were taken by D. Bennett, Visual Education Department, Medical College of Virginia, Virginia Commonwealth University, using a Ultra-Phot II, C. Ziess. Hydrolyzed regions appeared as dark areas on the negative. Total activity correlated with the size and darkness of these spots. Hydrolyzed areas were scanned from the negative at 540 nm with a densitometer and the absorbance was recorded. The area under each absorbance

peak was determined with a planimeter and was defined as relative total activity. Specific activity was the quotient of total activity and relative surface area of each cartilage slice. The relative surface areas were obtained by planimetry of magnified cartilage photographs. Statistical analysis of results was performed according to the method of Dunnett (85).

B. Determination of Age and Growth Hormone-dependent Lysosomal Enzyme Activities

1. Animals, hormone treatment, and tissue preparation - Normal and 14 day post-operative hypophysectomized (Hx) rats were used in hormone-related experiments. In age-related studies, Hx rats were used at least 14 days after operation. Rats were housed in a temperature and humidity controlled room with an alternating 12-h light and 12-h dark cycle. Rats were fed Purina Rat Chow ad libitium. Bovine growth hormone (bGH, NIH-GH-B-18), triiodo-L-thyronine (T_3), or a combination of both (GH + T_3) were dissolved in alkaline saline (3 drops of 0.1 N NaOH per 8 ml 0.9% NaCl). Hormone solutions (see Legends for doses) or alkaline saline were injected subcutaneously, one dose per day, into Hx rats, for four days. Rats were decapitated on the 5th day along with normal rats comparable in age (N age, normal age controls) or weight (N wt, normal weight controls) to Hx animals. Livers were perfused with cold saline (0.9% NaCl) in situ. Costal cartilage and livers were then cleaned of adhering tissue, weighed, and homogenized (1.5 min for liver and 45 sec for cartilage) in 0.005 M sodium phosphate buffer, pH 6.5, with a Polytron homogenizer.

The efficacy of homogenization was determined by a) fractionation of cartilage homogenate cathepsin D activity by centrifugation at

low (3,000 xg for 5 min) and high (48,000 xg for 2 h) speed, and b) treatment of samples with 0.2% Triton X-100 (for 1 min to 16 h at 4°) to release any latent activity.

2. Enzyme assays - Cathepsin D activity was measured by hydrolysis of acid-denatured hemoglobin using a slightly modified method of Anson (86). Optimal activity was found at about pH 3.4 using sodium acetate and sodium citrate buffers (Fig. 1-3) as would be expected from the literature (87). The highest apparent activity was found with sodium acetate buffer (Fig. 1). The pH of the reaction mixture was unaltered during the reaction period. In the standard assay, 125 ul of either 5% cartilage homogenates (w:v) or a 10% liver homogenate (w:v) was added to 125 ul, 1.35 M sodium acetate buffer, and 0.5 ml 4% acid-denatured hemoglobin. The assay mixture was adjusted to pH 3.4. Hemoglobin was denatured for 1 h at 37° at pH 1.8 prior to use. Samples were incubated at 37° for 0.5 to 1.5 h. After incubation, 1.25 ml 5% trichloroacetic acid was added and samples were placed on ice for 10 min. They were then centrifuged for 10 min at 8,000 x g. One ml of supernatant was removed and mixed with 2 ml 0.5 M NaOH after which 0.6 ml 0.66 N Folin-Cioculteau phenol reagent was added and the solution mixed. The absorbance at 660 nm was determined after 15 min. Tyrosine served as the reaction standard. Tyrosine concentration was linear (at least up to 25 ug) with absorbance at 660 nm, as shown in Fig 4. Activity of tissue homogenates (0-125 ul) and purified cathepsin D (0-0.72 ug) exhibited first-order kinetics and were inhibited by pepstatin but not by leupeptin (Figs. 5-7).

Cathepsin B activity was determined by the hydrolysis of N,-α-benzoyl-D,L-arginine-L-naphthylamide (BANA) (88a, b). Slightly different procedures were used for cartilage and liver determinations owing to

Figure 1. Effect of pH on cartilage cathepsin D activity using a sodium acetate buffer. Cartilage homogenates (125 ul, 5% homogenates (w:v) in .005 M sodium phosphate buffer, pH 6.5) were incubated with 125 ul 1.35 M sodium acetate buffer and 0.5 ml 4% acid-denatured hemoglobin for 1 h at 37°. Cathepsin D activity expressed as mg tyrosine equivalents/ml homogenate/h. Results are the mean of three experiments.

Hx = Hypophysectomized rats injected for four days with alkaline saline alone.

Hx-GH_{500} = Hx rats injected for four days with 500 ug bGH (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

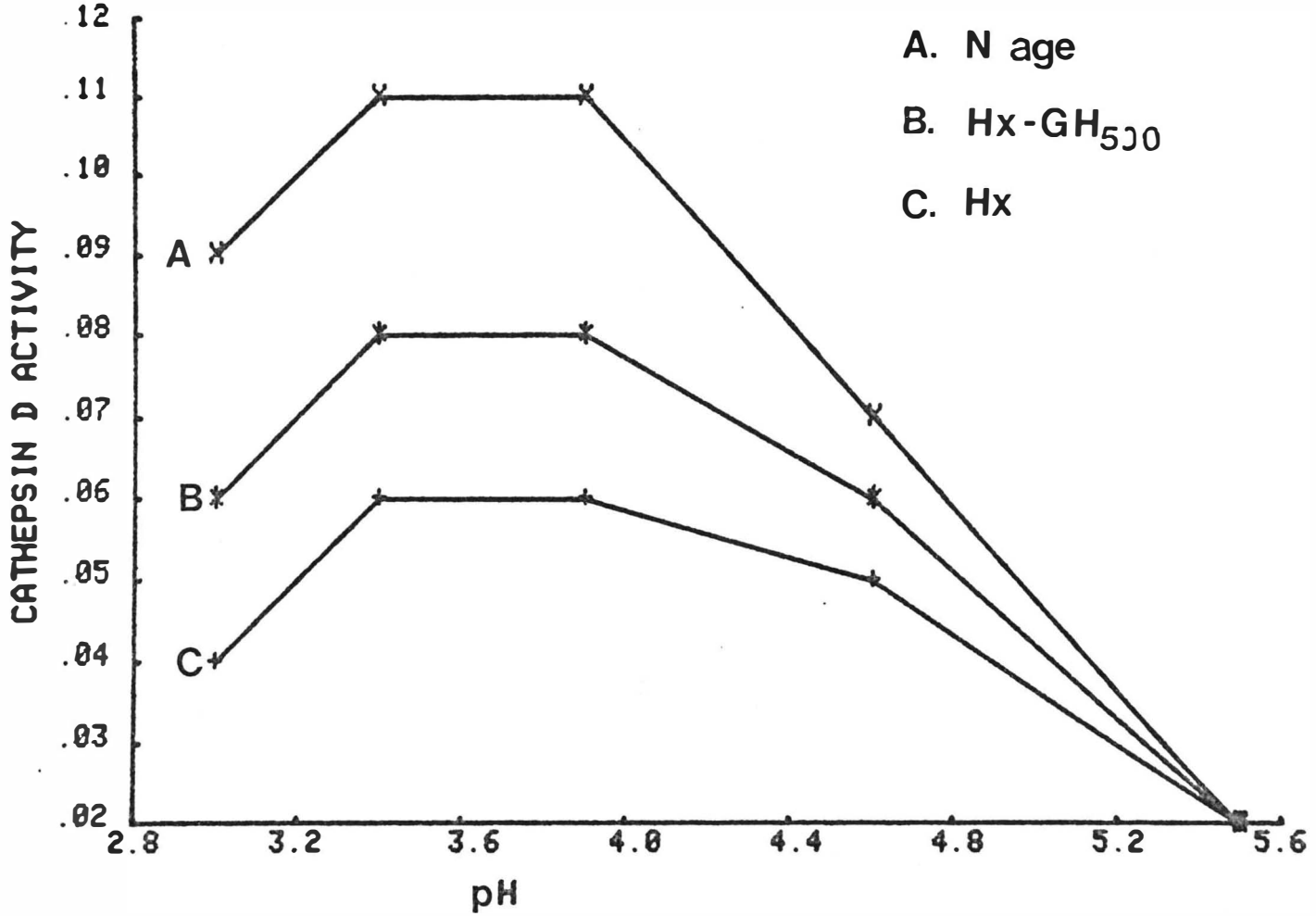

A. N age
B. Hx-GH500
C. Hx
CATHEPSIN D ACTIVITY
.12
.11
.10
.09
.08
.07
.06
.05
.04
.03
.02
A
B
C
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
pH

Figure 2. Effect of pH on cartilage cathepsin D activity using a sodium citrate buffer. Cartilage homogenates (125 ul, 5% homogenates (w:v) in .005 M sodium phosphate buffer, pH 6.5) were incubated with 125 ul 1.35 M sodium citrate buffer and 0.5 ml acid-denatured hemoglobin for 1 h at 37°. Cathepsin D activity expressed as ug tyrosine equivalents/ml homogenate/h. Results are the mean of three experiments.

Hx = Hypophysectomized rats injected for four days with alkaline saline alone.

Hx-GH$_{500}$ = Hx rats injected for four days with 500 ug bGH (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

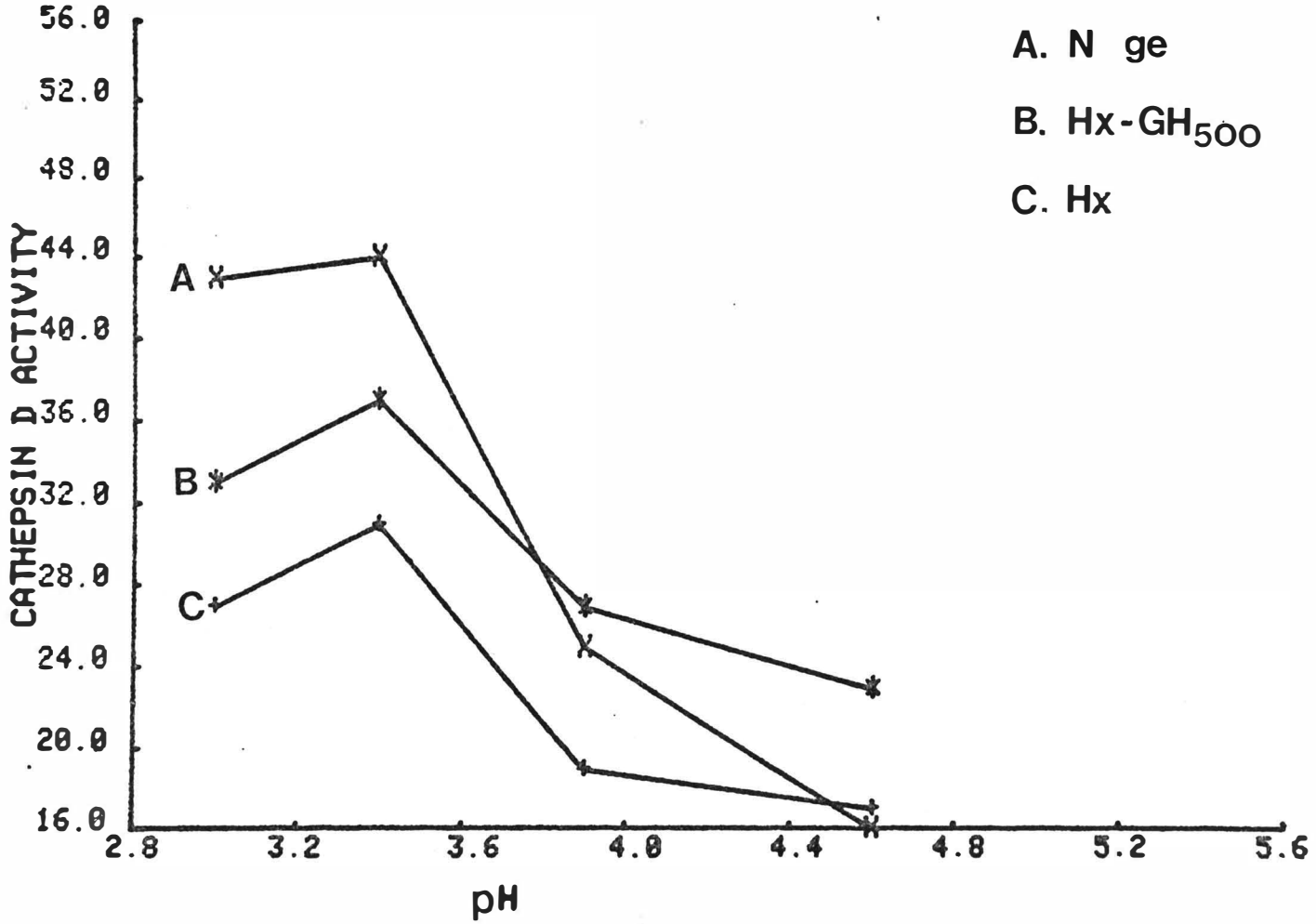

A. N ge
B. Hx-GH500
C. Hx
CATHEPSIN D ACTIVITY
56.0
52.0
48.0
44.0
40.0
36.0
32.0
28.0
24.0
20.0
16.0
A
B
C
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
pH

Figure 3. Effect of pH on liver cathepsin D activity. Liver homogenates (50 ul, 10% homogenates (w:v) in .005 M sodium phosphate buffer, pH 6.5) were incubated with 125 ul 1.35 M sodium acetate buffer and 0.5 ml 4% acid-denatured hemoglobin for 30 min at 37°. Cathepsin D activity expressed as mg Tyrosine equivalents/ml homogenate/h. Results are the mean of two values.

Hx = Hypophysectomized rats injected for four days with alkaline saline alone.

N age = Normal rats of comparable age to Hx rats.

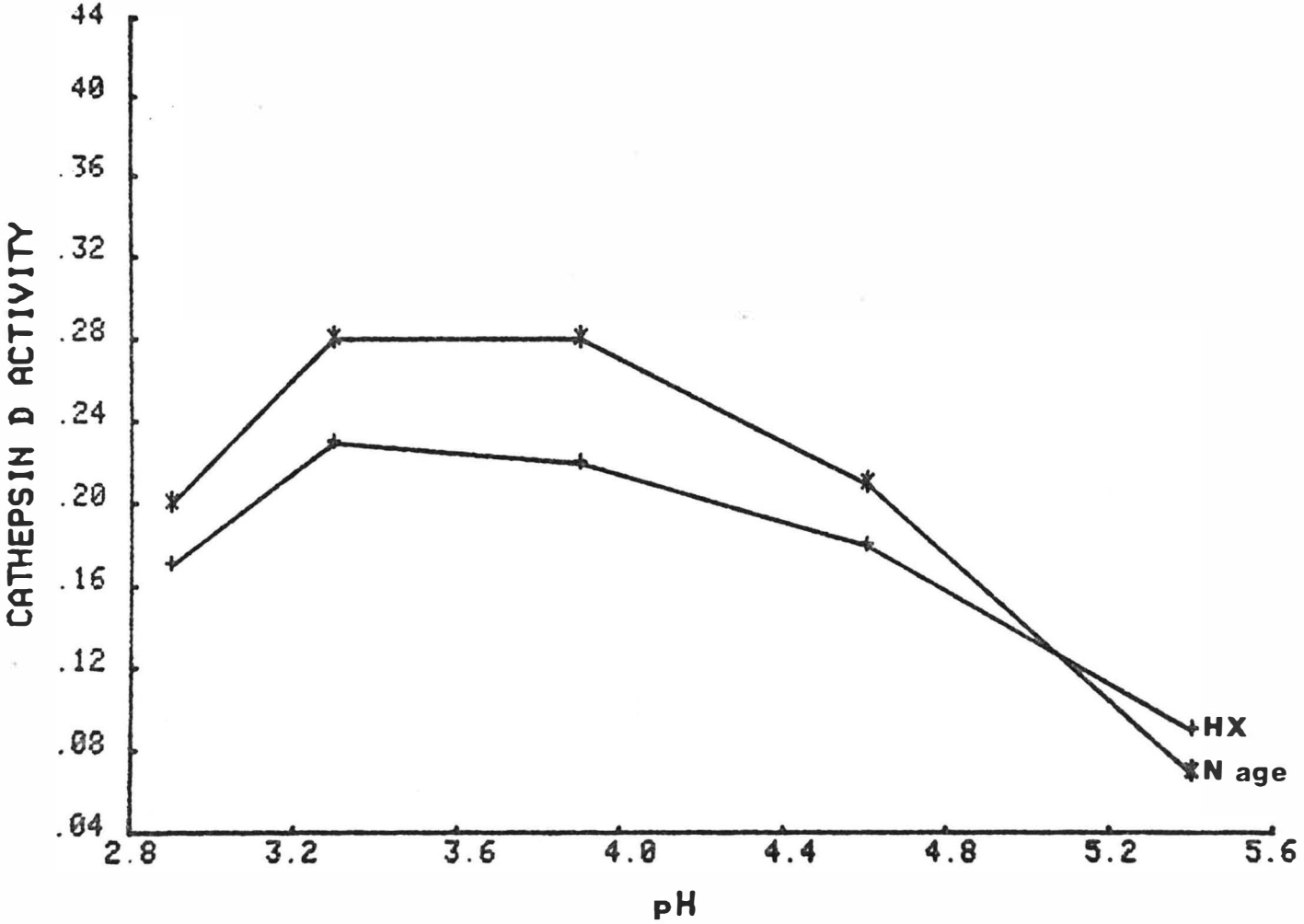

CATHEPSIN D ACTIVITY
44
40
.36
.32
.28
.24
.20
.16
.12
.08
.04
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
pH
HX
N age

Figure 4. Cathepsin D standard curve: linearity of absorbance versus tyrosine concentration. Tyrosine dissolved in 1 ml 5% trichloroacetic acid was mixed with 2 ml 0.5 M NaOH after which 0.66 N Folin-Cioculteau phenol reagent was added and the solution mixed. The absorbance at 660 nm was determined after 15 min. Values shown are from a representative experiment.

Linear correlation coefficient (r) = .999.

Regression slope (m) = .019.

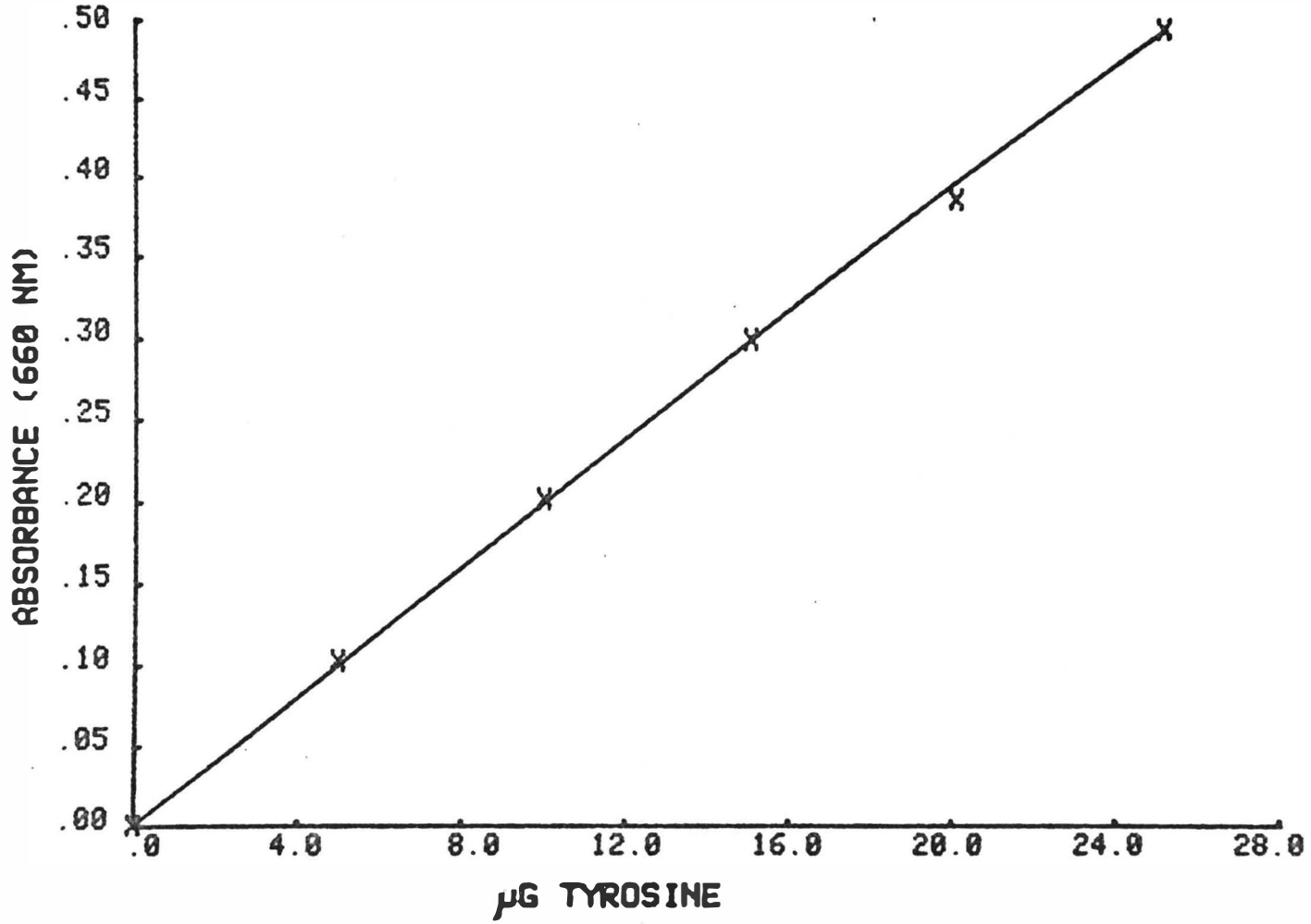

.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
.00
ABSORBANCE (660 NM)
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
µG TYROSINE

Figure 5. Effect of pepstatin and leupeptin on cathepsin D activity. Purified cathepsin D (0-0.75 ug) was incubated alone, in the presence of leupeptin (2.5 ug) or in the presence of pepstatin (0.5 ug - 2.5 ug). Test samples (125 ul total volume) were incubated with 125 ul 1.35 M sodium acetate buffer and 0.5 ml 4% acid-denatured hemoglobin (final pH = 3.4) for 1 h at 37°. Cathepsin D activity expressed as ug tyrosine equivalents/h. Values shown are from a representative experiment.

A = Cathepsin D alone.

B = Cathepsin D plus 2.5 ug leupeptin.

C = Cathepsin D plus 0.5 ug pepstatin.

D = Cathepsin D plus 2.5 ug pepstatin.

r = .999.

m = 13.

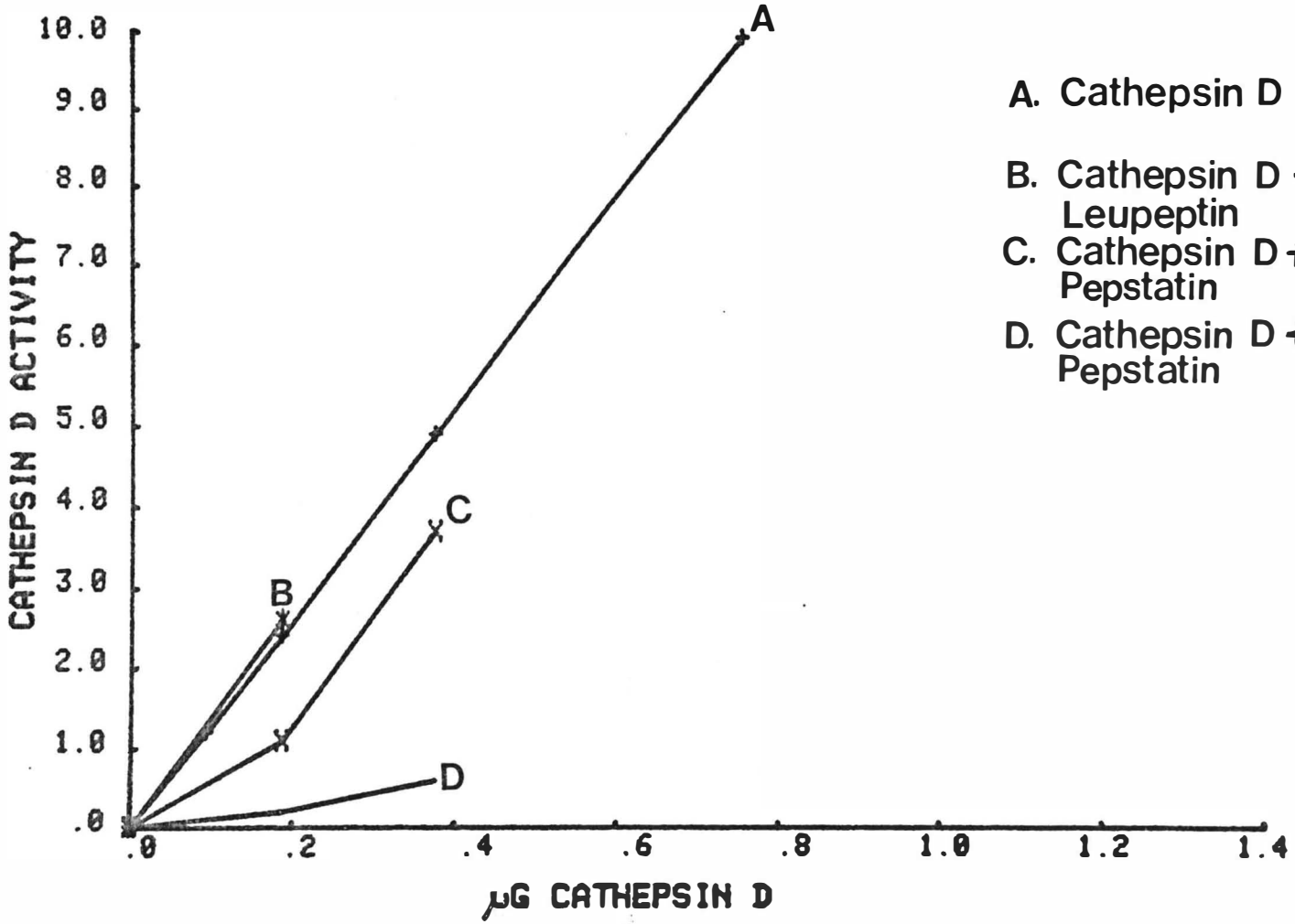

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
.0
CATHEPSIN D ACTIVITY
.0
.2
.4
.6
.8
1.0
1.2
1.4
µG CATHEPSIN D
A
B
C
D
A. Cathepsin D
B. Cathepsin D + 2.5
Leupeptin
C. Cathepsin D + 0.5
Pepstatin
D. Cathepsin D + 2.5
Pepstatin

Figure 6. Cathepsin D concentration curves using cartilage homogenates. Five % cartilage homogenates in .005 M sodium phosphate buffer, pH 6.5, were incubated with 125 ul 1.35 M sodium acetate buffer and 0.5 ml 4% acid-denatured hemoglobin (final pH = 3.4) for 1 h at 37°. Cathepsin D activity expressed as ug tyrosine equivalents/h. Values are from a representative experiment.

Hx = Hypophysectomized rats injected for four days with alkaline saline alone.

Hx + Pepstatin = Hx rat homogenates incubated with 2.5 ug pepstatin.

Hx + GH_{500} = Hx rats injected for four days with 500 ug bGH dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

N age + pepstatin = N age homogenates incubated with 2.5 ug pepstatin.

N wt = Normal rats of comparable weight to Hx rats.

*PEP = pepstatin.

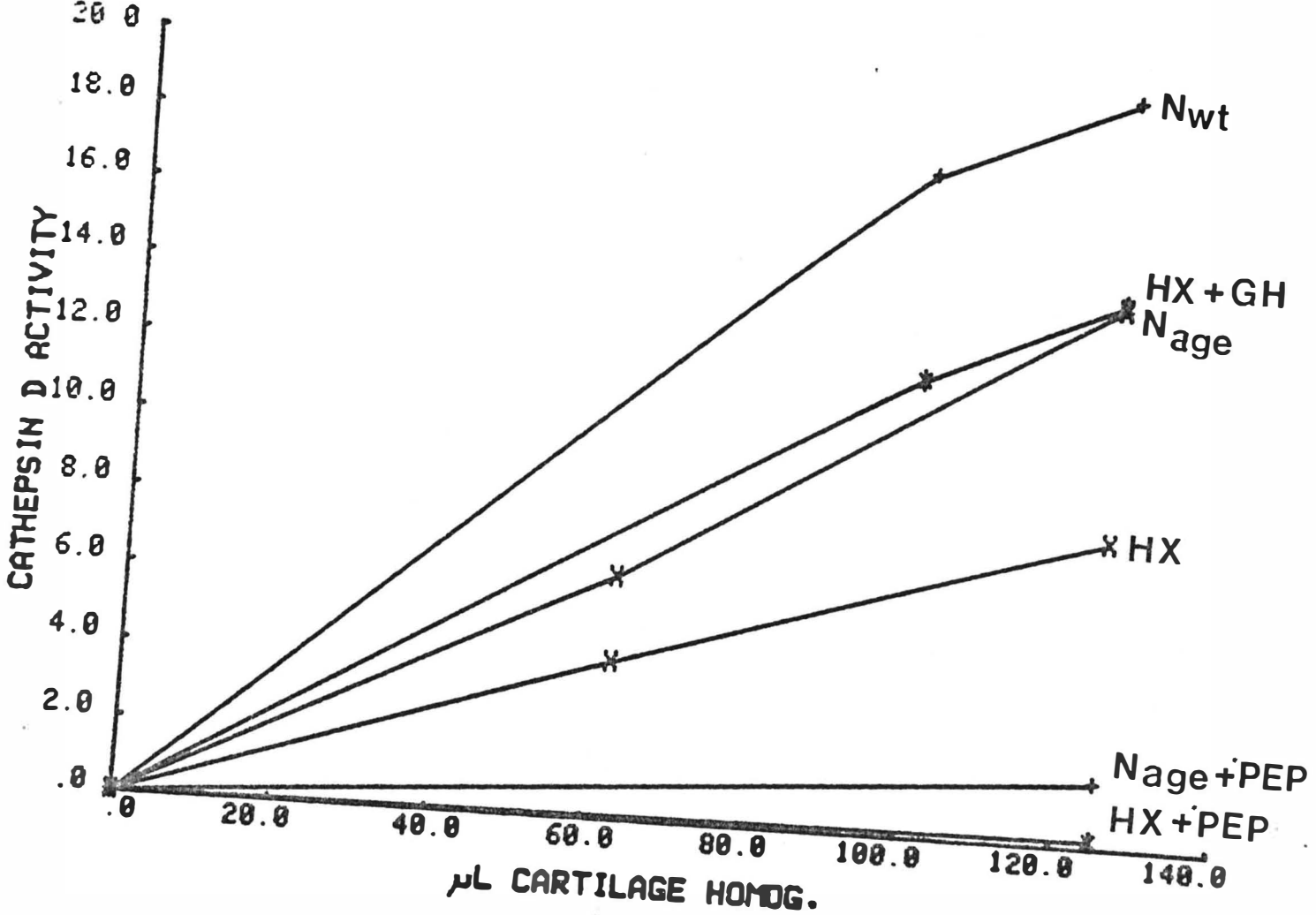
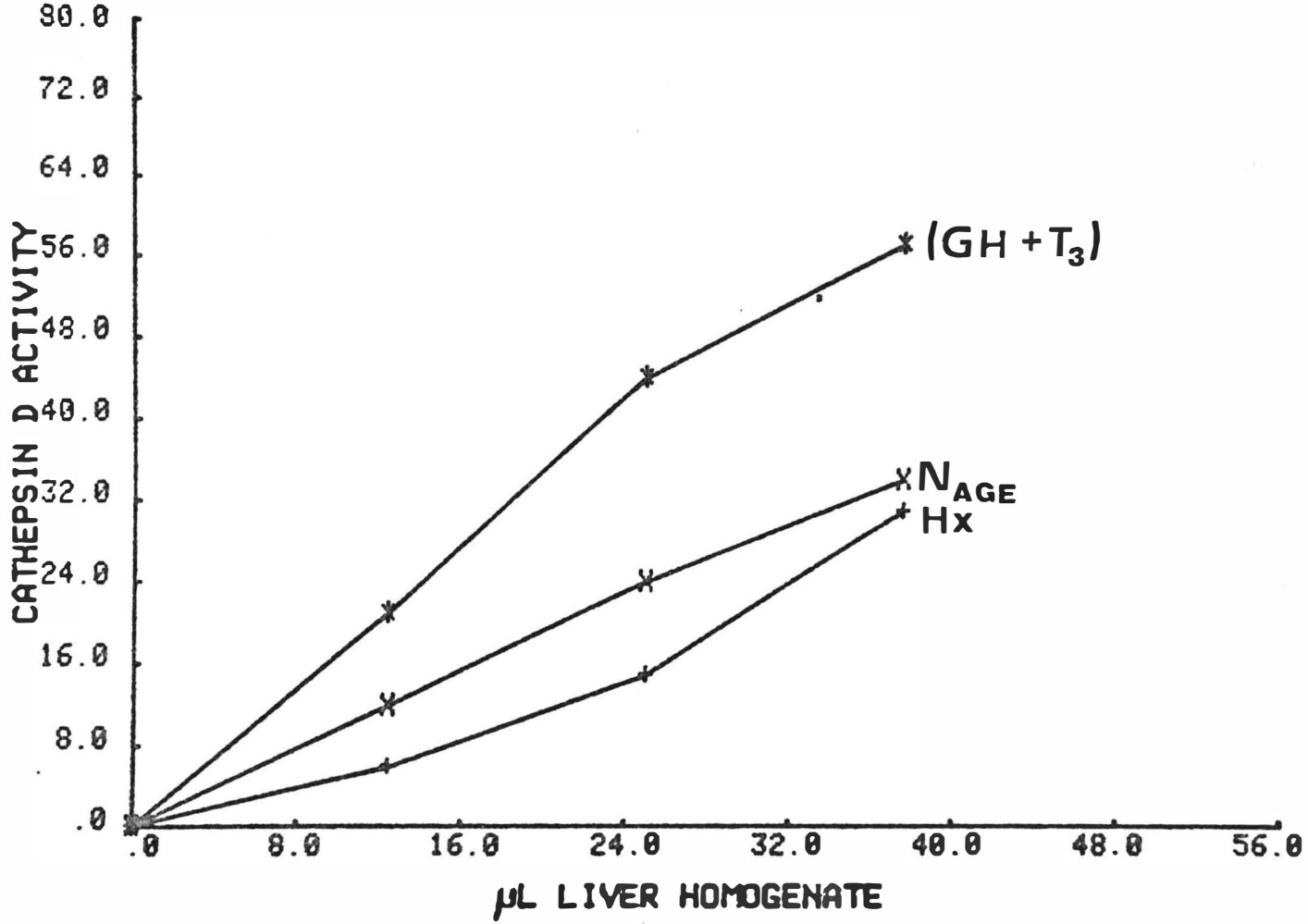

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
CATHEPSIN D ACTIVITY
.0
20.0
40.0
60.0
80.0
100.0
120.0
140.0
µL CARTILAGE HOMOG.
Nwt
HX + GH
Nage
HX
Nage + PEP
HX + PEP

Figure 7. Cathepsin D concentration curves using liver homogenates. Ten percent liver homogenates in 0.005 M sodium phosphate buffer, pH 6.5 were incubated with 125 ul 1.35 M sodium acetate buffer and 0.5 ml 4° acid-denatured hemoglobin (final pH = 3.4) for 30 min at 37°. Cathepsin D activity expressed as ug tyrosine equivalents/h. Values are from a representative experiment.

Hx = Hypophysectomized rats injected for four days with alkaline saline alone.

GH + T_3 = Hx rats injected for four days with 500 ug bGH and 12 ug T_3 (total doses) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.


80.0
72.0
64.0
56.0
48.0
40.0
32.0
24.0
16.0
8.0
.0
CATHEPSIN D ACTIVITY
(GH + T3)
NAGE
Hx
.0
8.0
16.0
24.0
32.0
40.0
48.0
56.0
µL LIVER HOMOGENATE

differences in pH optimum, relative tissue activities and EDTA requirements. Preliminary experiments showed a pH optimum of 5.0 for cartilage (Fig. 9) and 5.5 for liver (Fig. 8), as expected from the literature (89a, b). In the standard assay, liver cathepsin B activity was determined using a reaction mixture of 250 ul 2% homogenate and 0.75 ml of 0.2 M sodium acetate buffer, pH 5.8 containing 1.33 nM EDTA and 1.5 nM dithiothreitol. After preincubation of the reaction mixture for 5 min at 37°, 25 ul of BANA (40 mg/ml dimethyl sulphoxide) was added to initiate the reaction. After 30 min incubation at 37°, the reaction was terminated by adding 1 ml Fast-Garnet color reagent. Preparation of Fast-Garnet color reagent has been described previously (90). Approximately 20 min after addition of color reagent, samples were clarified by centrifugation at 6,000 x g for 10 min. Absorbance at 520 nm was then determined. Cartilage cathepsin B activity was assayed using 250 ul of a 5% homogenate (w:v) and 0.75 ml of 0.2 M sodium acetate buffer, pH 5.0 with 10 nM EDTA and 1.5 nM dithiothreitol. Color reagent was added after the reaction mixture was incubated for 2 h. The activity of tissue samples was dependent upon tissue concentration as shown in Fig. 10 and 11.

Acid phosphatase activity was detected by hydrolysis of p-nitrophenyl phosphate at pH 5.0 as described by Barrett (91). Sample homogenate (50 ul 5% cartilage homogenate and 0.5% liver homogenate) was added to 250 ul .005 M sodium phosphate buffer (pH 6.5) and 1.2 ml 0.2 M sodium acetate buffer, pH 5.0. After a 10 min preincubation at 37° to denature glucose-6-phosphatase, the reaction was started by the addition of 0.5 ml 32 mM p-nitrophenyl phosphate dissolved in water yielding a final 8 mM p-nitrophenyl phosphate reaction solution. After 30 min reaction at 37°, the reaction was terminated by the addition of 2.0 ml cold

Figure 8. Effect of pH on liver cathepsin B activity. Two percent liver homogenates (w:v) were incubated with 0.75 ml 0.2 M sodium acetate buffer, at various pH, containing 1.33 nM EDTA and 1.5 mM dithiothreitol. After 5 min preincubation at 37°, 25 ul of BANA (40 mg/ml dimethyl sulphoxide) was added to initiate the reaction. After 30 min incubation at 37°, the reaction was terminated by adding 1 ml Fast-Garnet color reagent. Activity expressed as mmoles BANA hydrolyzed $\times 10^{-1}$/h. Values are the mean of two experiments.

Hx = Hypophysectomized rats injected for four days with alkaline saline alone.

Hx-GH_{500} = Hx rats injected for four days with 500 ug bGH (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

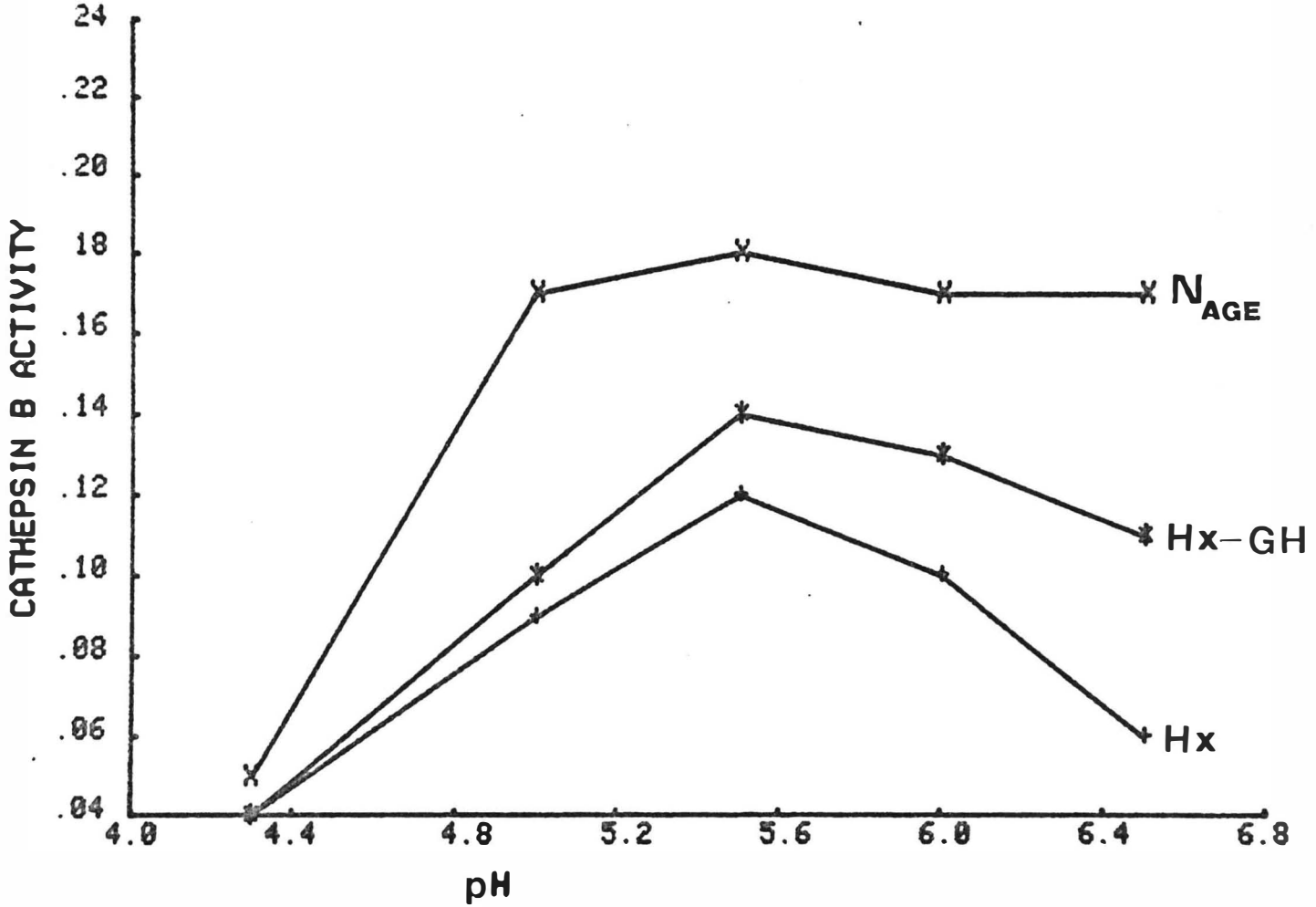

CATHEPSIN B ACTIVITY
24
.22
.20
18
.16
.14
.12
.10
.08
.06
.04
4.0
4.4
4.8
5.2
5.6
6.0
6.4
6.8
pH
N AGE
Hx-GH
Hx

Figure 9. Effect of pH on cartilage cathepsin B activity. Five percent cartilage homogenates were incubated with 0.75 ml 0.2 M sodium acetate buffer, at various pH, containing 10 mM EDTA and 1.5 mM dithiothreitol. After 5 min preincubation at 37°, 25 ul of BANA (40 mg/ml dimethyl sulphoxide) was added to initiate the reaction. After 2 h incubation at 37° the reaction was terminated by adding 1 ml Fast-Garnet color reagent. Activity expressed as umole BANA hydrolyzed/ml/h. Values are the mean of two experiments.

N_{15} = Normal rats about 15 weeks old.

N_{25} = Normal rats about 25 weeks old.

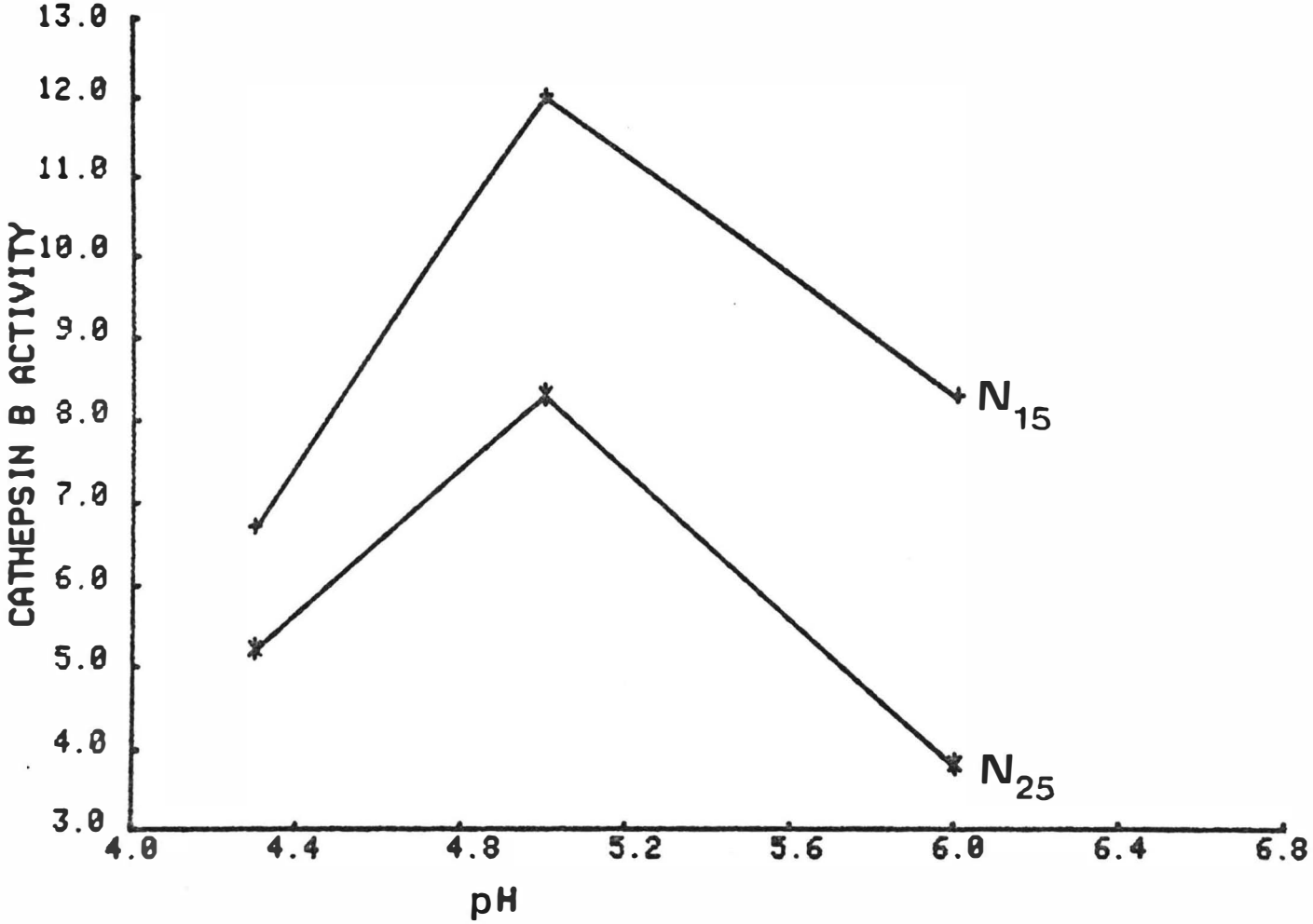

13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
CATHEPSIN B ACTIVITY
4.0
4.4
4.8
5.2
5.6
6.0
6.4
6.8
pH
N_{15}
N_{25}

Figure 10. Cathepsin B concentration curves using cartilage homogenates. Cartilage homogenates (0-125 ul brought to 250 ul with .005 M sodium phosphate buffer, pH 6.5) were incubated with 0.75 ml 0.2 M sodium acetate pH 5.0 containing 10 mM EDTA and 1.5 mM dithiothreitol. After 5 min preincubation at 37°, 25 ul of BANA (40 mg/ml dimethyl sulphoxide) was added to initiate the reaction. After 2 h incubation at 37°, the reaction was terminated by adding 1 ml Fast-Garnet color reagent. Activity expressed as umole BANA hydrolyzed/h. Values are from a representative experiment.

Hx = Hypophysectomized rats injected for four days with alkaline saline alone.

N age = Normal rats of comparable age to Hx rats.

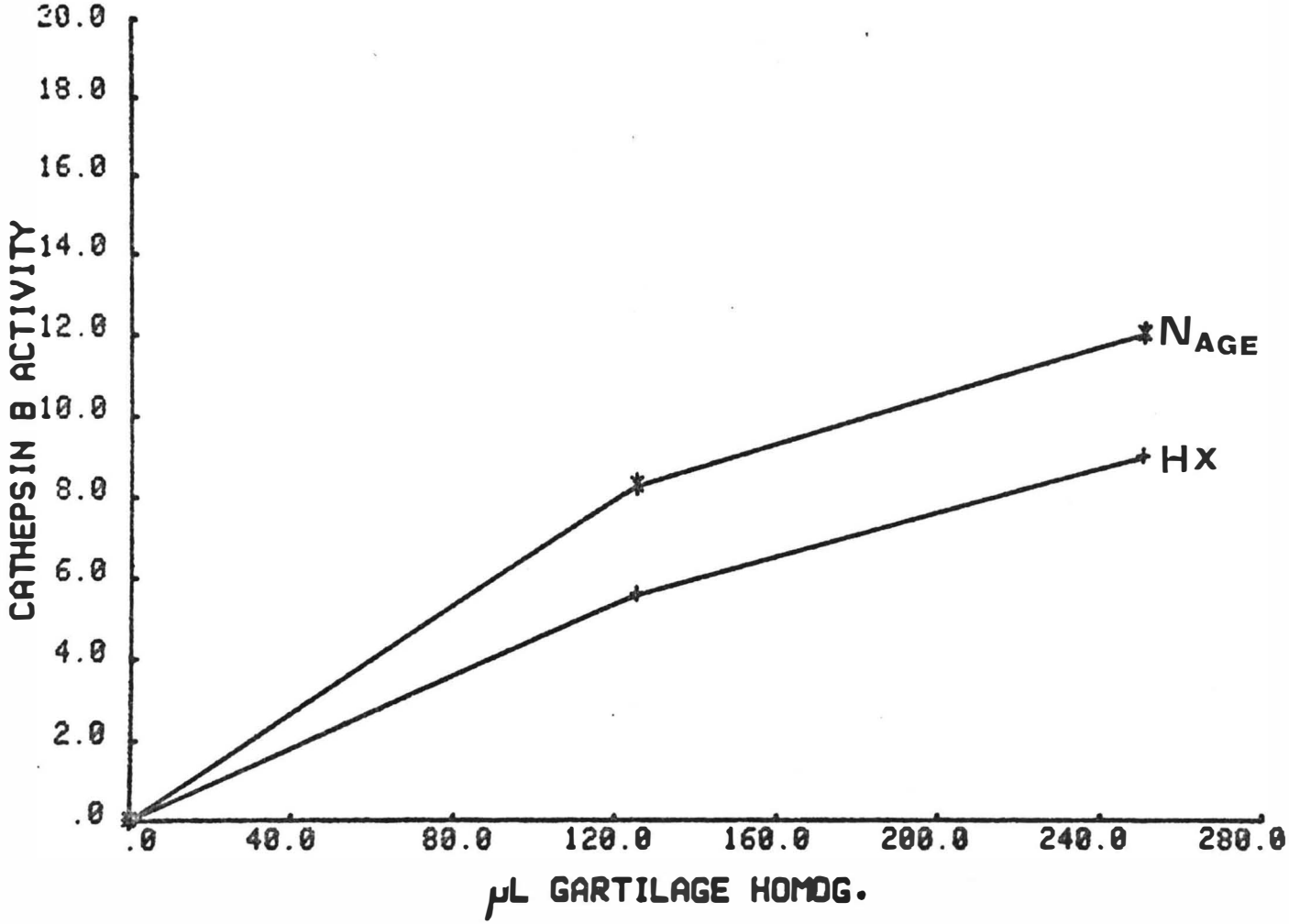

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
CATHEPSIN B ACTIVITY
.0
40.0
80.0
120.0
160.0
200.0
240.0
280.0
µL CARTILAGE HOMOG.
NAGE
HX

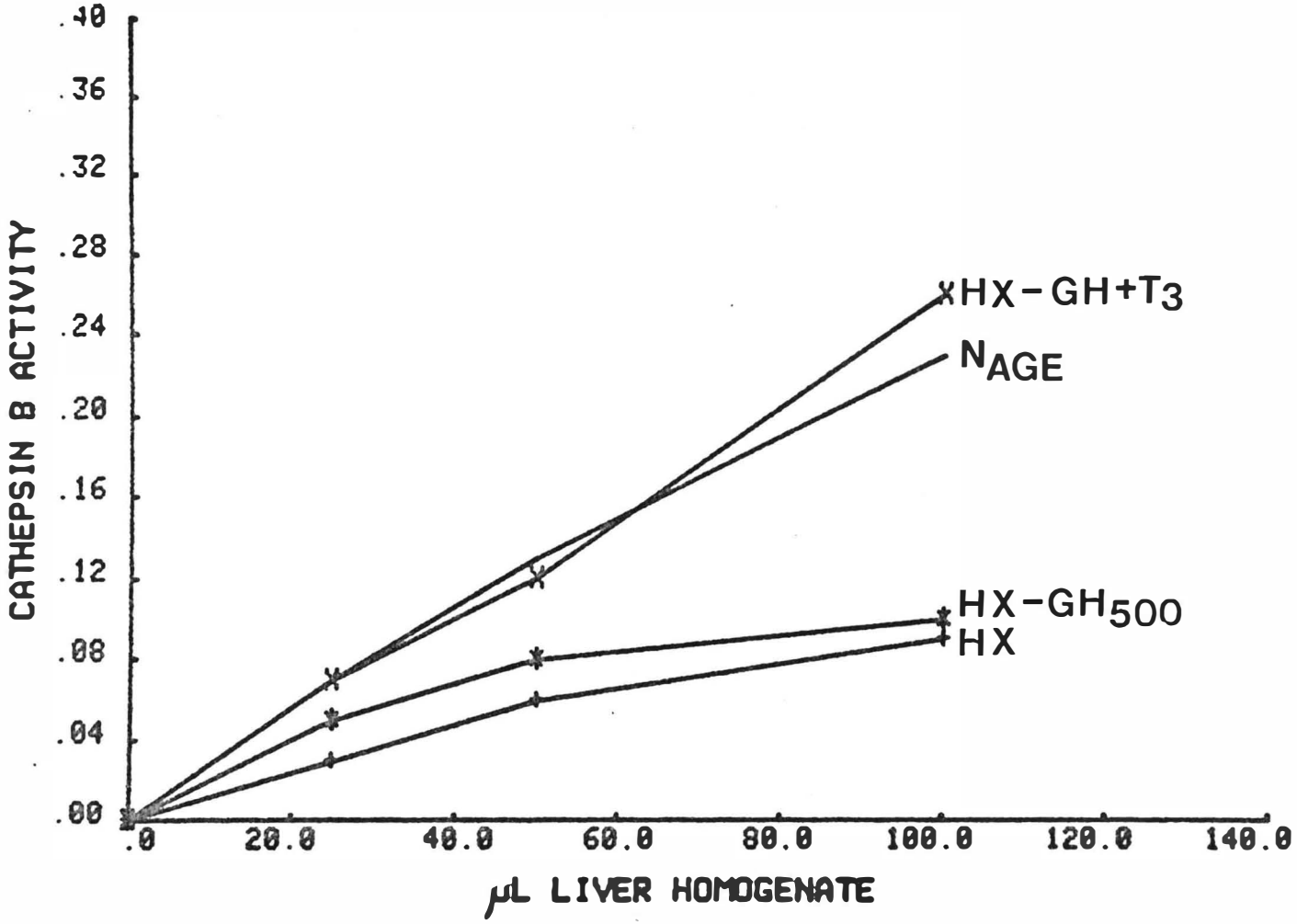
Figure 11. Cathepsin B concentration curves using liver homogenates. Liver homogenates (0-100 ul, 10% homogenates brought to 250 ul with .005 M sodium phosphate buffer, pH 6.5) were incubated with 0.75 ml 0.2 M sodium acetate buffer, pH 5.5 containing 1.33 mM EDTA and 1.5 mM dithiothreitol. After 5 min preincubation at 37°, 25 ul of BANA (40 mg/ml dimethyl sulphoxide) was added to initiate the reaction. After 30 min incubation at 37° the reaction was terminated by adding 1 ml Fast-Garnet color reagent. Activity expressed as umoles BANA hydrolyzed x 10^{-2}/h. Values are from a representative experiment.

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-GH_{500} = Hx rats injected for four days with 500 ug bGH (total dose) dissolved in alkaline saline.

Hx-(GH + T_3) = Hx rats injected for four days with 500 ug bGH and 12 ug T_3 (total doses) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.


.40
.36
.32
.28
.24
.20
.16
.12
.08
.04
.00
CATHEPSIN B ACTIVITY
.0
20.0
40.0
60.0
80.0
100.0
120.0
140.0
µL LIVER HOMOGENATE
HX-GH+T3
NAGE
HX-GH500
HX

Tris-phosphate reagent (1 M tris-HCl in 0.4 M K_2HPO_4, pH 8.5). Samples were centrifuged at 6,000 x g for 10 min to remove particulate material and the supernatants were read on a spectrophotometer at 420 nm. Preliminary experiments showed that maximal activity occurred at pH 5.0 for both cartilage and liver homogenates in agreement with the literature (91b) (Fig. 12 and 13). However, since liver tissue was approximately 10 times more active than cartilage tissue on a wet weight basis, different concentrations of homogenates were used in the reaction mixture as shown above. P-nitrophenol was used as standard. P-nitrophenol concentration was proportional to absorbance at 420 nm, as shown in Fig. 14. The activity of tissue homogenates showed first order kinetics (Fig. 15 and 16).

Enzyme activities were expressed on the basis of tissue weight, DNA content and protein content. DNA was determined by the diphenylamine reaction as described by Schneider (92). Calf thymus DNA was used as standard. Protein was determined by the Biuret procedure (93) using bovine serum albumin as the protein standard.

C. Determination of Tissue Wet Weights, Protein Concentration, and DNA Concentration

1. Determination of tissue wet weight - Liver and cartilage were removed from decapitated animals and placed in cold saline (0.9% NaCl) until homogenization. After tissues were cleaned of adhering material, the tissue was blotted and then weighed to the nearest mg on a Mettler balance.

2. Determination of DNA and protein content - Tissue homogenate protein and DNA were separated by differential solubility in hot TCA solution as described by Schneider (92). Five percent cartilage homogen-

Figure 12. Effect of pH on cartilage acid phosphatase activity. One percent cartilage homogenates (300 ul) were added to 1.2 ml 0.2 M sodium acetate buffer of various pH. After 10 min preincubation the reaction was initiated by the addition of 0.5 ml 32 mM p-nitrophenyl phosphate dissolved in water. After 30 min the reaction was terminated by addition of 2.0 ml cold Tris-phosphate reagent, pH 8.5. Activity expressed as mg p-nitrophenyl phosphate hydrolyzed/ml/h. Values are the mean of two experiments.

Hx = Hypophysectomized rats injected for four days with alkaline saline alone.

N age = Normal rats of comparable age to Hx rats.

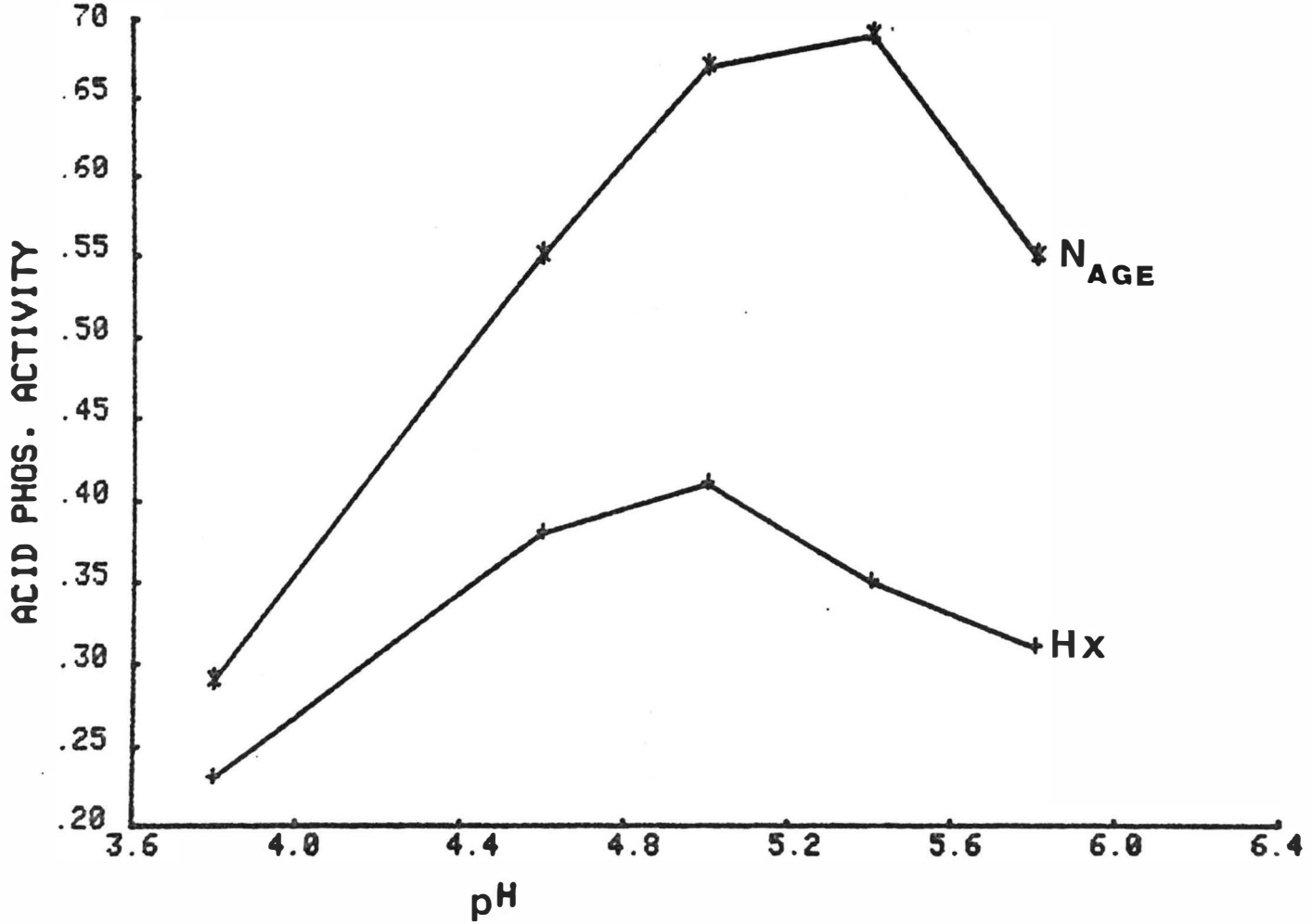

ACID PHOS. ACTIVITY
.70
.65
.60
.55
.50
.45
.40
.35
.30
.25
.20
3.6
4.0
4.4
4.8
5.2
5.6
6.0
6.4
pH
N AGE
Hx

Figure 13. Effect of pH on liver acid phosphatase activity. One percent liver homogenates (300 ul) were added to 1.2 ml 1.35 M sodium acetate buffer of various pH. After 10 min preincubation at 37°, the reaction was initiated by the addition of 0.5 ml 32 mM p-nitrophenyl phosphate dissolved in water. After 30 min incubation at 37° the reaction was terminated by the addition of cold Tris-phosphate reagent, pH 8.5. Activity was expressed as mg p-nitrophenyl phosphate hydrolyzed/h. Values are the mean of two experiments.

Hx = Hypophysectomized rats injected for four days with solvents alone.

Hx-GH_{500} = Hx rats injected for four days with 500 mg bGH (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

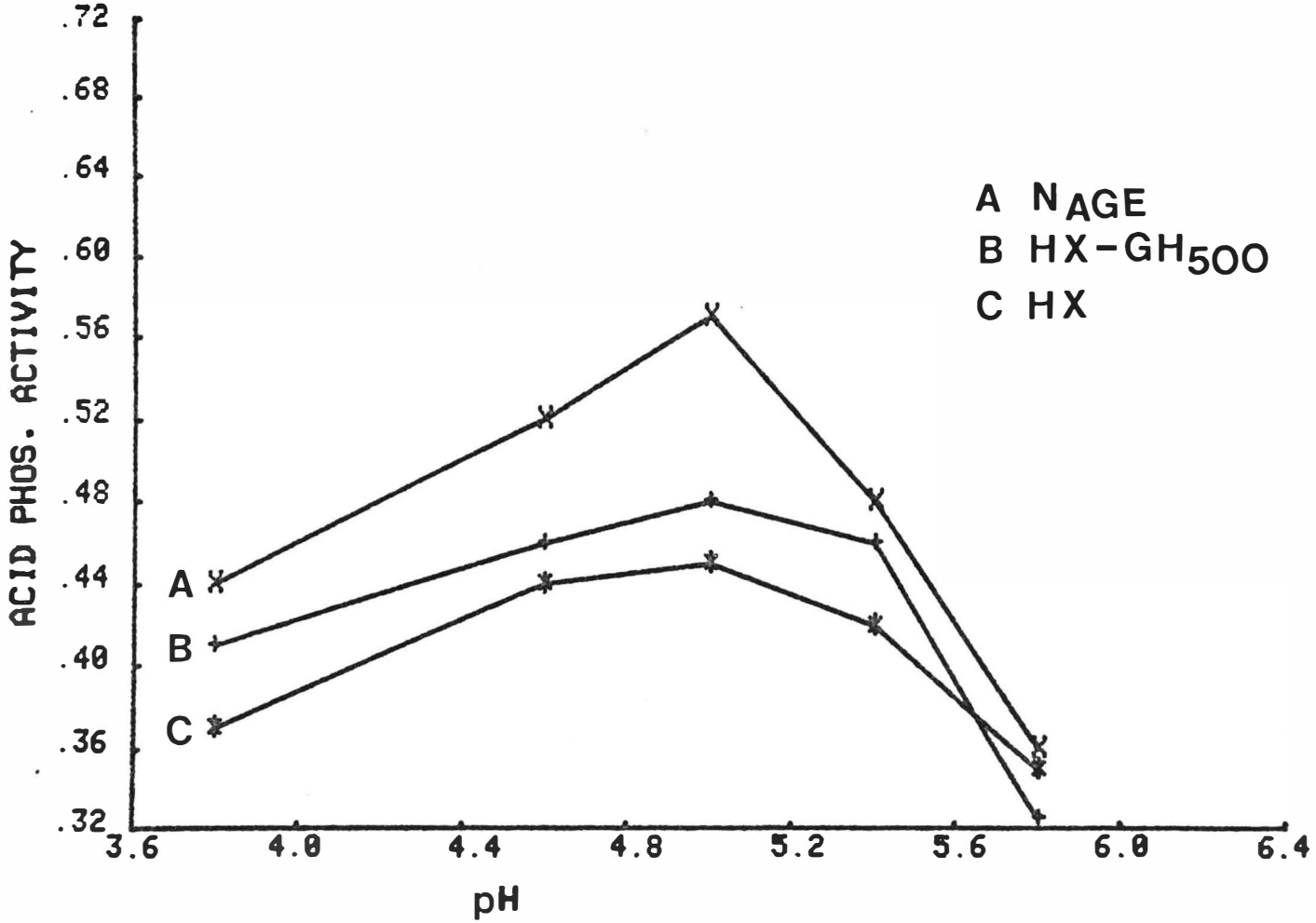

ACID PHOS. ACTIVITY
.72
.68
.64
.60
.56
.52
.48
.44
.40
.36
.32
3.6
4.0
4.4
4.8
5.2
5.6
6.0
6.4
pH
A
B
C
A N_{AGE}
B HX-GH_{500}
C HX

Figure 14. Acid phosphatase assay standard curve. P-nitrophenol (0-25 ug in 300 ul .005 M sodium phosphate, pH 6.5) was added to 1.2 ml 0.2 M sodium acetate buffer (pH 5.0), 0.5 ml 32 mM p-nitrophenyl phosphate dissolved in water, and 2.0 ml cold tris-phosphate reagent. Absorbance was measured at 420 nm. Values are from a representative experiment.

r = .999.

m = .012.

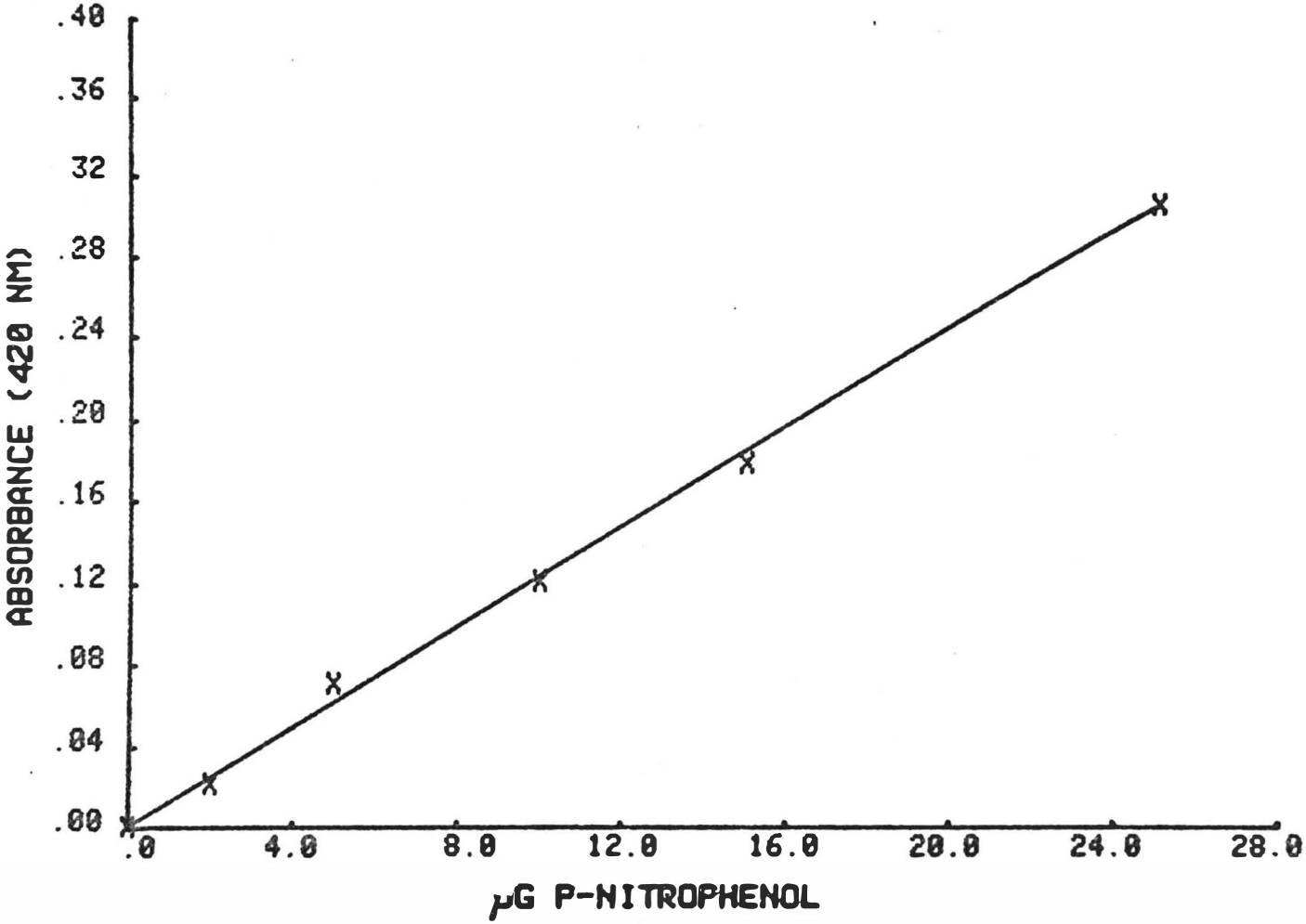

.40
.36
32
.28
.24
.20
.16
.12
.08
.04
.00
ABSORBANCE (420 NM)
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
µG P-NITROPHENOL

Figure 15. Dependence of acid phosphatase activity on cartilage homogenate concentration. Cartilage homogenate (0-200 ul Q S to 300 ul with .005 M sodium phosphate buffer, pH 6.5) were added to 1.2 ml sodium acetate buffer pH 5.0 and preincubated for 10 min at 37°. The reaction was initiated by the addition of 0.5 ml 32 mM p-nitrophenyl phosphate dissolved in water. After 2 h incubation at 37° the reaction was terminated by the addition of cold Tris-phosphate reagent, pH 8.5. Activity was expressed as mg p-nitrophenyl phosphate hydrolyzed/h. Values are from a representative experiment.

Hx = Hypophysectomized rats injected for four days with alkaline saline.

N wt = Normal rats of comparable weight to Hx rats.

N age = Normal rats of comparable age to Hx rats.

N_{48} = Normal rats about 48 weeks old.

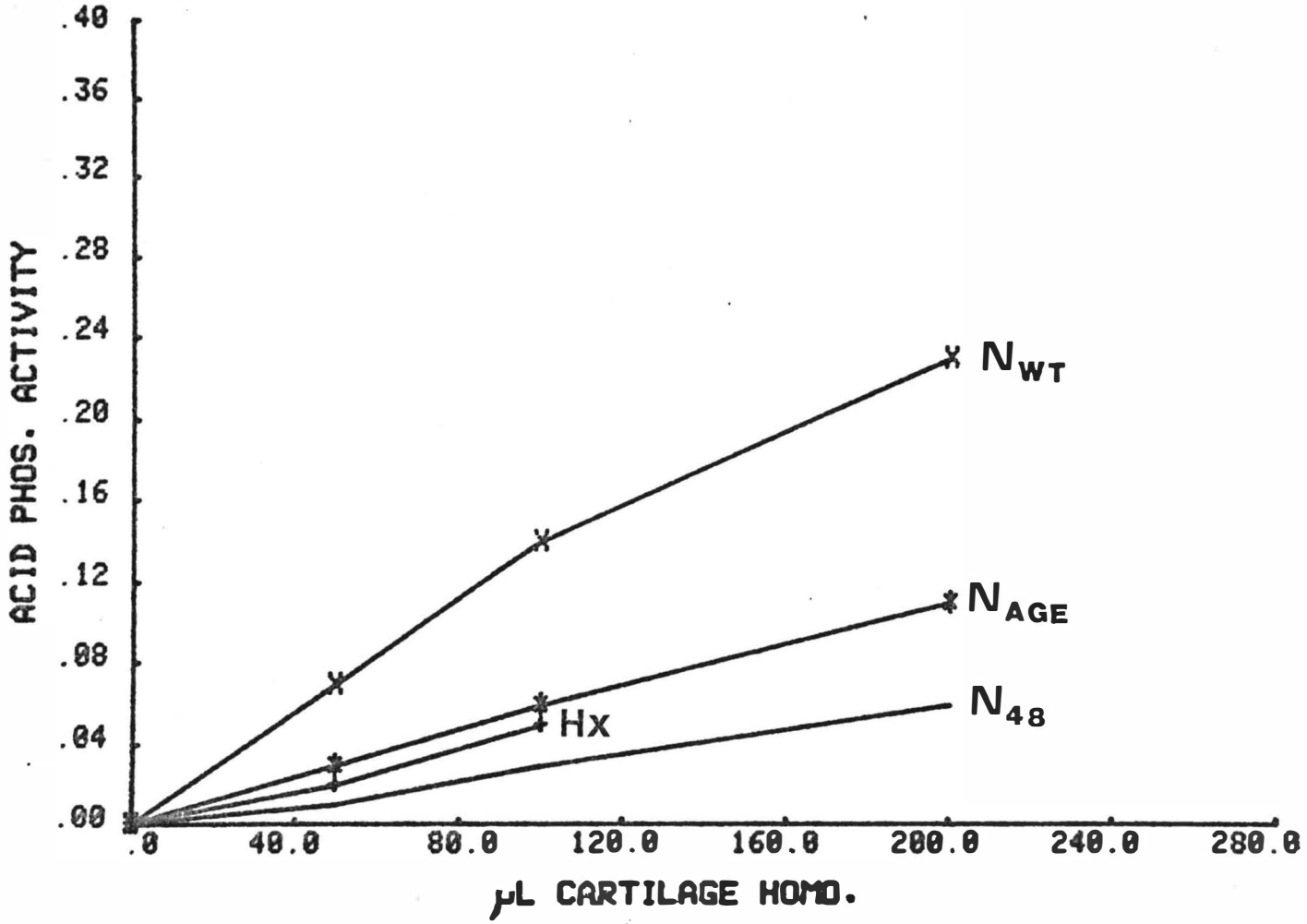
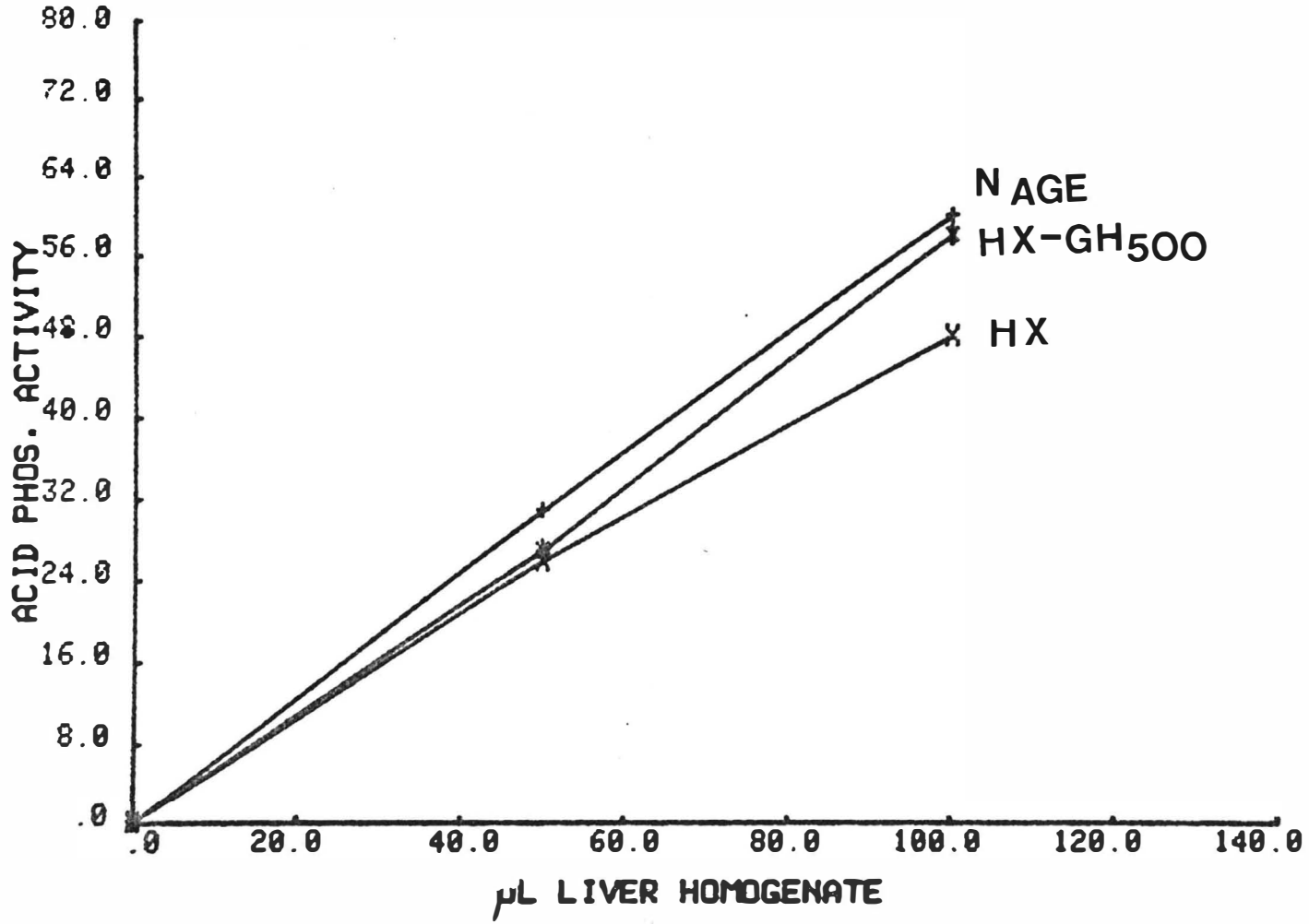

.40
.36
.32
.28
.24
.20
.16
.12
.08
.04
.00
ACID PHOS. ACTIVITY
.0
40.0
80.0
120.0
160.0
200.0
240.0
280.0
µL CARTILAGE HOMO.
N_WT
N_AGE
N_48
Hx

Figure 16. Dependence of acid phosphatase activity on liver homogenate concentrations. Liver homogenates (0-100 ul 0.5% liver homogenate brought to 300 ul with .005 M sodium phosphate buffer, pH 6.5) were added to 1.2 ml sodium acetate buffer pH 5.0 and preincubated 10 min at 37°. The reaction was initiated by the addition of 0.5 ml 32 mM p-nitrophenyl phosphate dissolved in water. After 30 min the reaction was terminated by the addition of cold Tris-phosphate reagent, pH 8.5. Activity was expressed as mg p-nitrophenyl phosphate hydrolyzed/h. Values are from a representative experiment.

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-GH_{500} = Hypophysectomized rats injected for four days with 500 ug bGH (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.


80.0
72.0
64.0
56.0
48.0
40.0
32.0
24.0
16.0
8.0
.0
ACID PHOS. ACTIVITY
.0
20.0
40.0
60.0
80.0
100.0
120.0
140.0
µL LIVER HOMOGENATE
N AGE
HX-GH500
HX

ates or 10% liver homogenates were placed in an equal volume of 10% TCA. After standing in ice for 20 min, the samples were centrifuged at 6,000 x g for 10 min and the supernatants were discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellets and mixed vigorously. Samples were then placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were carefully removed. Supernatants were then used for DNA determination and the sample pellet for protein determinations.

DNA content was determined by the diphenylamine reaction. Diphenylamine (1.0 g) was dissolved in 100 ml of glacial acetic acid and 2.75 ml concentrated sulfuric acid. Two ml of the diphenylamine reagent was added to 1 ml of sample supernatant or to 1 ml DNA standard dissolved in 5% TCA. Samples were then placed in a dry bath at 100° for 15 min. After cooling, absorbance was determined at 600 nm. Absorbance was linear with DNA content as shown in Fig. 17. The proportionality of tissue concentration to DNA content is shown in Table 1 and Fig. 18. The efficiency of the DNA recovery by the first hot TCA extraction is shown in Table 2.

Protein content was determined by the Biuret procedure. Sample pellets were dissolved in 0.5 ml 0.1 N NaOH. Two ml of biuret reagent were added to samples or to alkaline solutions of bovine serum albumin standards. After 20 min, absorbance at 540 nm was determined. Absorbance was linear with increasing concentration of bovine serum albumin as shown in Fig. 19. The proportionality of tissue concentration to protein concentration is shown in Fig. 20 and Table 3.

D. Statistical Analysis - Statistical analysis was performed

Figure 17. DNA standard curve. Calf thymus DNA (0-123 ug dissolved in 1 ml 5% trichloroacetic acid) was added to 2 ml diphenylamine reagent and incubated for 15 min at 100° in a dry bath. After cooling, absorbance was determined at 600 nm. Values are from a representative experiment.

r = .999.

m = .00127.

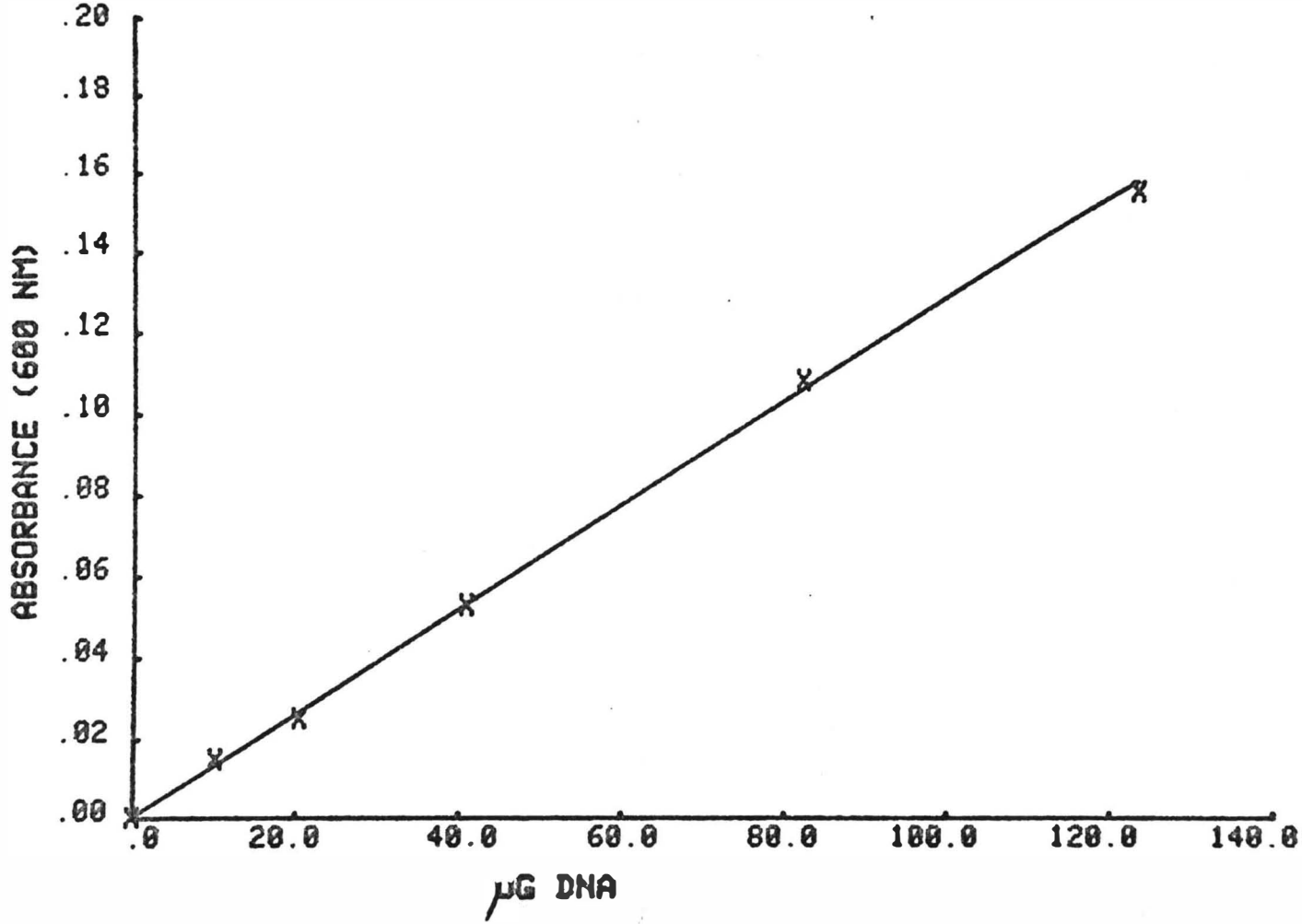

.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
.00
ABSORBANCE (600 NM)
.0
20.0
40.0
60.0
80.0
100.0
120.0
140.0
µG DNA

Table 1

Proportionality of Cartilage Homogenate Concentration and DNA Content

Experimental Group	200 ul 5% homogenate DNA content (ug)	400 ul 5% homogenate DNA content (ug)
Hx	11.3 ± 1.4 (4)	25.5 ± 1.5 (4)
Hx-(GH + T_3)	12.7 ± .9 (3)	25.7 ± 1.8 (3)
N age	7.5 ± .5 (2)	13.5 ± .3 (3)

Five percent cartilage homogenates (200 ul or 400 ul) were placed in an equal volume of 10% TCA and allowed to stand in ice for 20 min. Samples were centrifuged at 6,000 x g for 10 min and the supernatants wer discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellets and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were carefully removed. Supernatants were then used for DNA determinations using the diphenylamine reaction. Results are expressed as mean ± SE (number of samples).

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-(GH + T_3) = Hx rats injected for four days with 500 ug bGH and 12 ug T_3 (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

Figure 18. Proportionality of liver homogenate concentration and DNA content. Ten percent liver homogenates (75-300 ul) were placed in an equal volume of 10% TCA and allowed to stand in ice for 20 min. Samples were centrifuged at 6,000 x g for 10 min and the supernatants were discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellets and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were carefully removed. Supernatants were then used for DNA determinations using the diphenylamine reaction. Values are from a representative experiment.

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-(GH + T_3) = Hx rats injected for four days with 500 ug bGH and 12 ug T_3 (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

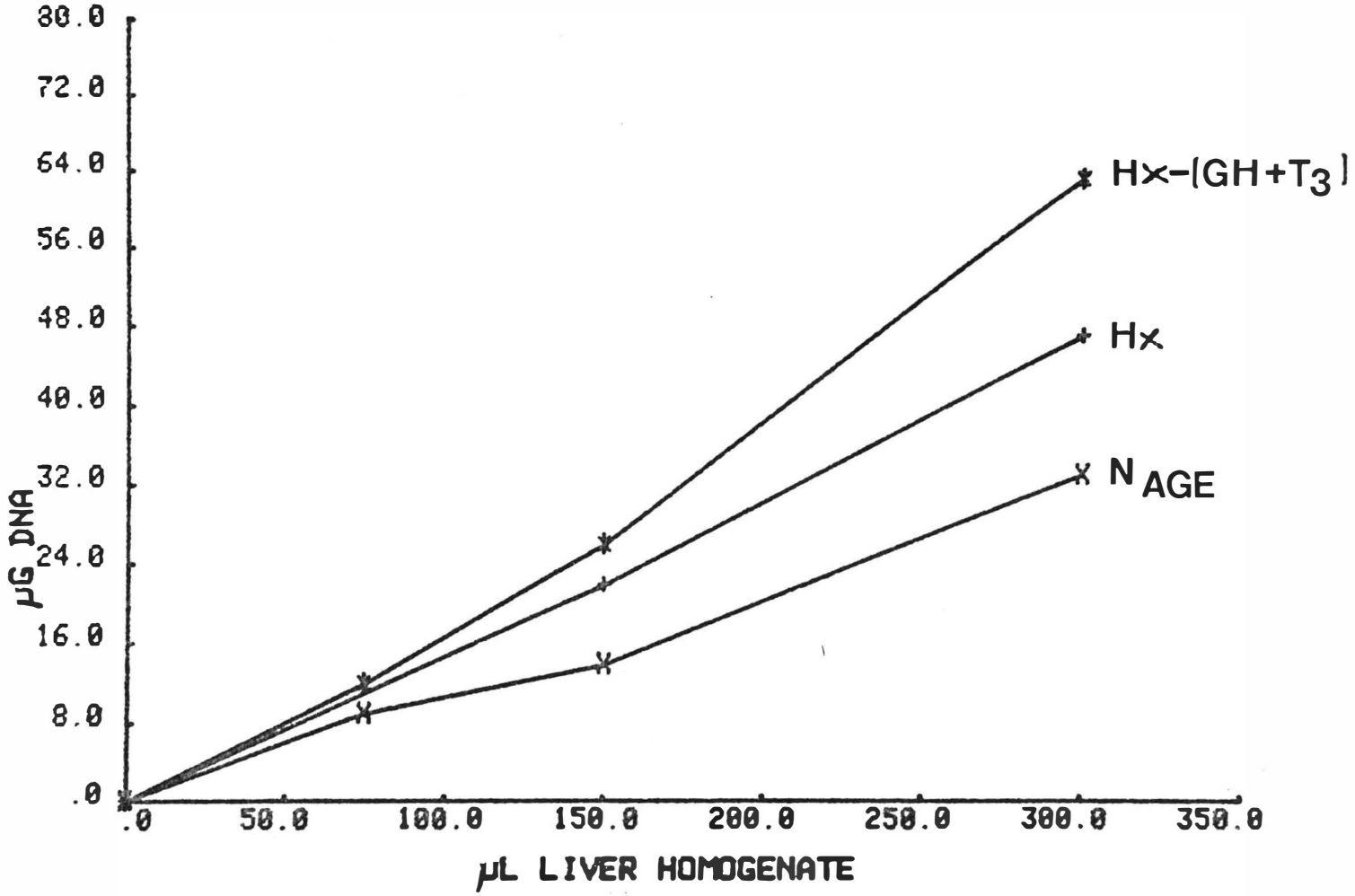

80.0
72.0
64.0
56.0
48.0
40.0
32.0
24.0
16.0
8.0
.0
µG DNA
.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
µL LIVER HOMOGENATE
Hx-(GH+T3)
Hx
N AGE

Table 2

Recovery of Cartilage DNA by Hot Trichloroacetic Acid Extraction

Experimental Group	DNA (ug extracted) 1st hot TCA wash	DNA (ug extracted) 2nd hot TCA wash	% DNA extracted in 2nd hot TCA wash
N wt	62 ± 3 (4)	5.1 ± .3 (2)	8%
N age	40 ± 2 (3)	~0 (2)	~0%
N_{48} wk	30 ± 2 (7)	3 ± 3 (2)	9%

Five percent cartilage homogenates (400 ul) were placed in 400 ul 10% TCA and allowed to stand on ice for 20 min. Samples were centrifuged at 5,000 x g for 10 min and the supernatants were discarded. This TCA precipitation was repeated once after which 1.0 ml 5% TCA was added to sample pellets and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were carefully removed. These supernatants were used for the 1st hot TCA wash DNA determinations. One ml 5% TCA was added to pellets and the hot TCA extraction procedure was repeated. The 2nd hot TCA wash supernatants were also used for DNA determinations. DNA was determined by the diphenylamine reaction. Results are expressed as mean ± SE (number of samples).

N wt = Normal rats about 3-4 weeks old.

N age = Normal rats about 6-8 weeks old.

N_{48} wk = Normal rats about 48 weeks old.

Figure 19. Protein standard curve. Bovine serum albumin (1-5 mg) was dissolved in 0.5 ml 0.1 N NaOH. Two ml biuret reagent were added to samples, and mixed. After 20 min absorbance at 540 nm was determined. Values are from a representative experiment.

r = .999.

m = .077.

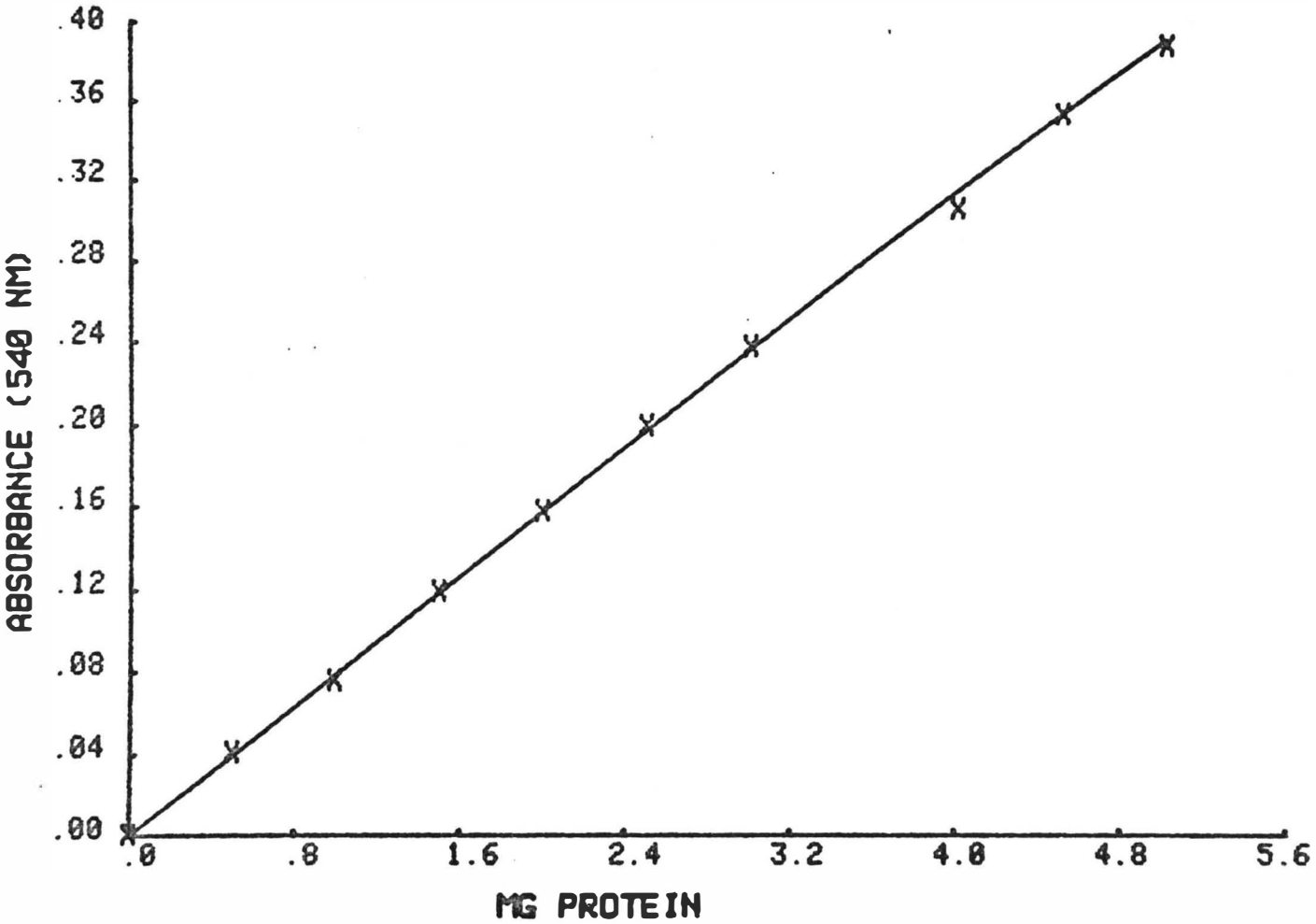

ABSORBANCE (540 NM)
.40
.36
.32
.28
.24
.20
.16
.12
.08
.04
.00
.0
.8
1.6
2.4
3.2
4.0
4.8
5.6
MG PROTEIN

Figure 20. Proportionality of liver homogenate concentration and protein content. Ten percent homogenates (75 ul - 200 ul) were placed in an equal volume of 10% TCA and allowed to stand on ice for 20 min. Samples were centrifuged at 6,000 x g for 10 min and the supernatants were discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellets and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were removed. Pellets were dissolved in 0.1 N NaOH and used for protein determinations using the Biuret procedure. Values are from a representative experiment.

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-(GH + T3) = Hx rats injected for four days with 500 ug bGH and 12 ug T3 (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

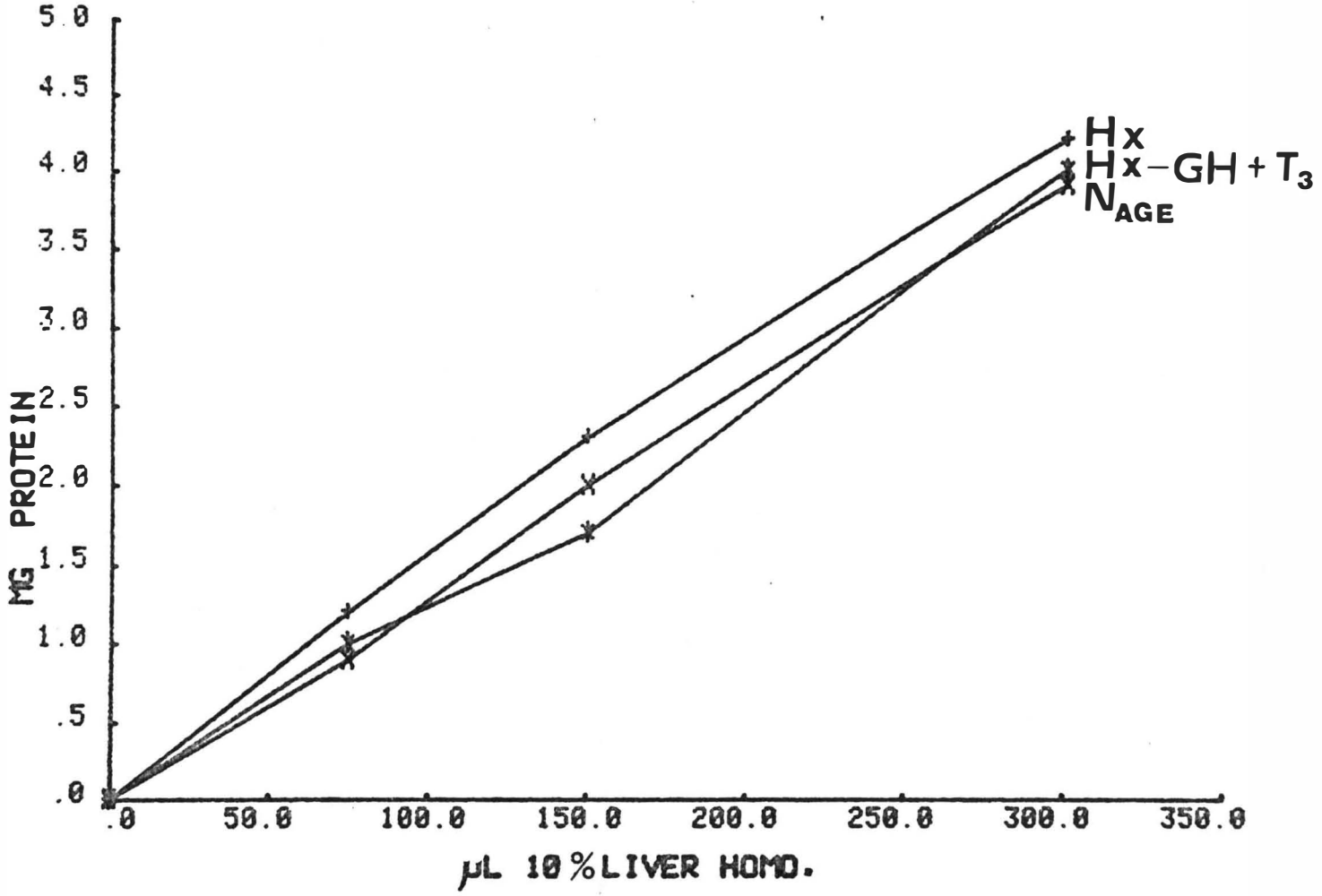

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
.0
MG PROTEIN
.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
µL 10% LIVER HOMO.
Hx
Hx−GH + T3
NAGE

Table 3

Proportionality of Cartilage Homogenate Concentration and Protein Content

Experimental Group	200 ul 5% homogenate protein content (ug)	400 ul 5% homogenate protein content (ug)
Hx	1.1 + .1 (4)	2.3 + .1 (4)
Hx-(GH + T_3)	1.3 + .1 (3)	2.7 + .4 (3)
N age	1.2 + .1 (2)	2.4 + .2 (2)

Five percent cartilage homogenates (200 ul or 400 ul) were placed in an equal volume of 10% TCA and allowed to stand on ice for 20 min. Samples were centrifuged at 6,000 x g for 10 min and supernatants were discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellet and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were removed. Pellets were dissolved in 0.5 ml 0.1 N NaOH and used for protein determinations using the Biuret procedure. Results are expressed as the mean $\pm$ SE (number of samples).

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-(GH + T_3) = Hx rats injected for four days with 500 ug bGH and 12 ug T_3 (total dose) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

according to the method of Dunnett (85). First, a one-way analysis of variance was used in each experiment to determine if intergroup differences were statistically demonstrated. If significant differences ($P < 0.05$) were found, Dunnett's analysis was conducted to determine which groups were significantly different from the control group. Where appropriate, linear regression analysis was performed and correlation coefficients determined (r = linear correlation coefficient, m = regression slope).

Chapter IV

RESULTS

A. Growth Rates of Experimental Animals

1. Effect of hormone-treatment on rat growth - The effect of hormone-treatment on rat body weight, liver wet weight, and costal cartilage wet weight (from ribs 2-5) is shown in Table 4. GH (500 ug total dose) significantly enhanced rat body weight and costal cartilage weight compared to Hx controls. GH also caused a slight but statistically insignificant increase in liver wet weight. However, GH did not alter the ratio of cartilage weight or liver weight to rat body weight. T_3 injected with GH produced about the same effects as GH alone. T_3 injected alone did not significantly alter these parameters. The N wt group had the largest ratio of liver weight to rat body weight. The effect of hormone treatment on costal cartilage, total DNA and protein content is shown in Table 5. GH (125 ug/day for 4 days) enhanced both DNA and protein content over Hx controls, but only the change in DNA content was statistically significant. T_3 injected with GH gave about the same results as GH administered alone. T_3 injected alone had no effect on DNA and protein content compared to Hx controls. On the other hand, liver DNA was enhanced by T_3 and (GH + T_3) but not affected by GH alone (Table 6). Protein content was not significantly enhanced by hormonal treatment.

2. Change in rat growth rate with age - The change in rat body weight with age is shown in Table 7. Although normal rat body weight increased with age, the growth rate decreased with age. Hx rat

Table 4

Effect of Hormone Treatment on Rat Body Weight, Liver Wet Weight, and Costal Cartilage Wet Weight

Experimental Group	Rat Weight (g)	Cartilage Weight (mg)	Liver Weight (g)	$\frac{\text{Cartilage wt (g)}}{\text{Rat wt (g)}}$	$\frac{\text{Liver wt (g)}}{\text{Rat wt (g)}}$
Hx	113 ± 2 (43)	147 ± 4	3.7 ± .1	1.3×10^{-3}	3.3×10^{-2}
Hx-GH$_{50\ ug}$	119 ± 3 (14)	164 ± 9	3.7 ± .2	1.4×10^{-3}	3.1×10^{-2}
Hx-GH$_{500\ ug}$	128 ± 2 (33)**	169 ± 5**	4.0 ± .2	1.3×10^{-3}	3.1×10^{-2}
Hx-T_3	111 ± 4 (8)	149 ± 14	3.5 ± .2	1.3×10^{-3}	3.2×10^{-2}
Hx-(GH + T_3)	117 ± 2 (17)	165 ± 7*	3.9 ± .2	1.4×10^{-3}	3.3×10^{-2}
N wt	116 ± 4 (11)	144 ± 5	5.2 ± .3	1.2×10^{-3}	4.5×10^{-2}

Rat weights were determined immediately prior to sacrifice. Livers were perfused in situ with cold isotonic saline prior to removal. Costal cartilage (from ribs 3-6) and liver were cleaned of adhering tissue, dried on Whatman 3 mm filter paper and weighed.

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-GH$_{50\ ug}$ = Hx rats injected for four days with 50 ug bGH (total dose) dissolved in alkaline saline.

Hx-GH$_{500\ ug}$ = Hx rats injected for four days with 500 ug bGH (total dose) dissolved in alkaline saline.

Hx-T_3 = Hx rats injected for four days with 12 ug T_3 (total dose) dissolved in alkaline saline.

Hx-(GH + T_3) = Hx rats injected for four days with 500 ug bGH and 12 ug T_3 (total dose) dissolved in alkaline saline.

N wt = Normal rats of comparable weight to Hx rats.

* Different from Hx rat values, $P < .05$ by Dunnett's analysis.

** Different from Hx rat values, $P < .01$ by Dunnett's analysis.

Table 5

Effect of Hormone Treatment on Costal Cartilage DNA and Protein Content

Experimental Group	DNA content (mg)	Protein content (mg)
Hx	.28 ± .02 (32)	10.0 ± 4 (35)
Hx-$GH_{500\ ug}$	.38 ± .04 (24)*	11.5 ± .7 (27)
Hx-T_3	.29 ± .03 (8)	9.1 ± .9 (8)
Hx-(GH + T_3)	.35 ± .03 (17)	11.4 ± .7 (17)
N wt	.22 ± .02 (11)	9.8 ± 1.1 (11)

Five percent cartilage homogenates (400 ul) were placed in an equal volume of 10% TCA and allowed to stand on ice for 20 min. Samples were centrifuged at 6,000 x g for 10 min and supernatants were discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellet and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were removed. Pellets were dissolved in 0.5 ml 0.1 N NaOH and used for protein determinations using the biuret procedure. Supernatants were used for DNA determinations using the diphenylamine reaction. Results are expressed as total DNA and protein content, mean ± SE (number of experimental samples).

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-$GH_{500\ ug}$ = Hx rats injected for four days with 500 ug bGH (total dose) dissolved in alkaline saline.

Hx-T_3 = Hx rats injected for four days with 12 ug T_3 (total dose) dissolved in alkaline saline.

Hx-(GH + T_3) = Hx rats injected for four days with 500 ug bGH and 12 ug T_3 (total dose) dissolved in alkaline saline.

N wt = Normal rats of comparable weight to Hx rats.

* Different from Hx rat values, $P < .05$ by Dunnett's analysis.

Table 6

Effect of Hormone Treatment on Liver DNA and Protein Content

Experimental Group	DNA content (mg)	Protein content (mg)
Hx	9.6 ± .4 (31)	565 ± 25 (31)
Hx-GH_{500}	10.5 ± .6 (28)	658 ± 39 (28)
Hx-T_3	13.4 ± .7 (8)**	576 ± 47 (8)
Hx-(GH + T_3)	14.5 ± .8 (13)**	586 ± 35 (13)
N wt	10.3 ± 1.6 (8)	730 ± 84 (8)

Ten percent liver homogenates (300 ul) were placed in an equal volume of 10% TCA and allowed to stand on ice for 20 min. Samples were centrifuged at 6,000 x g for 10 min and supernatants were discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellet and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were removed. Pellets were dissolved in 0.5 ml 0.1 N NaOH and used for protein determinations using the biuret procedure. Supernatants were used for DNA determinations using the diphenylamine reaction. Results are expressed as total DNA and protein content, mean ± SE (number of experimental samples).

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-GH_{500} = Hx rats injected for four days with 500 ug bGH (total dose) dissolved in alkaline saline.

Hx-T_3 = Hx rats injected for four days with 12 ug T_3 (total dose) dissolved in alkaline saline.

Hx-(GH + T_3) = Hx rats injected for four days with 500 ug bGH and 12 ug T_3 (total dose) dissolved in alkaline saline.

N wt = Normal rats of comparable weight to Hx rats.

** Different from Hx rat values, $P < .01$ by Dunnett's analysis.

Table 7

Rat Growth Rate with Age

Experimental Group Age (weeks)	Age Range (weeks)	Average Body Weight (g)	Growth Rate (g/week)
Normal rats:			
4.5	3- 6	95 ± 5 (21)	
8	7- 9	268 ± 22 (14)**	50
15	13-18	565 ± 21 (13)**	42
25	20-36	723 ± 19 (10)**	16
Hypophysectomized rats:			
4.5	3- 6	107 ± 4 (6)	
8	7- 9	123 ± 7 (9)	4.6
15	13-17	132 ± 4 (11)^{x}	1.3
25	20-36	176 ± 11 (6)^{xx}	4.4

Rat body weights were determined immediately prior to animal sacrifice. Results are the mean ± SE (number of rats). Age (in weeks) represents the approximate average age of rats in the experimental group. Age range represents the minimum and maximum age of rats in the experimental group.

** Different from normal rats about 4.5 weeks old, $P < .01$ by Dunnett's analysis.

^{x} Different from hypophysectomized rats about 4.5 weeks old, $P < .05$ by Dunnett's analysis.

^{xx} Different from hypophysectomized rats about 4.5 weeks old, $P < .01$ by Dunnett's analysis.

body weight increased much more slowly with age, and the growth rate did not clearly decrease with age. The change in costal cartilage and liver wet weight with rat age is shown in Table 8. Like rat body weight, although costal cartilage weight increased with age in the normal animals, the rate of growth was inversely proportional to rat age. Cartilage and liver growth was greatly retarded in the Hx animals. The rate of cartilage and liver growth in Hx animals decreased with age, but not as profoundly as in normal animals. The effect of age on DNA and protein content in rat costal cartilage is shown in Table 9. Although the total content of these macromolecules increases with age, the rate of increase was retarded. A similar trend was found in cartilage DNA of Hx rats while cartilage protein content of Hx rats remained essentially constant. Liver DNA and protein content followed much the same pattern as that noted in cartilage as shown on Table 10. Although total content of DNA and protein increased with normal rat age, the rate of growth decreased with age. In Hx rats, liver DNA and protein content did not greatly change with rat age.

B. Effectiveness of Homogenization - The effectiveness of homogenization was determined by centrifugational and detergent studies. As shown in Table 11, low speed centrifugation (3,000 x g for 5 min) pelleted most of the homogenate protein, but little cathepsin D activity. On the average, about 80% of the activity remained in the supernatant fraction. In several experiments 100% of the activity remained in the supernatant. The relative activities of experimental groups were essentially unaltered by centrifugation. In fact, determination of supernatant acid hydrolase activities was often used as an internal check on results obtained with homogenates. Even after ultracentrifugation (48,000

Table 8

Growth Rate of Rat Liver and Costal Cartilage with Age

Experimental Group Age (weeks)	Costal Cartilage Wet Weight (mg)	Costal Cartilage Growth Rate (mg/week)	Liver Wet Weight (g)	Liver Growth Rate (g/week)
Normal rats:				
4.5	142 ± 9 (24)		4.1 ± .5 (16)	
		44		2.5
8	296 ± 26 (17)**		12.9 ± 1.2 (11)**	
		13		0.8
15	386 ± 27 (16)**		18.6 ± .9 (13)**	
		6		~0
25	442 ± 41 (11)**		19.0 ± 1.1 (11)**	
Hypophysectomized rats:				
4.5	138 ± 10 (6)		3.9 ± .8 (6)	
		2		0.2
8	144 ± 10 (9)		4.6 ± .4 (9)	
		2		0.1
15	156 ± 22 (11)		5.2 ± .2 (13)	
		~0		0.1
25	140 ± 11 (6)		6.0 ± .6 (6)**	

Livers were perfused in situ with cold isotonic saline prior to removal. Costal cartilage (from ribs 3-6) and livers were cleaned of adhering tissue, wiped dry in Whatman 3 MM filter paper and weighed. Rat ages represent the approximate average age of the experimental group. The age range in each group is shown in Table 7.

Table 9

Effect of Rat Age on Costal Cartilage DNA and Protein Content

Experimental Group Age (weeks)	DNA Content (ug)	DNA Content Change (ug/wk)	Protein Content (mg)	Protein Content Change (mg/wk)
Normal rats:				
4.5	411 ± 31 (19)		17 ± 3 (18)	
		54		5
8	601 ± 52 (15)*		33 ± 6 (15)	
		35		4
15	846 ± 81 (12)**		60 ± 13 (12)**	
		~0		1
25	848 ± 84 (12)**		65 ± 11 (12)**	
Hypophysectomized rats:				
4.5	227 ± 25 (5)		15 ± 2 (5)	
		5		~0
15	286 ± 37 (6)		16 ± 3 (6)	
		1		~0
25	300 ± 45 (6)		18 ± 3 (6)	

Five percent cartilage homogenates (400 ul) were placed in an equal volume of 10% TCA and allowed to stand in ice for 20 min. Samples were centrifuged at 6,000 x g for 10 min and supernatants were discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellet and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were removed. Pellets were dissolved in 0.5 ml 0.1 N NaOH and used for protein determinations using the biuret procedure. Supernatants were used for DNA determinations using the diphenylamine reaction. Results are expressed as total DNA and protein content, mean ± SE (number of experimental samples). Rat ages represent the approximate average age of the experimental group. The age range in each group is shown in Table 7.

Normal rats:
4.5 = Normal rats about 4.5 weeks old.
8 = Normal rats about 8 weeks old.
15 = Normal rats about 15 weeks old.
25 = Normal rats about 25 weeks old.

Hypophysectomized rats (Hx rats):
4.5 = Hx rats about 4.5 weeks old.
15 = Hx rats about 15 weeks old.
25 = Hx rats about 25 weeks old.

Table 10

Effect of Rat Age on Liver DNA and Protein Content

Experimental Group Age (weeks)	Total DNA (mg)	DNA Content Change (mg/wk)	Total Protein (g)	Protein Content Change (g/wk)
Normal rats:				
4.5	15 ± 2 (11)		1.1 ± .3 (11)	
		1.4		.2
8	20 ± 1 (9)		1.9 ± .5 (9)	
		1.5		.1
15	31 ± 3 (9)**		2.5 ± .2 (9)*	
		0.4		.1
25	35 ± 3 (11)**		3.2 ± .5 (11)**	
Hypophysectomized rats:				
4.5	10 ± 1 (16)		.9 ± .1 (6)	
		0.2		~0
15	12 ± 1 (9)		.9 ± .1 (9)	
		~0		~0
25	11 ± 1 (6)		1.3 ± .2 (6)	

Ten percent liver homogenates (300 ul) were placed in an equal volume of 10% TCA and allowed to stand in ice for 20 min. Samples were centrifuged at 6,000 x g for 10 min and supernatants were discarded. This TCA precipitation was repeated one time after which 1.0 ml 5% TCA was added to sample pellet and mixed vigorously. Samples were placed in a dry bath at 90° for 15 min with occasional mixing. Samples were centrifuged at 6,000 x g for 10 min and supernatants were removed. Pellets were dissolved in 0.5 ml 0.1 N NaOH and used for protein determinations using the biuret procedure. Supernatants were used for DNA determinations using the diphenylamine reaction. Results are expressed as total DNA and protein content, mean ± SE (number of experimental samples). Rat ages represent the approximate average age of the experimental group. The age range in each group is shown in Table 7.

Normal rats:
4.5 = Normal rats about 4.5 weeks old.
8 = Normal rats about 8 weeks old.
15 = Normal rats about 15 weeks old.
25 = Normal rats about 25 weeks old.

Hypophysectomized rats (Hx rats):
4.5 = Hx rats about 4.5 weeks old.
15 = Hx rats about 15 weeks old.
25 = Hx rats about 25 weeks old.

Table 11

Fractionation of Cartilage Cathepsin D Activity
by Low Speed Centrifugation

Experimental Group	Homogenate Protein (mg/ml)	Supernatant Protein (ug/ml)	Cathepsin D Activity (ug Tyr/ml/h)	
			Homogenate	Supernatant (% Homogenate)
Hx	6.1 ± .7 (6)	74 ± 7	45 ± 10	37 ± 3 (82%)
Hx-GH$_{500}$	6.4 ± .4 (6)	83 ± 1	86 ± 9	64 ± 9 (75%)
N wt	3.9 ± .6 (10)	54 ± 7	98 ± 12	77 ± 7 (79%)
N age	4.1 ± .7 (8)	59 ± 5	73 ± 9	61 ± 8 (84%)
N old	5.0 ± .3 (11)	51 ± 6	26 ± 4	22 ± 4 (85%)

Cartilage 5% homogenates were centrifuged at 3,000 x g for 5 min. The supernatant was removed and .005 M sodium phosphate buffer, pH 6.5 (one half the original volume of homogenate) was added to pellets, mixed and centrifuged as before. Supernatants were pooled and the volume was adjusted to the original homogenate volume with .005 M sodium phosphate buffer, pH 6.5 prior to cathepsin D and protein determinations.

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx-GH$_{500}$ = Hx rats injected for four days with bGH (500 ug total dose) dissolved in alkaline saline.

N wt = Normal rats of comparable weight to Hx rats.

N age = Normal rats of comparable age to Hx rats.

N old = Normal rats greater than 25 weeks old.

x g for 2 h) of low speed supernatants most of the cathepsin D activity was recovered in the soluble fraction (Table 12).

Triton X-100, a detergent, has previously been shown to disrupt lysosomal integrity and release latent acid hydrolase activity (94). To establish with confidence that differential lysosomal disruption was not altering detectable enzyme activities, Triton X-100 (0.2%) was added to homogenates and low speed supernatant samples. Triton X-100 had little or no effect on cartilage activities or on the purified cathepsin D control as shown in Table 13.

C. Effect of Growth Hormone and Rat Age on Cartilage Protease Activity Using a Nonspecific Gelatin Membrane Substrate

1. Time and temperature-dependence of protease activity - Optimal cartilage protease activity on gelatin membranes was previously shown to occur at pH 4.0, with little or no activity at neutral or alkaline pH (84b). Therefore, we used pH 4.0 in the standard assay and found substantial activity with 16 u thick sections of rat costal cartilage tissue. Protease activity, at this pH, probably represents the effect of one or more of the lysosomal, acid proteases called cathepsins. As shown in Table 14, protease activity at pH 4.0 was time-dependent.

The effect of temperature on cartilage protease activity is shown in Table 15. After preincubation at various temperatures, cartilage was frozen, sectioned, and incubated with gelatin substrate at 37°, pH 4.0. Optimal activity was obtained at 37° preincubation. Activity increased between 4° and 37°, indicating a temperature-dependent process may be required for full expression of activity. Temperatures greater than 55° greatly reduced activity, presumably due to enzyme denaturation. These results cannot be attributed to temperature-dependent changes in

Table 12

Ultracentrifugation of Cartilage Cathepsin D Activity

Experimental Group	Cathepsin D Activity (% Low Speed Supernatant Activity)			
	Low Speed Supernatant	Low Speed Pellet	Ultracentrifugation Supernatant	Ultracentrifugation Pellet
N age	100 ± 4	None[a]	72 ± 16	None
N old	100 ± 16	None	88 ± 17	None

Cartilage 5% homogenates were centrifuged at 3,000 x g for 5 min. The resulting supernatants (low speed supernatants) were then centrifuged at 48,000 x g for 2 h. These supernatants (ultracentrifugation supernatant) and pellet (ultracentrifugation pellet, suspended in .005 M sodium phosphate buffer, pH 6.5), as well as the 3,000 x g supernatant and pellet (low speed pellet, suspended in .005 M sodium phosphate buffer, pH 6.5) were then assayed for cathepsin D activity. Values are the average of four samples from two experiments and are expressed as % low speed supernatant activity.

N age = Normal rats about 8 weeks old.

N old = Normal rats over 25 weeks old.

[a]None = Not detectable.

Table 13

Effect of Triton X-100 on Cartilage Cathepsin D Activity

Experimental Group	Cathepsin D Activity (ug Tyr equivalents/h) No Triton	0.2% Triton for 1 min. before assay	0.2% Triton for 16 h at 4°
Cartilage homogenates:			
N 3-8	8.7 ± .1	9.6 ± .1	9.0 ± .2
N 48	4.6 ± .1	5.3 ± .1	5.3 ± .1
Cartilage low speed supernatants:			
Hx	3.6 ± .1	3.6 ± .1	3.9 ± .3
Hx-GH_{500}	4.4 ± .1	4.5 ± .1	4.5 ± .1
Liver homogenate:			
Hx	10.5 ± .3	10.4 ± .1	12.3 ± .7
N 3-8	8.2 ± .4	8.4 ± .4	8.0 ± 1.0
Purified cathepsin D:			
0.25 ug	19.6 ± .1	22.2 ± .1	17.8 ± .1

Cartilage 5% homogenates, cartilage low speed (3,000 x g for 5 min) supernatants, liver 10% homogenates and purified cathepsin D were pre-incubated for 16 h at 4° in the absence or presence of 0.2% (final concentration) Triton X-100. Just 1 min prior to assay 0.2% (final concentration) Triton X-100 was added to indicated samples. Samples were assayed for cathepsin D activity using 100 ml cartilage samples, 50 ul liver samples and 0.25 mg cathepsin D. Results are expressed as the mean ± SE of two samples.

Table 14

Time-dependence of Cartilage Protease Activity

Experimental Groups	Relative Protease Activities at 37°	
	2 h	5 h
Hx	15 ± 3 (7)	100 ± 11 (5)**
Hx + 45 ug bGH	43 ± 16 (6)	226 ± 23 (7)**
N wt	83 ± 15 (8)	443 ± 29 (7)**

Cartilage sections were placed on India-ink stained gelatin membranes and incubated at 37°, pH 4.0 for times indicated above. Values (mean ± SE (n) of two experiments) are expressed as relative protease activities (Hx, 5 h activity = 100%). Statistical differences between time points were determined by Dunnett's analysis.

Hx = Hypophysectomized rats.

Hx + 45 ug bGH = Hx injected with bGH, 45 ug total dose.

N wt = Normal rats matched in weight to Hx.

** Different from 2 h activity of the same experimental group, $P < .01$.

Table 15

Effect of Temperature on Protease Activity

Temperature	Relative Protease Activity
4°	60 ± 4 (6)[a]
23°	75 ± 5 (6)
37°	100 ± 10 (4)
55°	58 ± 4 (4)[a]
75°	29 ± 8 (4)[a]
100°	46 ± 7 (7)[a]

Acid protease activity of normal age control rat cartilage was determined after 3 h preincubation at various temperatures in 0.9% NaCl. One h preincubation resulted in essentially the same relative activities. Values (mean ± SE (n) of 1-2 experiments) are expressed as relative specific activities.

[a]Different from activity at 37°, 3 h preincubation, $P < .01$.

gelatin substrate structure since only cartilage tissue, without gelatin substrate, was preincubated.

2. Growth hormone-dependence of protease activity - As shown in Fig. 21, hypophysectomy greatly reduced cartilage protease activity. Normal rat cartilage appeared to have approximately 2-4 fold more activity than that of Hx rat cartilage. Injection of bGH for four days (1.5 to 500 ug total dose) into Hx rats enhanced protease activity approximately to levels found in normal rats (Fig. 21). Significant stimulation occurred at 45 ug and 500 ug bGH, total dose. Linear regression analysis indicated that GH injected animals had a much greater growth rate than Hx rats injected with alkaline saline.

3. Age-dependence of protease activity - Cartilage of the normal weight control group had greater specific activity than that of normal age control rats, indicating a probable age-dependence of the protease activity (Fig. 21). Further investigation of this phenomenon confirmed that the younger (4 week old), more rapidly growing animals, had higher protease activity than that of older, less rapidly growing animals (Table 16). Specific activity in lieu of total activity, was used in the comparison because of substantial differences in cartilage slice cross-sectional areas.

D. Relationship of Certain Lysosomal Hydrolases to Age and Growth Hormone-dependent Growth Rate

1. Effect of growth hormone treatment on certain lysosomal enzyme activities - Hypophysectomy greatly reduced cartilage cathepsin D (Table 17) and acid phosphatase (Table 18) activities, but had no effect on cathepsin B activity relative to the N age control (Table 19). Approximately 50-100% more cathepsin D and acid phosphatase activities were

Figure 21. Cartilage protease activity of normal, Hx and bGH-treated Hx rats. Hx rats (21 days post operative) were injected for four days with a solution of bGH (NIH-GH-B-18, 1.5-500 ug total dose) in alkaline saline or with solvent alone. Costal cartilage was removed on day 5 and immediately froze, sliced and assayed for proteolytic activity. Results are expressed as percent of Hx control average specific activity. Values are the average of four experiments. The SE averaged $\leq$ 13% of the mean.

N age = Normal rats of comparable age to Hx rats.

N wt = Normal rats of comparable weight to Hx rats.

*Different from Hx rat values, $P < .05$ of the mean.

**Different from Hx rat values, $P < .01$.

$r = .98$.

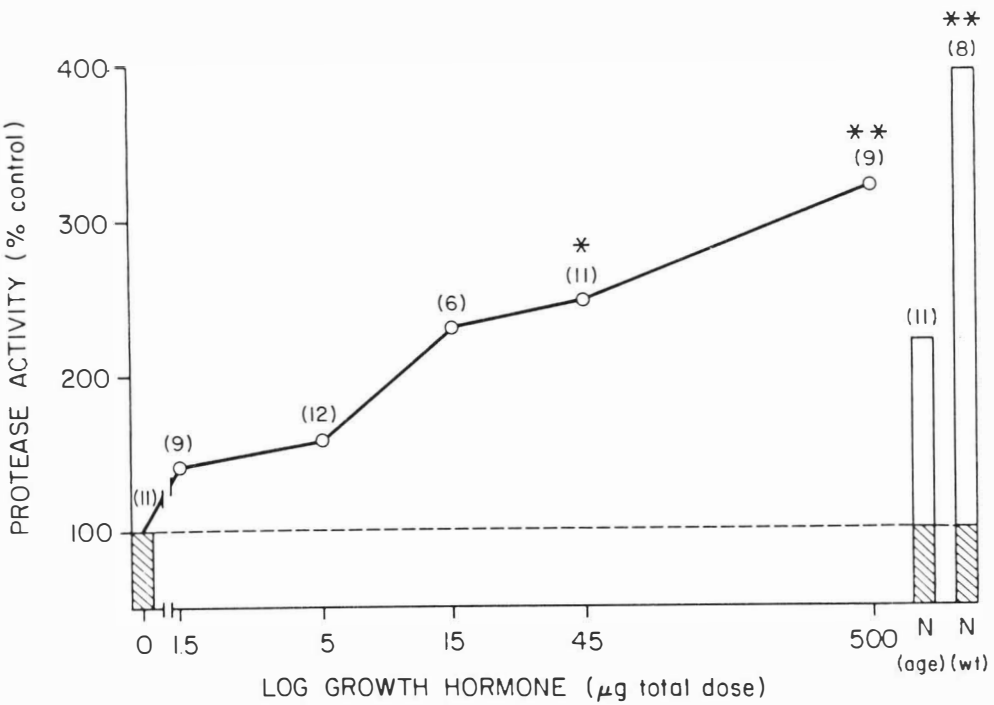


Table 16

Correlation of Rat Age, Body Weight and Growth Velocity with Cartilage Protease Activity

Age (weeks)	Body Weight Range (g)	Growth Velocity[c]	Protease Specific Activity (%)
4[a]	90-110	6.1	100 ± 7 (8)
5	115-130	4.7	76 ± 4 (8)**
6	160-170	3.4	75 ± 4 (8)**
8[b]	270-290	2.9	56 ± 3 (13)**

Costal cartilage protease activity of normal rats differing in age and weight was determined. Protease values (mean + SE (n) of 3-4 experiments) are expressed as % of the average activity of the 4-week old rats. Growth velocity values (mean of 16 or more rats per age group) were obtained from information supplied by Zivic-Miller Laboratories. Statistical differences were determined by Dunnett's analysis.

[a]Rats of equivalent weight to Hx animals.

[b]Rats of equivalent age to Hx animals.

[c]Mean % increase of body weight per day.

** Different from activity of 4 week old rats, $P < .01$.

Table 17

Effect of Hypophysectomy and Hormone Treatment on Cartilage Cathepsin D Activity

Experimental Group	Enzyme Activity (% Hx) per weight tissues	per protein content	per DNA content
Hx	100 ± 2 (32)	100 ± 2	100 ± 3
Hx + $GH_{50\ ug}$	121 ± 9 (8)	107 ± 9	120 ± 13
Hx + $GH_{500\ ug}$	157 ± 7 (20)**	153 ± 8**	147 ± 7**
Hx + T_3	115 ± 5 (10)	120 ± 9	122 ± 7
Hx + (GH + T_3)	155 ± 4 (12)**	137 ± 7**	145 ± 5**
N age	155 ± 10 (17)**	152 ± 19**	198 ± 26**
N wt	198 ± 11 (10)**	189 ± 13**	185 ± 17**

Costal cartilage cathepsin D activity of normal, Hx and hormone-treated Hx rats were determined. Hx rats (2 weeks post operative) were injected for 4 days with either bGH (NIH-GH-B-18, 50 to 500 ug total dose) in alkaline saline, 500 ug bGH plus T_3 (12 ug total dose) in alkaline saline, T_3 12 ug (total dose) in alkaline saline or with solvent alone. After the treatment period, costal cartilage was removed, cleaned of adhering tissue, homogenized in 0.005 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (yielding a 5% homogenate) and assayed for enzymatic activity, DNA content, and protein content. Results are expressed as % of the Hx activity ± SE (n = number of rats).

Hx = Hypophysectomized rats injected for four days with solvent alone.

Hx + $GH_{50\ ug}$ = Hypophysectomized rats injected for four days with 50 ug bGH (total dose) dissolved in alkaline saline.

Hx + $GH_{500\ ug}$ = Hypophysectomized rats injected for four days with 500 ug bGH dissolved in alkaline saline.

Hx + T_3 = Hypophysectomized rats injected for four days with T_3 (12 ug total dose) dissolved in alkaline saline.

Hx + (GH + T_3) = Hypophysectomized rats injected for four days with 500 ug bGH in combination with 12 ug T_3, dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

N wt = Normal rats of comparable weight to Hx rats.

**Different from Hx rat values, $P < .01$ by Dunnett's analysis.

Table 18

Effect of Hypophysectomy and Hormone Treatment on Cartilage Acid Phosphatase Activity

Experimental Group	Enzyme Activity (% Hx) per weight tissues	per protein content	per DNA content
Hx	100 ± 3 (27)	100 ± 3	100 ± 3
Hx + $GH_{500\ ug}$	134 ± 9 (17)**	132 ± 10*	128 ± 8*
Hx + $GH_{2.0\ mg}$	150 ± 13 (9)**	159 ± 17**	140 ± 15*
Hx + (GH + T_3)	153 ± 6 (6)**	128 ± 14	132 ± 7
N age	148 ± 8 (8)**	146 ± 15*	169 ± 22**
N wt	168 ± 7 (7)**	183 ± 11**	163 ± 9**

Costal cartilage acid phosphatase activity of normal, Hx and hormone-treated Hx rats were determined. Hx rats (2 weeks post operative) were injected for 4 days with either bGH (NIH-GH-B-18, .5 or 2 mg total dose) in alkaline saline, 500 ug bGH plus T_3 (12 ug total dose) in alkaline saline, T_3 12 ug (total dose) in alkaline saline or with solvent alone. After the treatment period, costal cartilage was removed, cleaned of adhering tissue, homogenized in 0.005 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (yielding a 5% homogenate) and assayed for enzymatic activity, DNA content, and protein content. Results are expressed as % of the Hx activity ± SE (n = number of rats).

Hx = Hypophysectomized rats injected for four days with solvent alone.

Hx + $GH_{500\ ug}$ = Hypophysectomized rats injected for four days with 500 ug bGH dissolved in alkaline saline.

Hx + $GH_{2.0\ mg}$ = Hypophysectomized rats injected for four days with 2.0 mg bGH dissolved in alkaline saline.

Hx + (GH + T_3) = Hypophysectomized rats injected for four days with 500 ug bGH in combination with 12 ug T_3, dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

N wt = Normal rats of comparable weight to Hx rats.

*Different from Hx rat values, $P < .05$ by Dunnett's analysis.

**Different from Hx rat values, $P < .01$ by Dunnett's analysis.

Table 19

Effect of Hypophysectomy and Hormone Treatment on Cartilage Cathepsin B Activity

Experimental Group	Enzyme Activity (% Hx) per weight tissue	per protein content	per DNA content
Hx	100 ± 3 (17)	100 ± 5	100 ± 5
Hx + $GH_{500\ ug}$	101 ± 6 (10)	98 ± 9	94 ± 7
Hx + $GH_{2.0\ mg}$	103 ± 5 (4)	101 ± 6	99 ± 7
Hx + (GH + T_3)	117 ± 10 (4)	99 ± 15	102 ± 7
N age	102 ± 12 (4)	95 ± 2	106 ± 5
N wt	145 ± 3 (11)**	151 ± 14**	145 ± 10**

Costal cartilage cathepsin B activity of normal, Hx and hormone-treated Hx rats were determined. Hx rats (2 weeks post operative) were injected for 4 days with either bGH (NIH-GH-B-18, .5 or 2 mg total dose) in alkaline saline, 500 ug bGH plus T_3 (12 ug total dose) in alkaline saline, T_3 12 ug (total dose) in alkaline saline or with solvent alone. After the treatment period, costal cartilage was removed, cleaned of adhering tissue, homogenized in 0.005 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (yielding a 5% homogenate) and assayed for enzymatic activity, DNA content, and protein content. Results are expressed as % of the Hx activity ± SE (n = number of rats).

Hx = Hypophysectomized rats injected for four days with solvent alone.

Hx + $GH_{500\ ug}$ = Hypophysectomized rats injected for four days with 500 ug bGH dissolved in alkaline saline.

Hx + $GH_{2.0\ mg}$ = Hypophysectomized rats injected for four days with 2.0 mg bGH dissolved in alkaline saline.

Hx + (GH + T_3) = Hypophysectomized rats injected for four days with 500 ug bGH in combination with 12 ug T_3, dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

N wt = Normal rats of comparable weight to Hx rats.

**Different from Hx rat values, $P < .01$ by Dunnett's analysis.

found in normal animals compared with Hx animals. Treatment of Hx rats with bGH (500 ug or more total dose) enhanced cathepsin D and acid phosphatase activities towards normal levels, as shown in Table 17 and 18. Significant stimulation of these activities was attained with 500 ug bGH. Interestingly, however, GH had no effect on cartilage cathepsin B activity (Table 19). T_3, alone or in combination with BH, little or no apparent effect on cartilage acid hydrolase activities (Tables 17-19).

Pepstatin inhibits cathepsin D by binding with high affinity to the active site of the enzyme stoichiometrically (95). Pepstatin titration experiments were used to determine if the difference in cathepsin D activity between normal and Hx rat cartilage was a reflection of enzyme level. As shown in Table 20, greater amounts of pepstatin were required to produce 50% inhibition of rat cartilage activity of normal and hormone-treated Hx rats than to produce 50% inhibition of Hx activity. This observation suggests that there are a greater number of cathepsin D molecules in normal rat cartilage than in Hx cartilage.

The effect of hypophysectomy and GH-treatment on liver enzyme activities is shown in Tables 21-23. Hypophysectomy had little or no effect on liver cathepsin D (Table 21) and acid phosphatase activities (Table 23) (particularly when compared to N age controls), but appeared to decrease cathepsin B activity (Table 22). Thus, the effects of hypophysectomy on cartilage versus liver lysosomal enzyme activities were quite different. Also unlike cartilage tissue, treatment of Hx rats with GH had no effect on the three lysosomal enzymes in liver (Tables 21-23). T_3, alone and in combination with bGH enhanced hepatic cathepsin D (Table 21). T_3 in combination with bGH increased liver acid phosphatase (Table 23) activity and slightly enhanced cathepsin B (Table 22) activity of Hx

Table 20

Titration of Cartilage Cathepsin D Activity with Pepstatin

Experimental Group	Enzyme Activity (% Hx)	Concentration of pepstatin producing 50% inhibition of cathepsin D activity (% Hx)
Hx	100 ± 4 (8)	100 ± 3 (7)
Hx+$GH_{500\ ug}$	159 ± 15 (8)**	152 ± 7 (7)*
Hx+(GH + T_3)	161 ± 10 (8)**	164 ± 17 (6)**
N age	211 ± 15 (8)**	235 ± 19 (7)**

Hx rats were treated as described below. The concentration of pepstatin required to produce 50% inhibition of cartilage homogenate cathepsin D activity was determined. In each assay, several concentrations of pepstatin were added to a constant amount of cartilage homogenate. Results are expressed as % Hx ± SE (n = number of cartilage samples analyzed).

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx + $GH_{500\ ug}$ = Hx rats injected for four days with 500 ug (total dose) bGH dissolved in alkaline saline.

Hx + (GH + T_3) = Hx rats injected for four days with 500 ug bGH plus 12 ug T_3 (dose doses) dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

*Different from Hx rat values, $P < .05$ by Dunnett's analysis.

**Different from Hx rat values, $P < .01$ by Dunnett's analysis.

Table 21

Effect of Hypophysectomy and Hormone Treatment on Liver Cathepsin D Activity

Experimental Group	Enzyme Activity (% Hx) per weight tissue	per protein content	per DNA content
Hx	100 + 3 (57)	100 + 3	100 + 3
Hx + $GH_{50\ ug}$	94 + 9 (9)	97 + 12	96 + 8
Hx + $GH_{500\ ug}$	108 + 5 (33)	104 + 5	105 + 4
Hx + $GH_{2.0\ mg}$	82 + 10 (4)	94 + 9	96 + 8
Hx + T_3	155 + 18 (10)**	172 + 18**	120 + 13
Hx + (GH + T_3)	171 + 16 (17)**	172 + 13**	137 + 12**
N age	110 + 8 (22)	113 + 6	136 + 8
N wt	135 + 15 (16)**	146 + 17**	158 + 26**

Liver cathepsin D activity of normal, Hx and hormone-treated Hx rats were determined. Hx rats were treated as described below. Livers were perfused with cold 0.9% NaCl in situ, homogenized in 0.005 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (yielding a 10% homogenate) and assayed for enzymatic activity, DNA content, and protein content. Results are expressed at % of Hx activity $\pm$ SE (n = number of rats).

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx + $GH_{50\ ug}$ = Hx rats injected for four days with 50 ug bGH (total dose) dissolved in alkaline saline.

Hx + $GH_{500\ ug}$ = Hx rats injected for four days with 500 ug bGH dissolved in alkaline saline.

Hx + $GH_{2.0\ mg}$ = Hx rats injected for four days with 2.0 mg bGH dissolved in alkaline saline.

Hx + T_3 = Hx rats injected for four days with T_3 (12 ug total dose) dissolved in alkaline saline.

Hx + (GH + T_3) = Hx rats injected for four days with 500 ug bGH plus 12 ug T_3, dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

N wt = Normal rats of comparable weight to Hx rats.

**Different from Hx rat values, $P < .01$ by Dunnett's analysis.

Table 22

Effect of Hypophysectomy and Hormone Treatment on Liver Cathepsin B Activity

Experimental Group	Enzyme Activity (% Hx) per weight tissue	per protein content	per DNA content
Hx	100 ± 4 (20)	100 ± 4	100 ± 3
Hx + $GH_{500\ ug}$	110 ± 7 (12)	108 ± 7	106 ± 7
Hx + $GH_{2.0\ mg}$	96 ± 9 (4)	100 ± 6	112 ± 7
Hx + T_3	123 ± 11 (4)	131 ± 11 (4)	100 ± 8 (4)
Hx + (GH + T_3)	130 ± 9 (10)*	130 ± 8	110 ± 11
N age	168 ± 14 (10)**	171 ± 14**	206 ± 20**
N wt	170 ± 15 (11)**	171 ± 20**	198 ± 35**

Liver cathepsin B activity of normal, Hx and hormone-treated Hx rats were determined. Hx rats were treated as described below. Livers were perfused with cold 0.9% NaCl in situ, homogenized in 0.005 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (yielding a 10% homogenate) and assayed for enzymatic activity, DNA content, and protein content. Results are expressed at % of Hx activity ± SE (n = number of rats).

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx + $GH_{500\ ug}$ = Hx rats injected for four days with 500 ug bGH dissolved in alkaline saline.

Hx + $GH_{2.0\ mg}$ = Hx rats injected for four days with 2.0 mg bGH dissolved in alkaline saline.

Hx + T_3 = Hx rats injected for four days with T_3 (12 ug total dose) dissolved in alkaline saline.

Hx + (GH + T_3) = Hx rats injected for four days with 500 ug bGH plus 12 ug T_3, dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

N wt = Normal rats of comparable weight to Hx rats.

*Different from Hx rat values, $P < .05$ by Dunnett's analysis.

**Different from Hx rat values, $P < .01$ by Dunnett's analysis.

Table 23

Effect of Hypophysectomy and Hormone Treatment on Liver Acid Phosphatase Activity

Experimental Group	Enzyme Activity (% Hx) per weight tissue	per protein content	per DNA content
Hx	100 ± 3 (20)	100 ± 3	100 ± 3
Hx + $GH_{500\ ug}$	95 ± 15 (8)	93 ± 13	87 ± 13
Hx + $GH_{2.0\ mg}$	99 ± 9 (4)	100 ± 6	108 ± 5
Hx + (GH + T_3)	183 ± 19 (6)**	181 ± 16**	142 ± 20*
N age	107 ± 5 (10)	129 ± 7	130 ± 7
N wt	120 ± 5 (13)	137 ± 18*	136 ± 16*

Liver acid phosphatase activity in normal, Hx and hormone-treated Hx rats were determined. Hx rats were treated as described below. Livers were perfused with cold 0.9% NaCl in situ, homogenized in 0.005 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (yielding a 10% homogenate) and assayed for enzymatic activity, DNA content, and protein content. Results are expressed at % of Hx activity ± SE (n = number of rats).

Hx = Hypophysectomized rats injected for four days with alkaline saline.

Hx + $GH_{500\ ug}$ = Hx rats injected for four days with 500 ug bGH dissolved in alkaline saline.

Hx + $GH_{2.0\ mg}$ = Hx rats injected for four days with 2.0 mg bGH dissolved in alkaline saline.

Hx + (GH + T_3) = Hx rats injected for four days with 500 ug bGH plus 12 ug T_3, dissolved in alkaline saline.

N age = Normal rats of comparable age to Hx rats.

N wt = Normal rats of comparable weight to Hx rats.

*Different from Hx rat values, $P < .05$ by Dunnett's analysis.

**Different from Hx rat values, $P < .01$ by Dunnett's analysis.

rats while bGH alone had no effect. The percent stimulation of acid hydrolases by T_3 (alone or in combination with bGH) injected rats was lower when expressed per DNA content (compared to per protein content or per wet weight).

2. Relationship of rat age to lysosomal enzyme activities - The relationship of cartilage lysosomal enzyme activities to rat age is shown in Figs. 22-31. A striking decrease in cathepsin D (Figs. 22-23), acid phosphatase (Figs. 26-27) and cathepsin B activities (Figs. 24-25) was found in normal rats between one and 25 weeks of age, particularly when expressed per protein content. When expressed per DNA, the age-dependency of lysosomal enzymes was less dramatic but still statistically significant. The percent change of cathepsin D activity and acid phosphatase activity with normal rat age appeared to be nearly identical, while the change in cathepsin B activity was slightly more pronounced with age (Fig. 28 and 29). Statistical analysis revealed that the relative cathepsin B activity was significantly different from that noted with cathepsin D and acid phosphatase at one or more groups. The change of cartilage lysosomal enzyme activities with Hx rat age were less pronounced than in normal animals and in many cases statistically insignificant (Fig. 22-27). Also unlike results of experiments using normal animals, Hx rat cartilage cathepsin D and acid phosphatase activities were not clearly parallel with changing rat age (Figs. 30 and 31).

Pepstatin titration experiments were used to determine if the change in normal cartilage cathepsin D activity with rat age was a reflection of enzyme concentration. As shown in Table 24, more pepstatin was required to inhibit young rat cartilage activity than to inhibit that of older animals.

Figure 22. Age-dependence of cartilage cathepsin D activity (per protein content) of normal and hypophysectomized rats. Enzyme activities were determined in 5% cartilage homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

[+]N = Normal rats.

[x]Hx = Hypophysectomized rats.

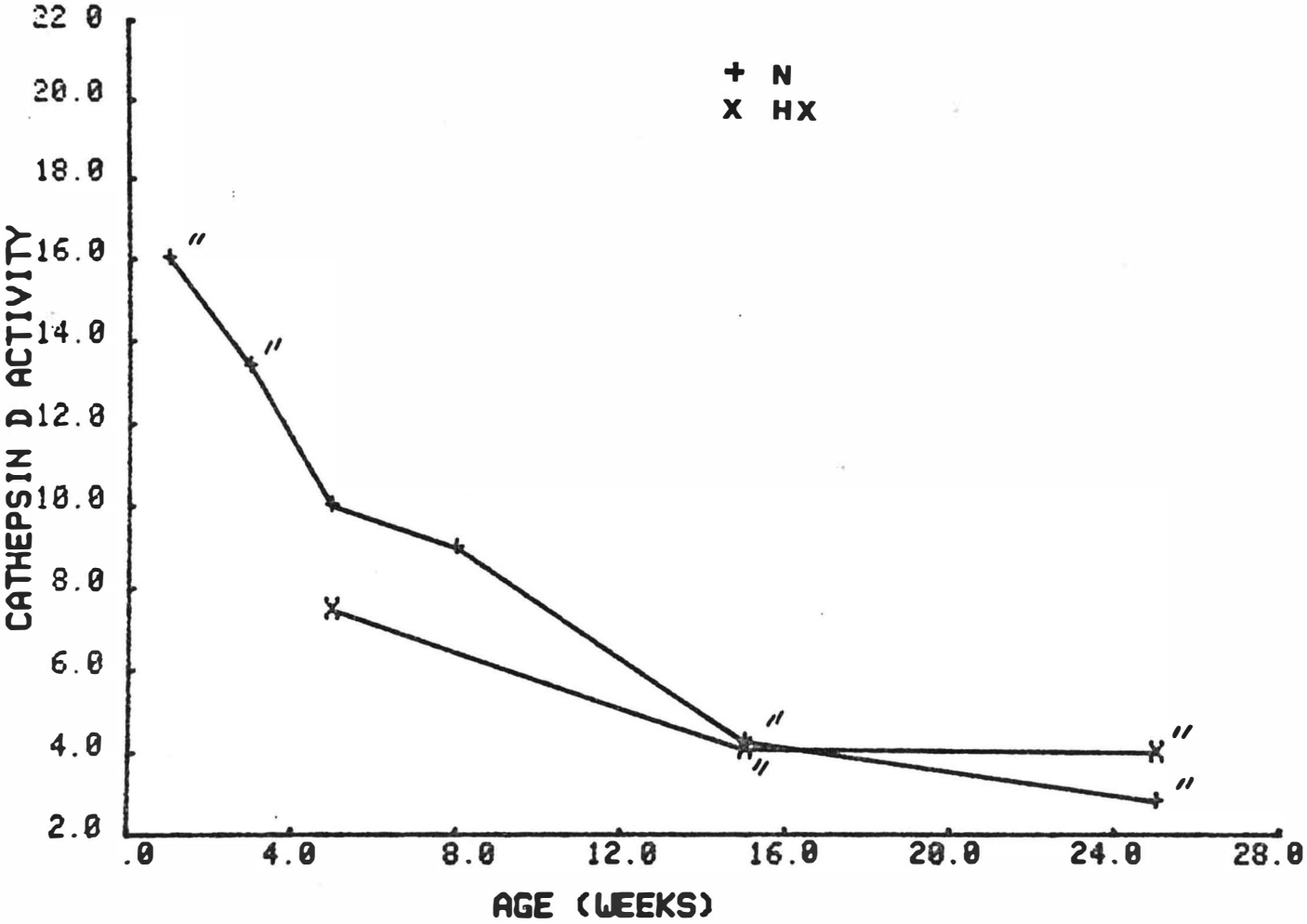

+ N
X HX
CATHEPSIN D ACTIVITY
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 23. Age-dependence of cartilage cathepsin D activity (per DNA content) of normal and hypophysectomized rats. Enzyme activities were determined in 5% cartilage homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

[+]N = Normal rats.

[x]Hx = Hypophysectomized rats.

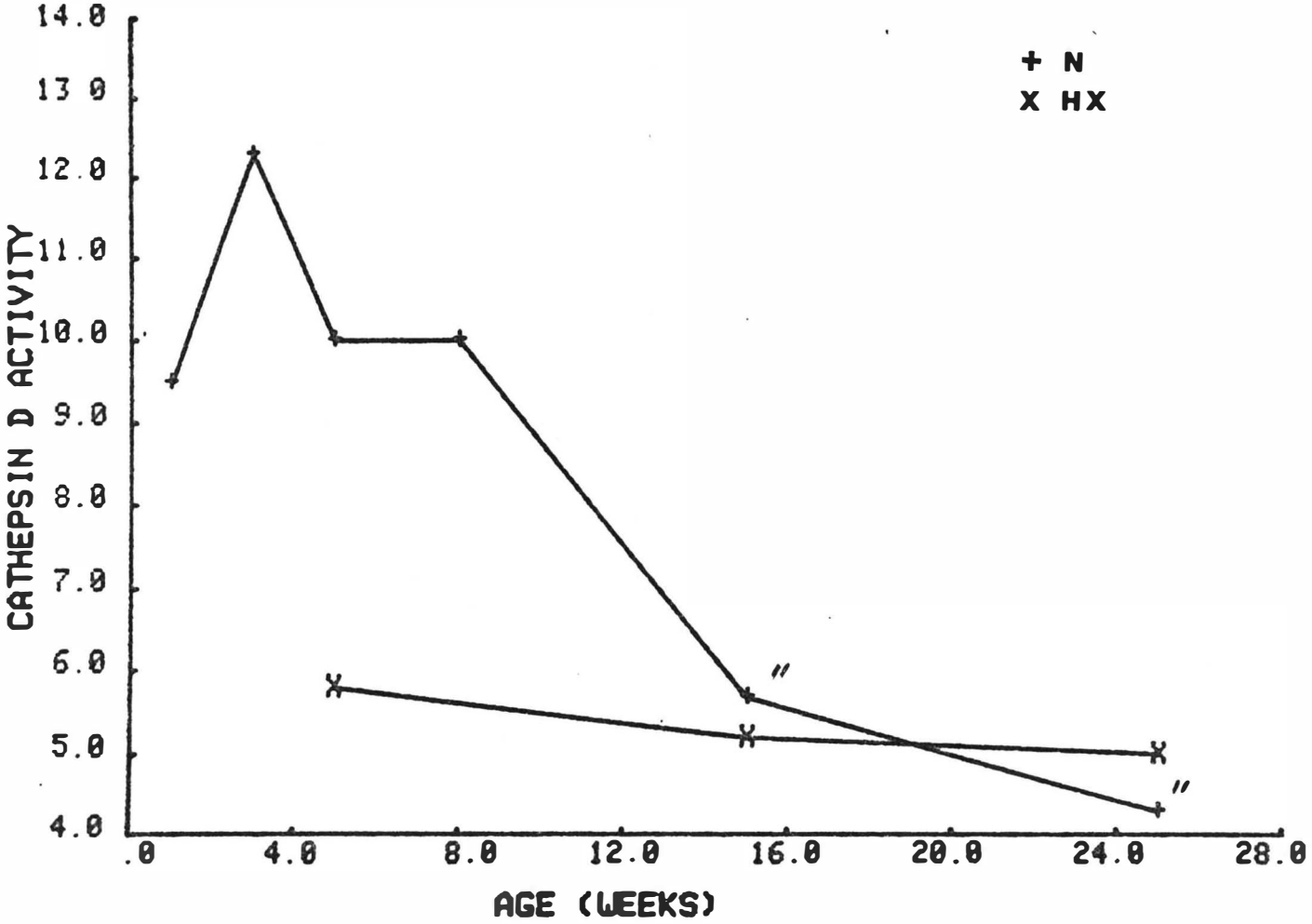

+ N
X HX
CATHEPSIN D ACTIVITY
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 24. Age-dependence of cartilage cathepsin B activity (per protein content) of normal and hypophysectomized rats. Enzyme activities were determined in 5% cartilage homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[1]Different from values of the comparable rats 4.5 weeks old, $P < .05$ by Dunnett's analysis.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

[+]Normal rats.

[x]Hx = Hypophysectomized rats.

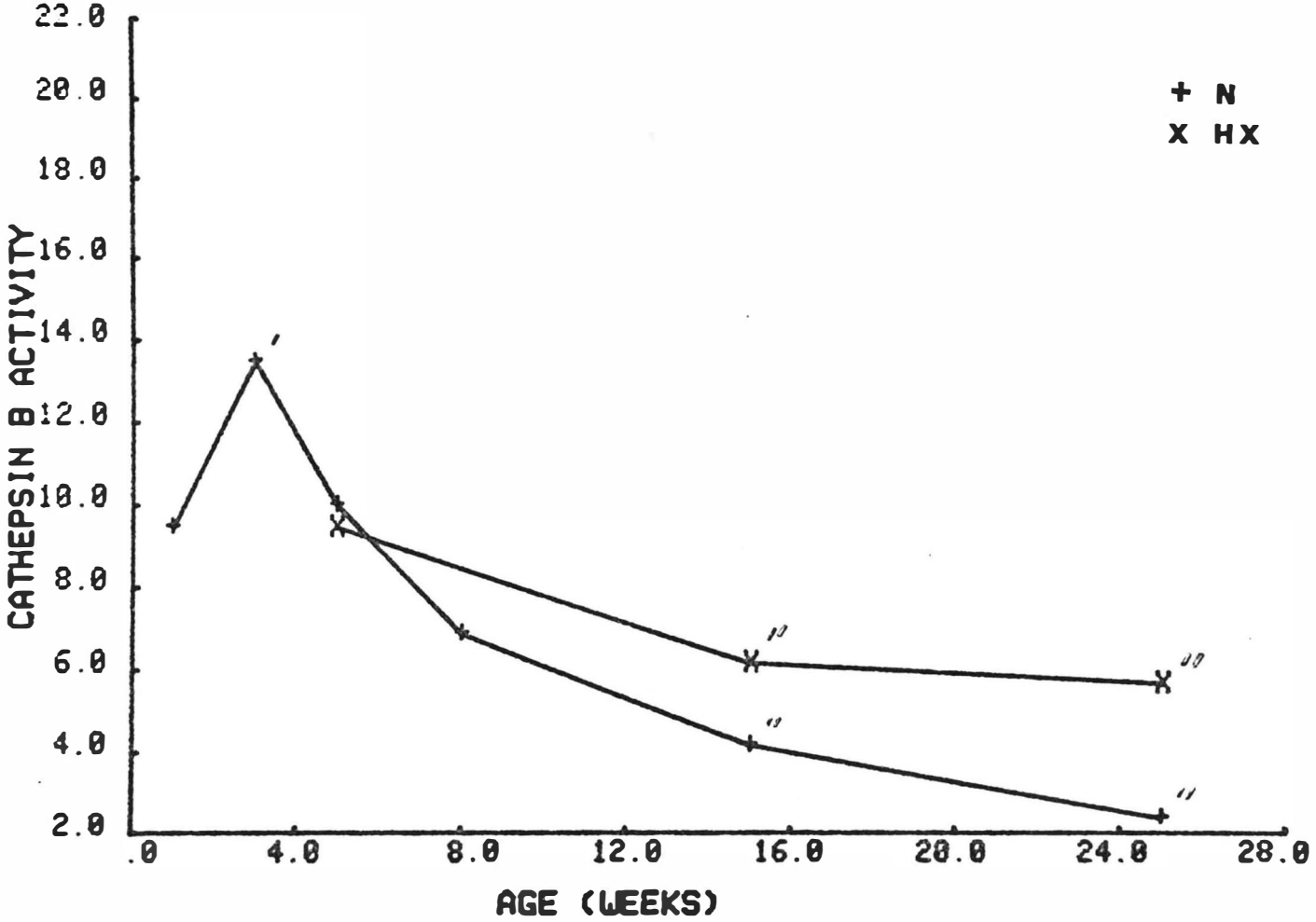

22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
CATHEPSIN B ACTIVITY
+ N
X HX
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 25. Age-dependence of cartilage cathepsin B activity (per DNA content) of normal and hypophysectomized rats. Enzyme activities were determined in 5% cartilage homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[1]Different from values of the comparable rats 4.5 weeks old, $P < .05$ by Dunnett's analysis.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

[+]N = Normal rats.

[x]Hx = Hypophysectomized rats.

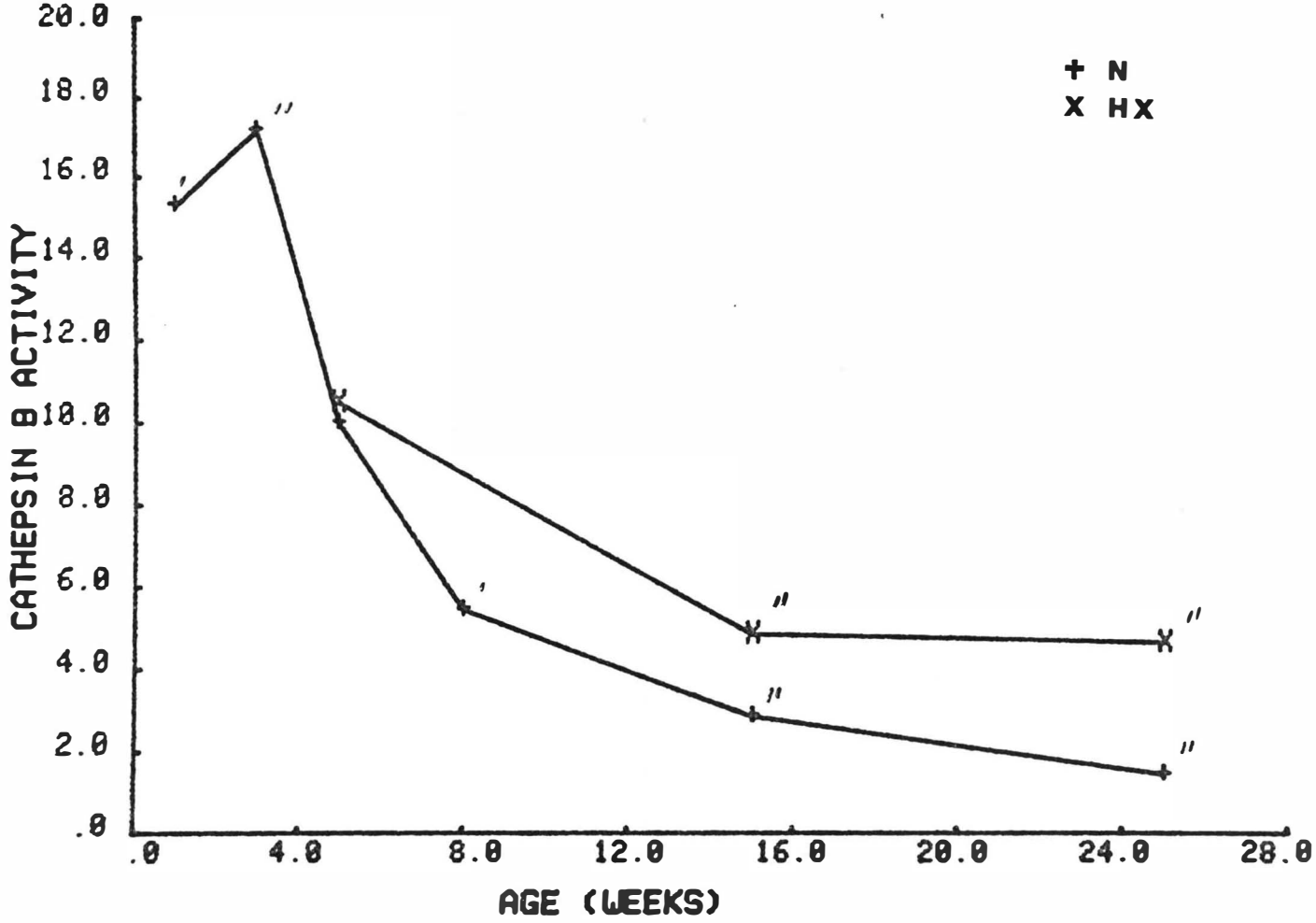

+ N
X HX
CATHEPSIN B ACTIVITY
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 26. Age-dependence of cartilage acid phosphatase activity (per protein content) of normal and hypophysectomized rats. Enzyme activities were determined in 5% cartilage homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed as percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

[+]N = Normal rats.

[x]Hx = Hypophysectomized rats.

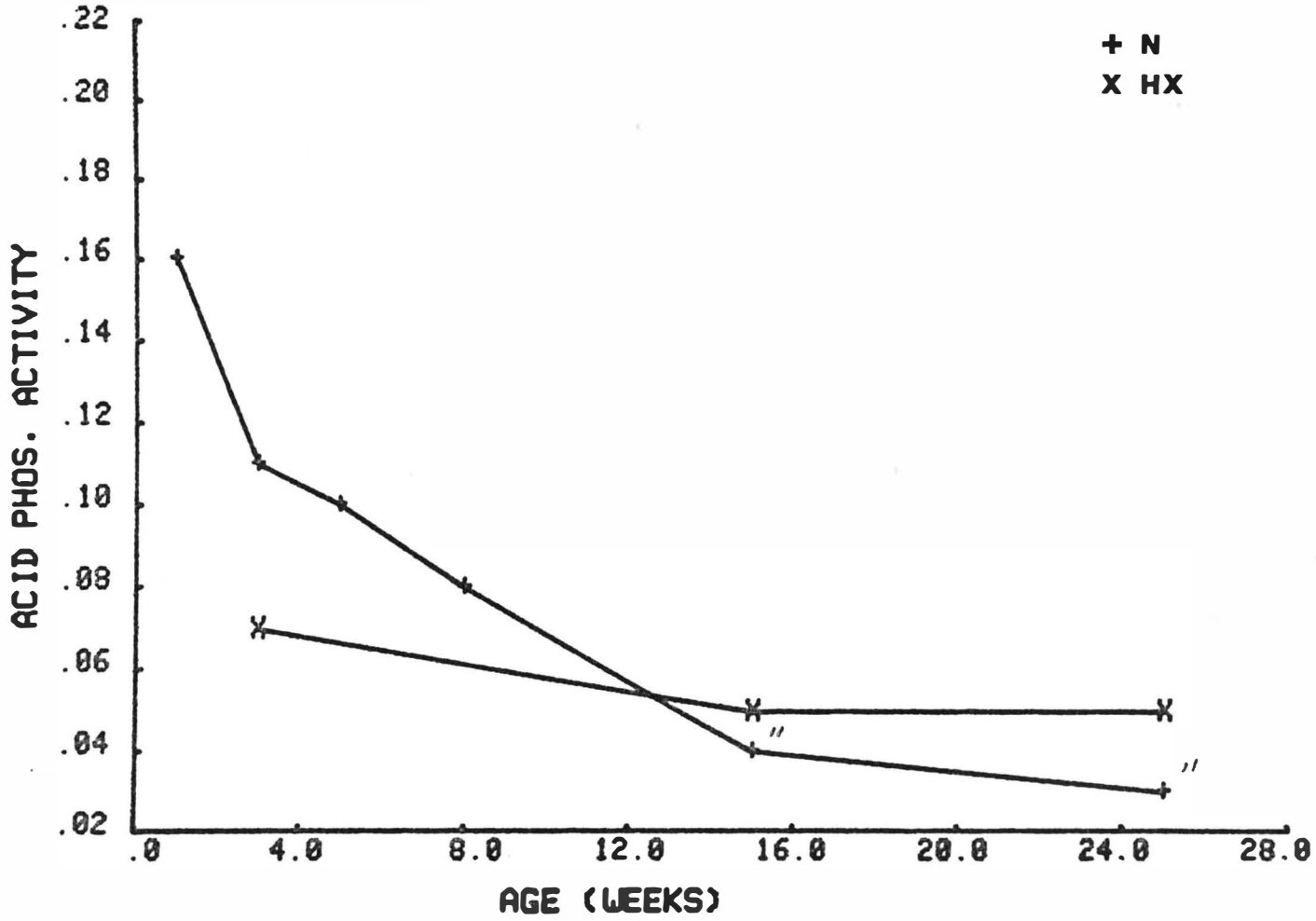

+ N
X HX
ACID PHOS. ACTIVITY
.22
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 27. Age-dependence of cartilage acid phosphatase activity (per DNA content) of normal and hypophysectomized rats. Enzyme activities were determined in 5% cartilage homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

[+]N = Normal rats.

[x]Hx = Hypophysectomized rats.

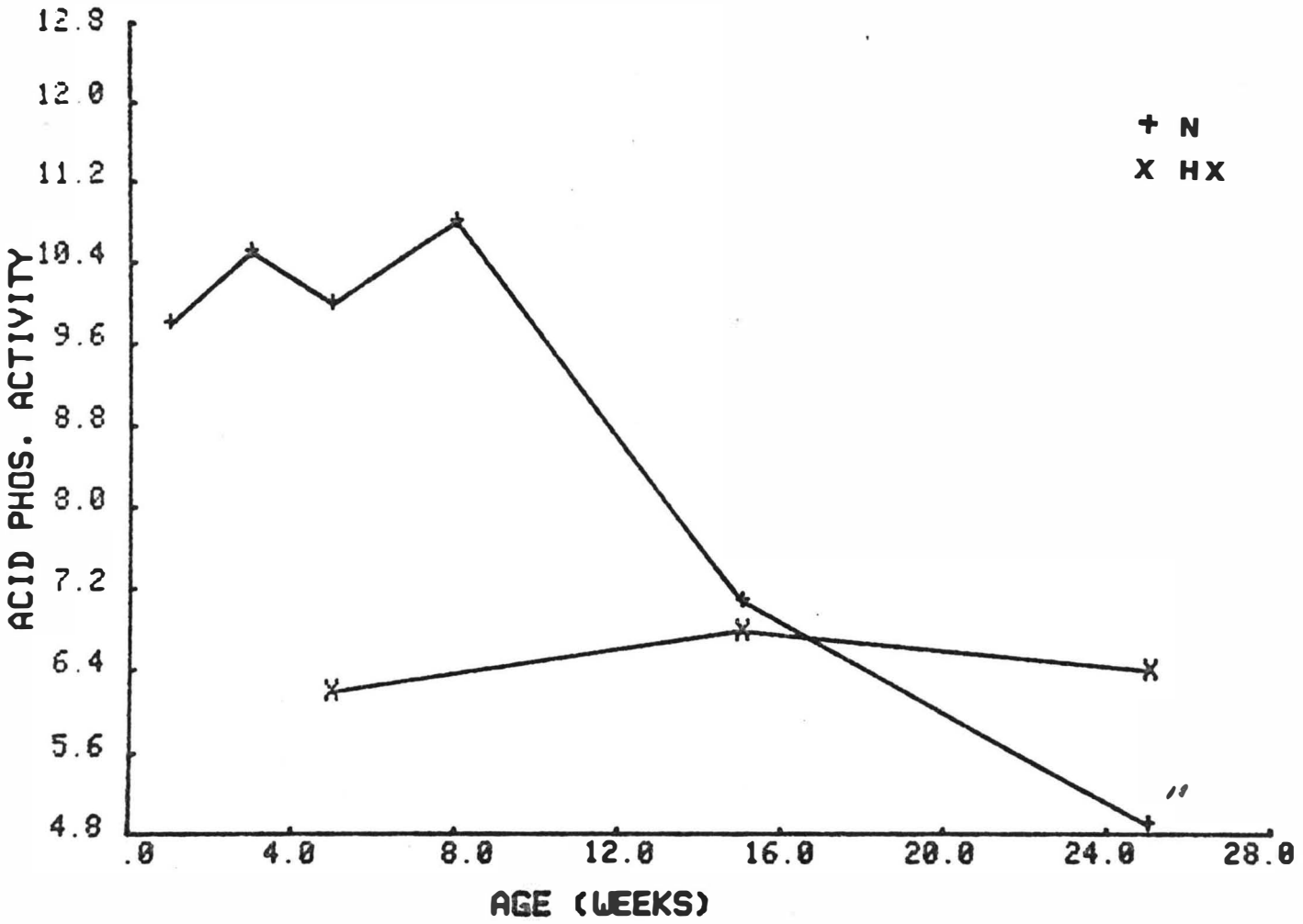

12.8
12.0
11.2
10.4
9.6
8.8
8.0
7.2
6.4
5.6
4.8
ACID PHOS. ACTIVITY
+ N
X HX
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 28. Direct comparison of three cartilage acid hydrolase activities (per protein content) as a function of rat age. Enzyme activities were determined in 5% cartilage homogenates of normal rats differing in age. Results are expressed as percent x 10^{-1} of activity of normal rats 5 weeks of age. The SE averaged $\leq$ 12% of the mean.

[1]Different from cathepsin B values in the same age group, $P < .05$ by Dunnett's analysis.

D = Cathepsin D values.

B = Cathepsin B values.

AP = Acid phosphatase values.

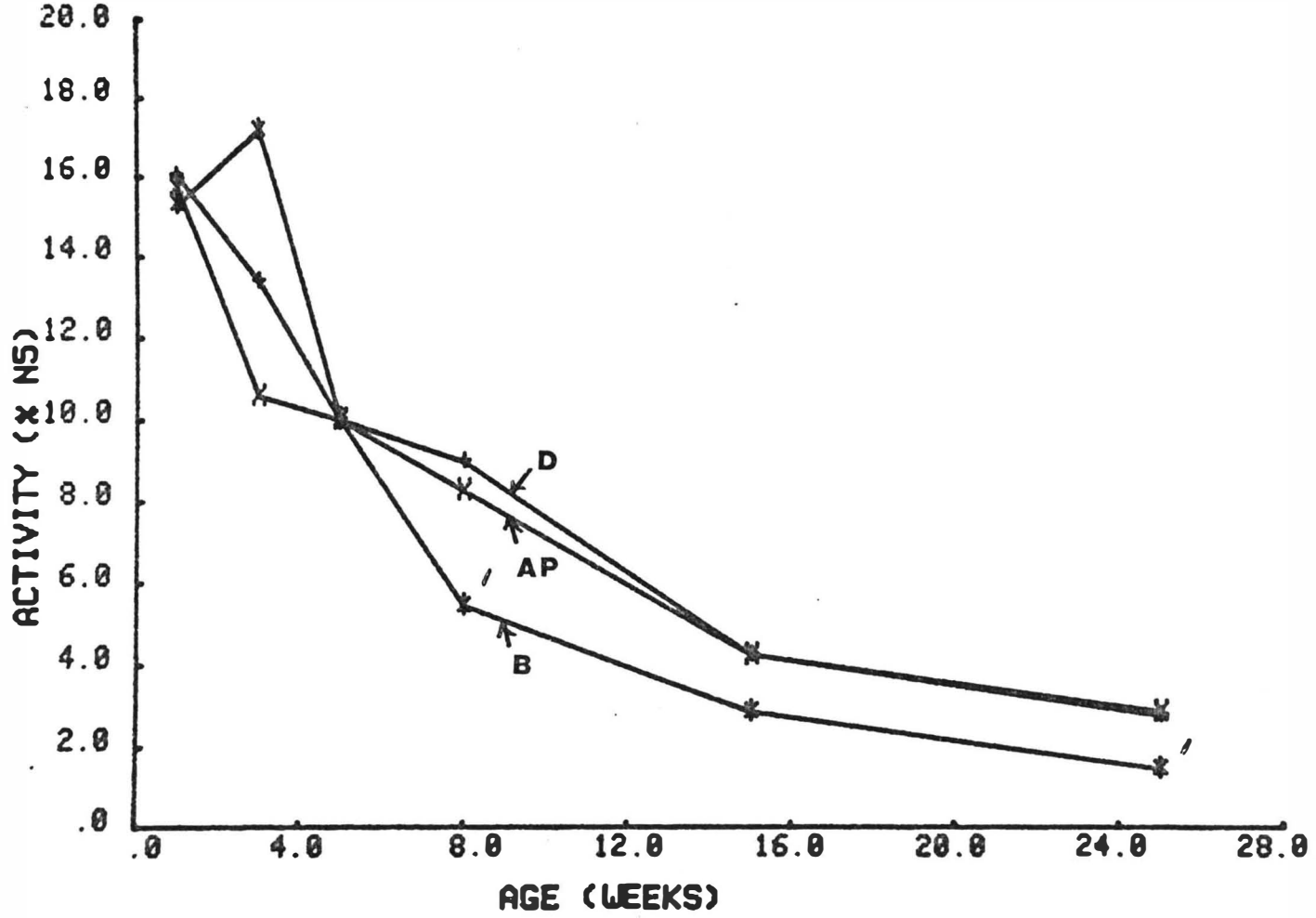

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
ACTIVITY (x NS)
D
AP
B
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 29. Direct comparison of three cartilage acid hydrolase activities (per DNA content) as a function of rat age. Enzyme activities were determined in 5% cartilage homogenates of normal rats differing in age. Results are expressed as percent x 10^{-1} of activity of normal rats 5 weeks of age. The SE averaged $\leq$ 12% of the mean.

[1]Different from cathepsin B values in the same age group, $P < .05$ by Dunnett's analysis.

D = Cathepsin D values.

B = Cathepsin B values.

AP = Acid phosphatase values.

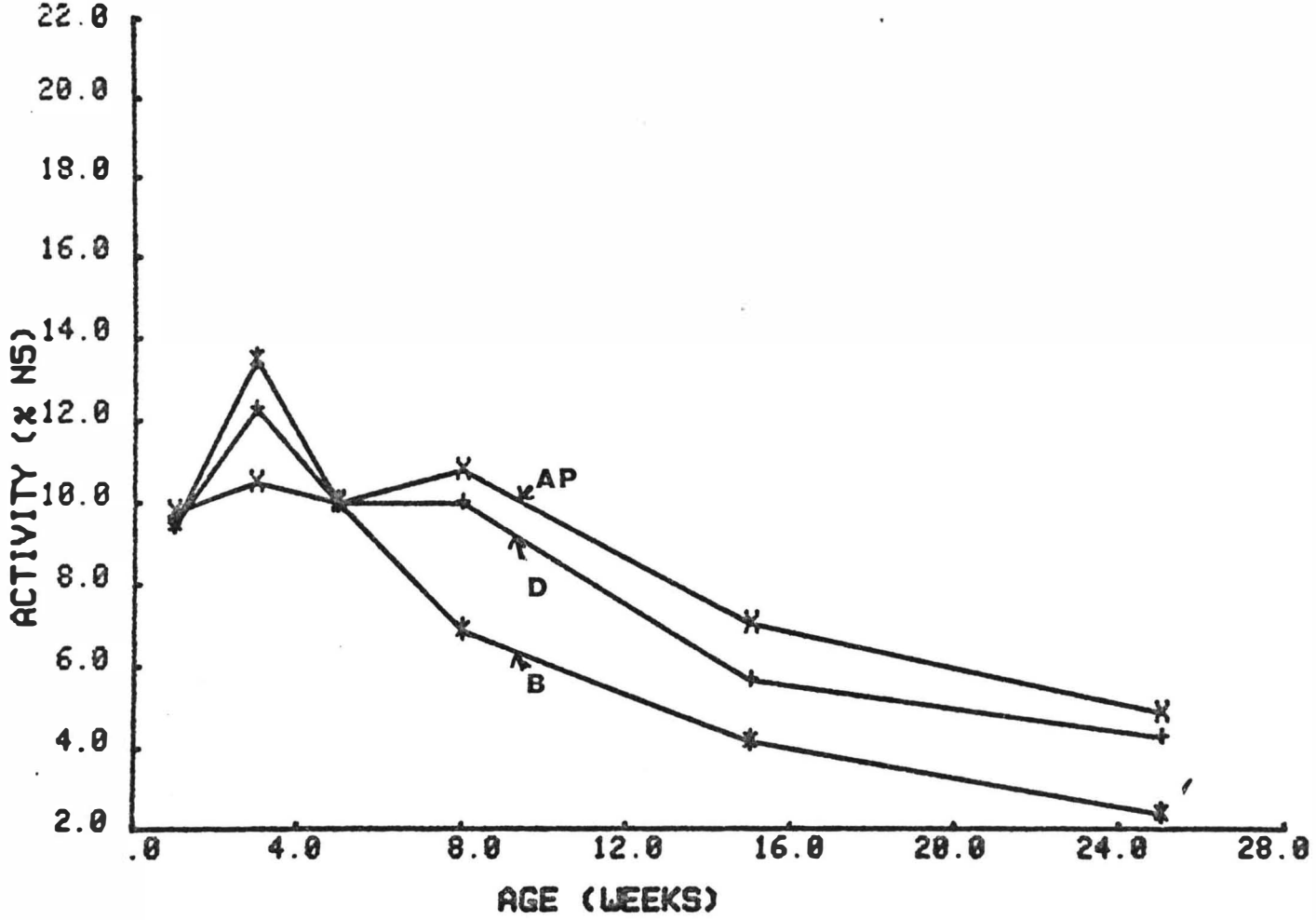

22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
ACTIVITY (% NS)
AP
D
B
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 30. Direct comparison of three cartilage acid hydrolase activities (per protein content) as a function of hypophysectomized (Hx) rat age. Enzyme activities were determined in 5% cartilage homogenates of Hx rats differing in age. Results are expressed as percent x 10^{-1} of hypophysectomized rats 5 weeks of age. The SE averaged $\leq$ 12% of the mean.

[1]Different from cathepsin D values of the same age group, $P < .05$ by Dunnett's analysis.

D = Cathepsin D values.

B = Cathepsin B values.

AP = Acid phosphatase values.

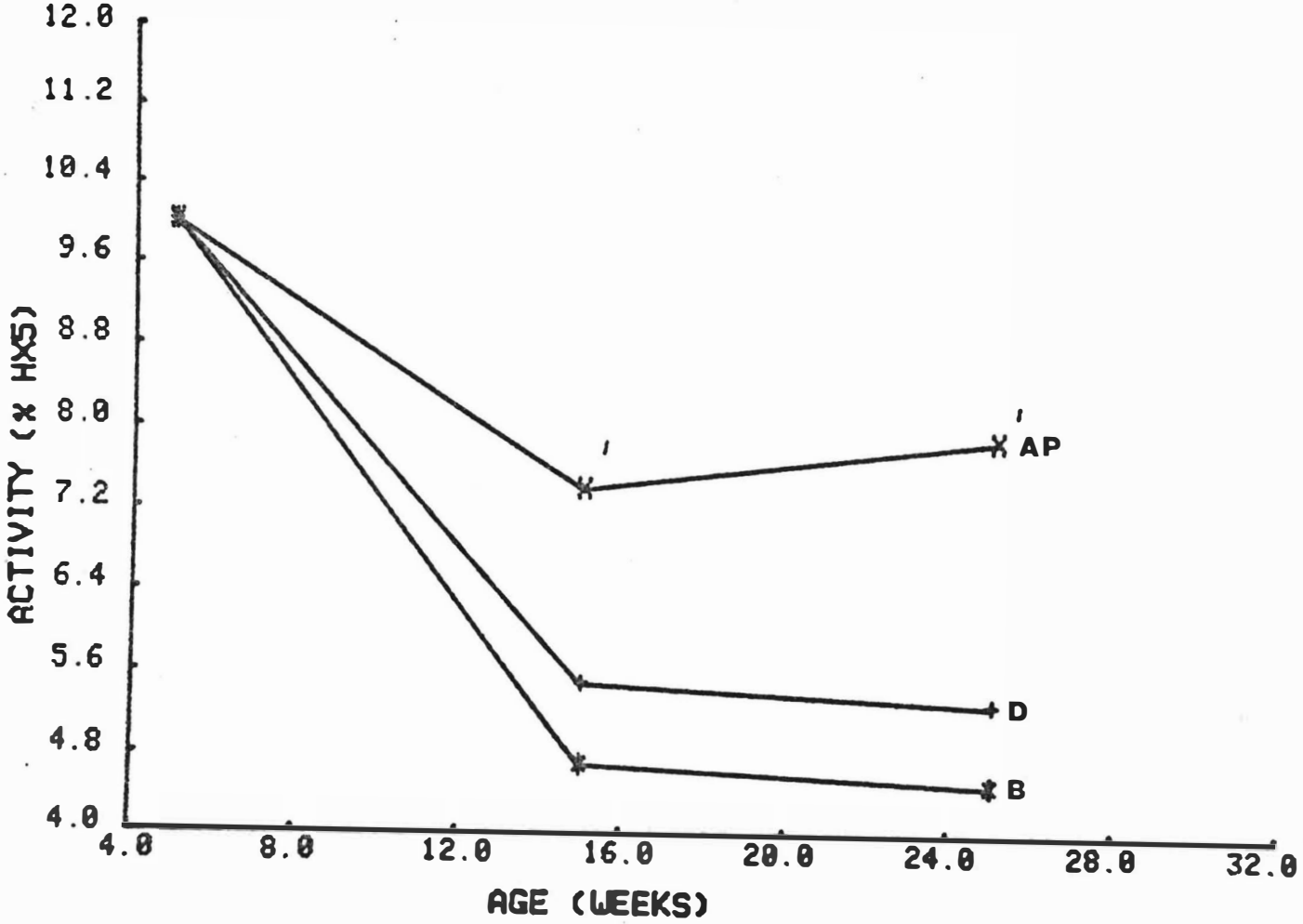

12.0
11.2
10.4
9.6
8.8
8.0
7.2
6.4
5.6
4.8
4.0
ACTIVITY (x HX5)
AP
D
B
4.0
8.0
12.0
16.0
20.0
24.0
28.0
32.0
AGE (WEEKS)

Figure 31. Direct comparison of three cartilage acid hydrolase activities (per DNA content) as a function of hypophysectomized (Hx) rat age. Enzyme activities were determined in 5% cartilage homogenates of Hx rats differing in age. Results are expressed as percent x 10^{-1} of hypophysectomized rats 5 weeks of age. The SE averaged $\leq$ 12% of the mean.

[1]Different from cathepsin D values of the same age group, $P < .05$ by Dunnett's analysis.

D = Cathepsin D values.

B = Cathepsin B values.

AP = Acid phosphatase values.

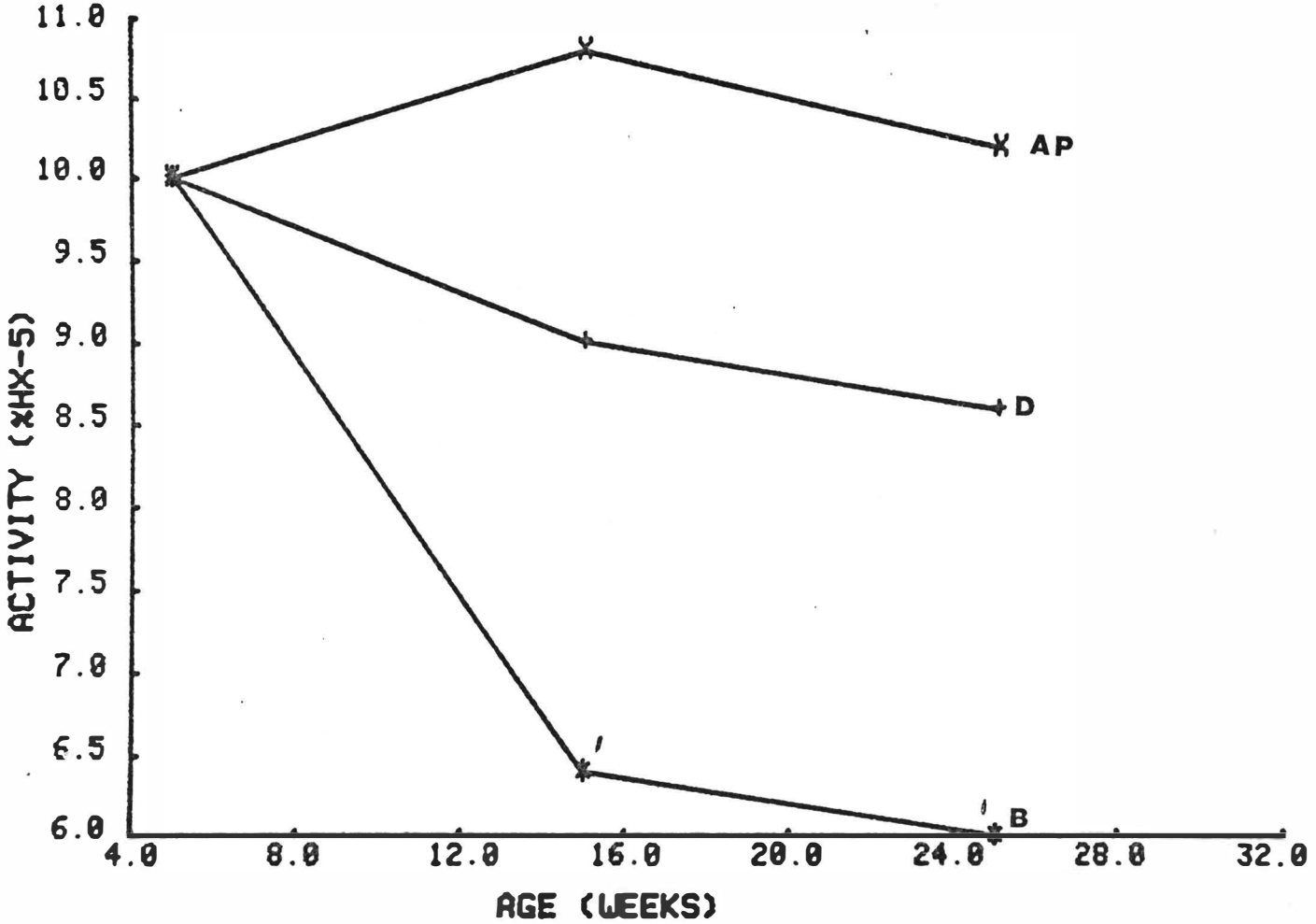

11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
ACTIVITY (xHX-5)
4.0
8.0
12.0
16.0
20.0
24.0
28.0
32.0
AGE (WEEKS)
AP
D
B

Table 24

Pepstatin Titration of Cartilage Cathepsin D Activity from Rats Varying in Age

Experimental Group	Enzyme Activity (% N_{3-8})	Concentration of pepstatin producing 50% inhibition of cathepsin D activity (% N_{3-8})
N_{3-8}	100 ± 2 (6)	100 ± 5 (6)
N_{25-38}	42 ± 5 (6)**	43 ± 7 (5)**

The concentration of pepstatin required to produce 50% inhibition of cartilage homogenate cathepsin D activity was determined. In each assay, several concentrations of pepstatin were added to a constant amount of cartilage homogenate. Results are expressed as % N_{3-8} activity ± SE (n = number of cartilage samples analyzed).

N_{3-8} = Normal rats 3-8 weeks of age.

N_{25-38} = Normal rats 25-38 weeks of age.

**Different from N_{3-8} values, $P < .01$ by Dunnett's analysis.

The effect of rat age on liver lysosomal enzyme activities is shown in Figs. 32-37. Compared with cartilage tissue, the change in liver enzyme activity with rat aging was relatively small. Basically the normal liver activities remained constant over the age range studied or displayed increasing activity from one week to about eight weeks of age, followed by a plateauing of the activity (or a gradually decreasing activity from about 15 weeks to 25 weeks of age). Thus, the change of normal liver lysosomal enzymes was quite different from that observed with normal cartilage tissue. However, similar to cartilage results, Hx rat liver activities remained statistically unaltered over the age range studied.

Figure 32. Age-dependence of liver cathepsin D activity (per protein content) of normal and hypophysectomized rats. Enzyme activities were determined from 10% liver homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

No statistical differences were found (compared to comparable rats 4.5 weeks old).

N = Normal rats.

Hx = Hypophysectomized rats.

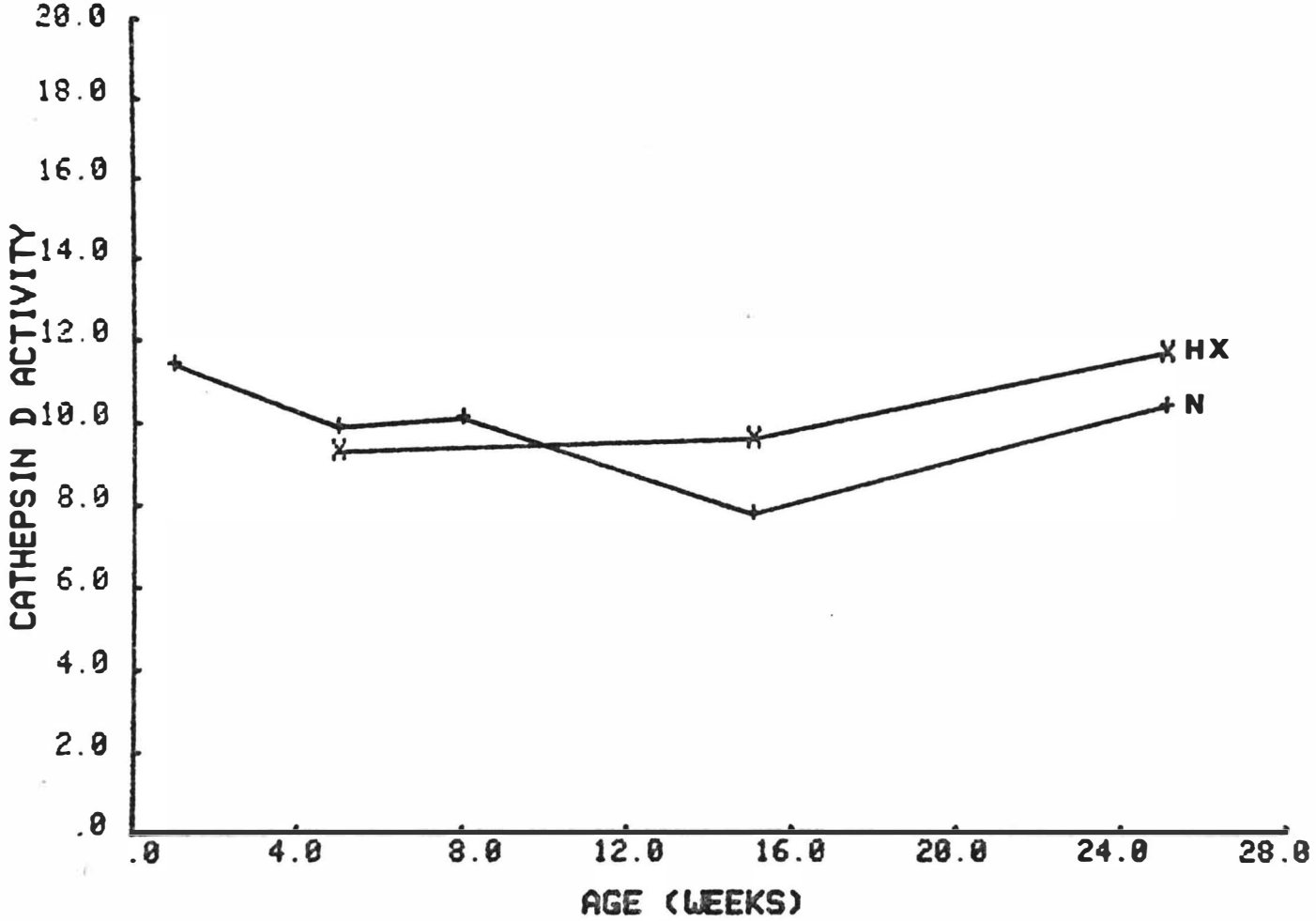

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
CATHEPSIN D ACTIVITY
HX
N
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)

Figure 33. Age-dependence of liver cathepsin D activity (per DNA content) of normal and hypophysectomized rats. Enzyme activities were determined from 10% liver homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

No statistical differences were found (compared to comparable rats 4.5 weeks old).

N = Normal rats.

Hx = Hypophysectomized rats.

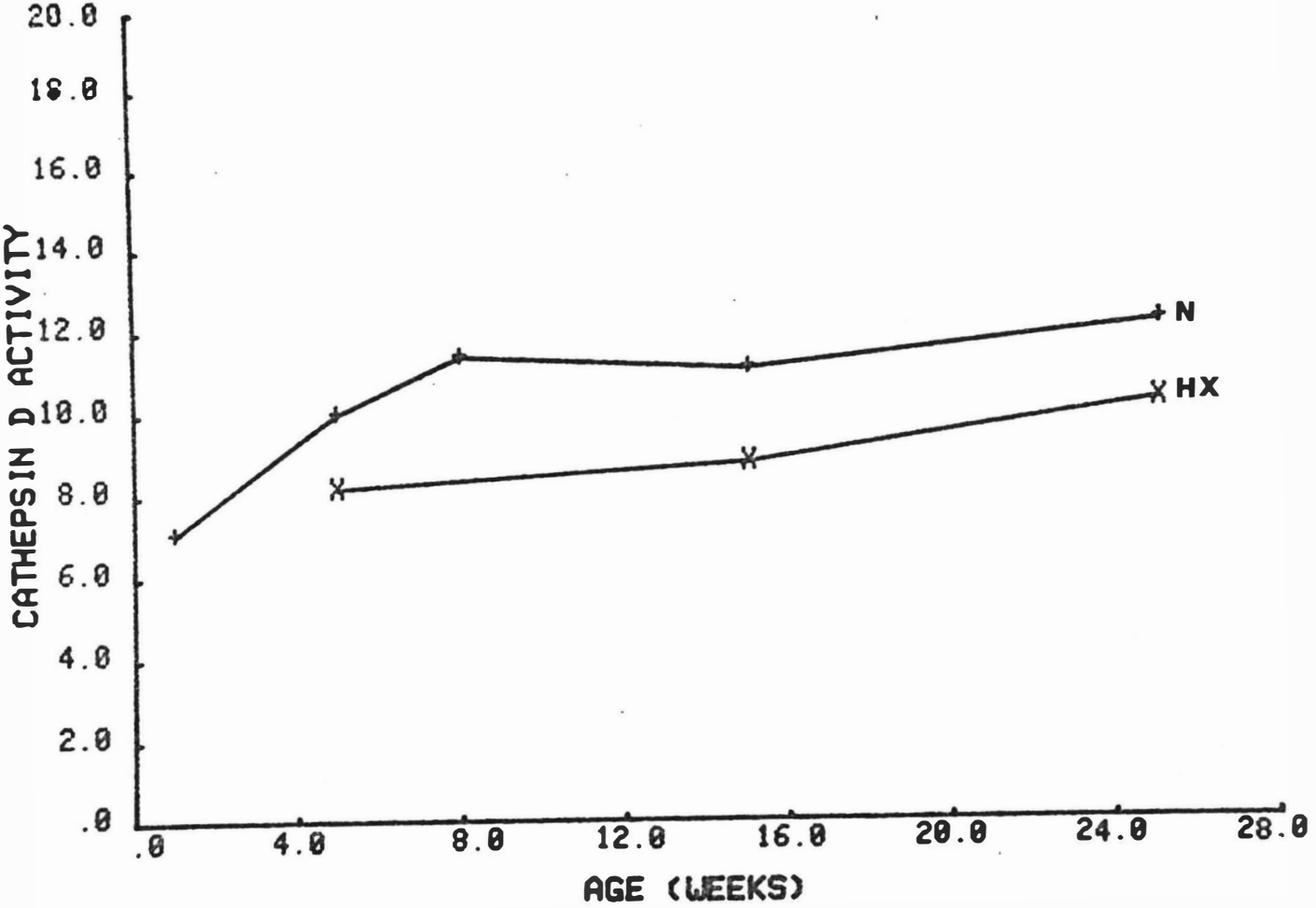

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
CATHEPSIN D ACTIVITY
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)
N
HX

Figure 34. Age-dependence of liver cathepsin B activity (per protein content) of normal and hypophysectomized rats. Enzyme activities were determined from 10% liver homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[1]Different from values of the comparable rats 4.5 weeks old, $P < .05$ by Dunnett's analysis.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

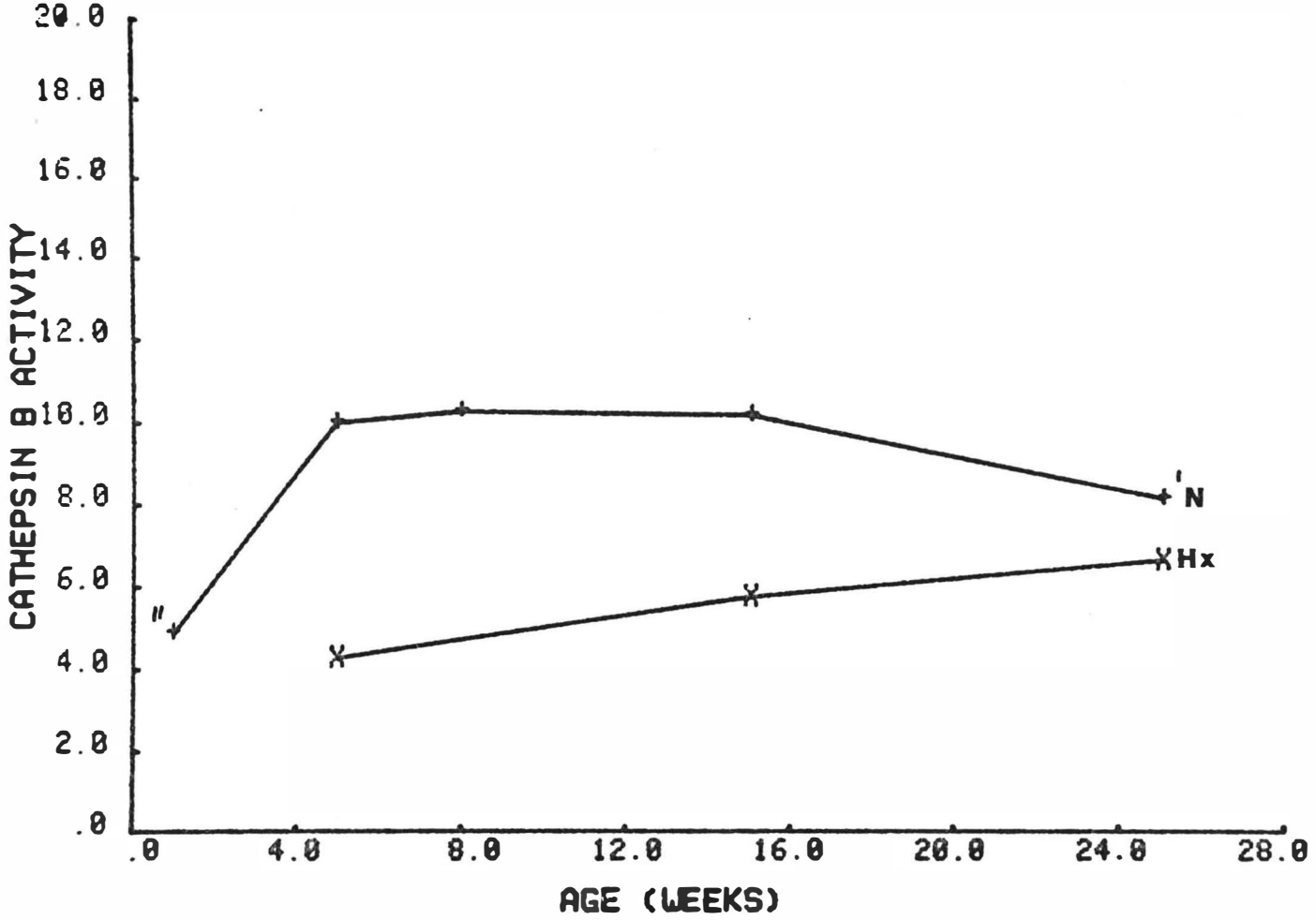

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
CATHEPSIN B ACTIVITY
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)
N
Hx

Figure 35. Age-dependence of liver cathepsin B activity (per DNA content) or normal and hypophysectomized rats. Enzyme activities were determined from 10% liver homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed as percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

N = Normal rats.

Hx = Hypophysectomized rats.

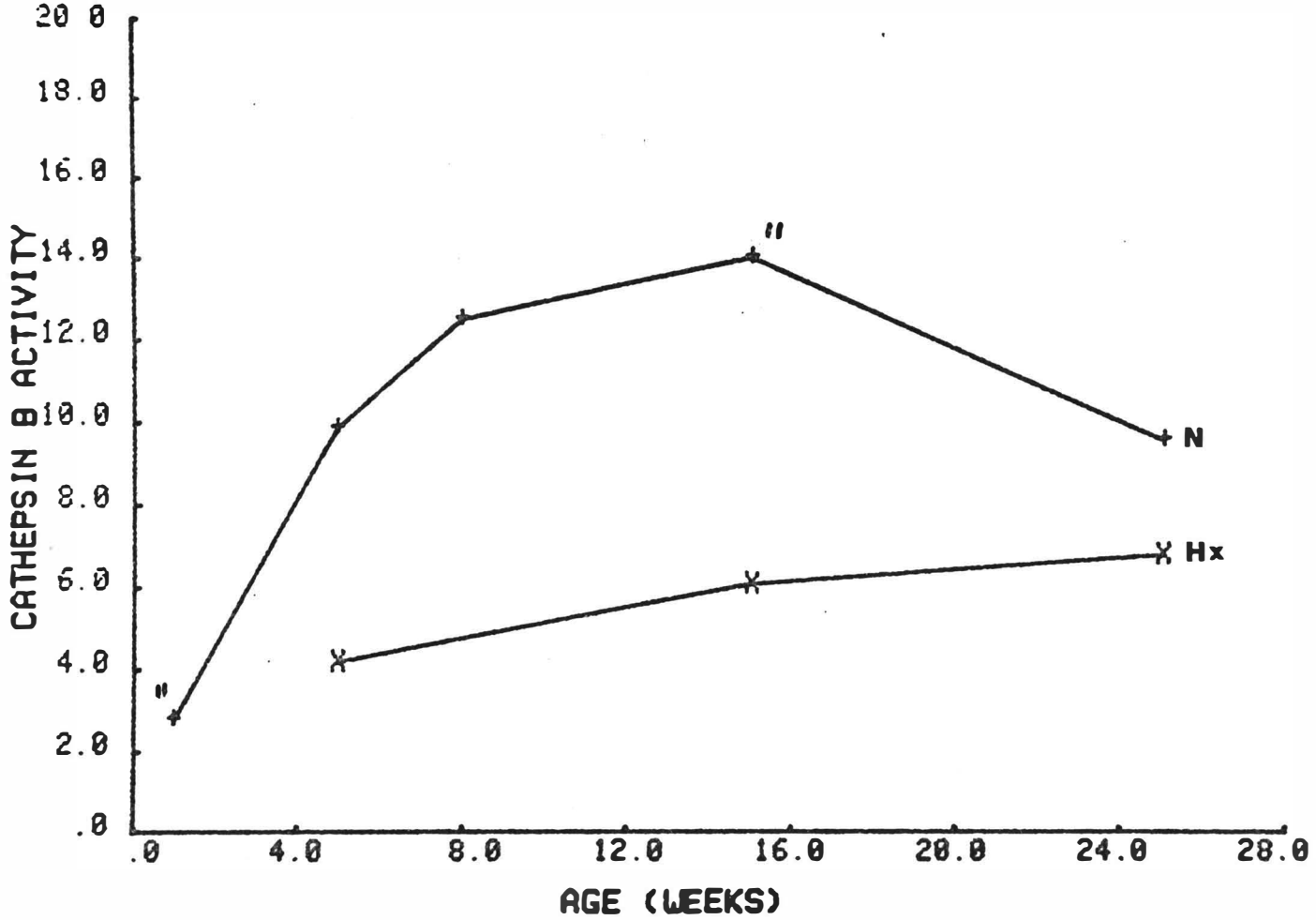

20 0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
CATHEPSIN B ACTIVITY
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)
N
Hx

Figure 36. Age-dependence of liver acid phosphatase activity (per protein content) of normal and hypophysectomized rats. Enzyme activities were determined from 10% liver homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed as percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[1]Different from values of the comparable rats 4.5 weeks old, $P < .05$ by Dunnett's analysis.

N = Normal rats.

Hx = Hypophysectomized rats.

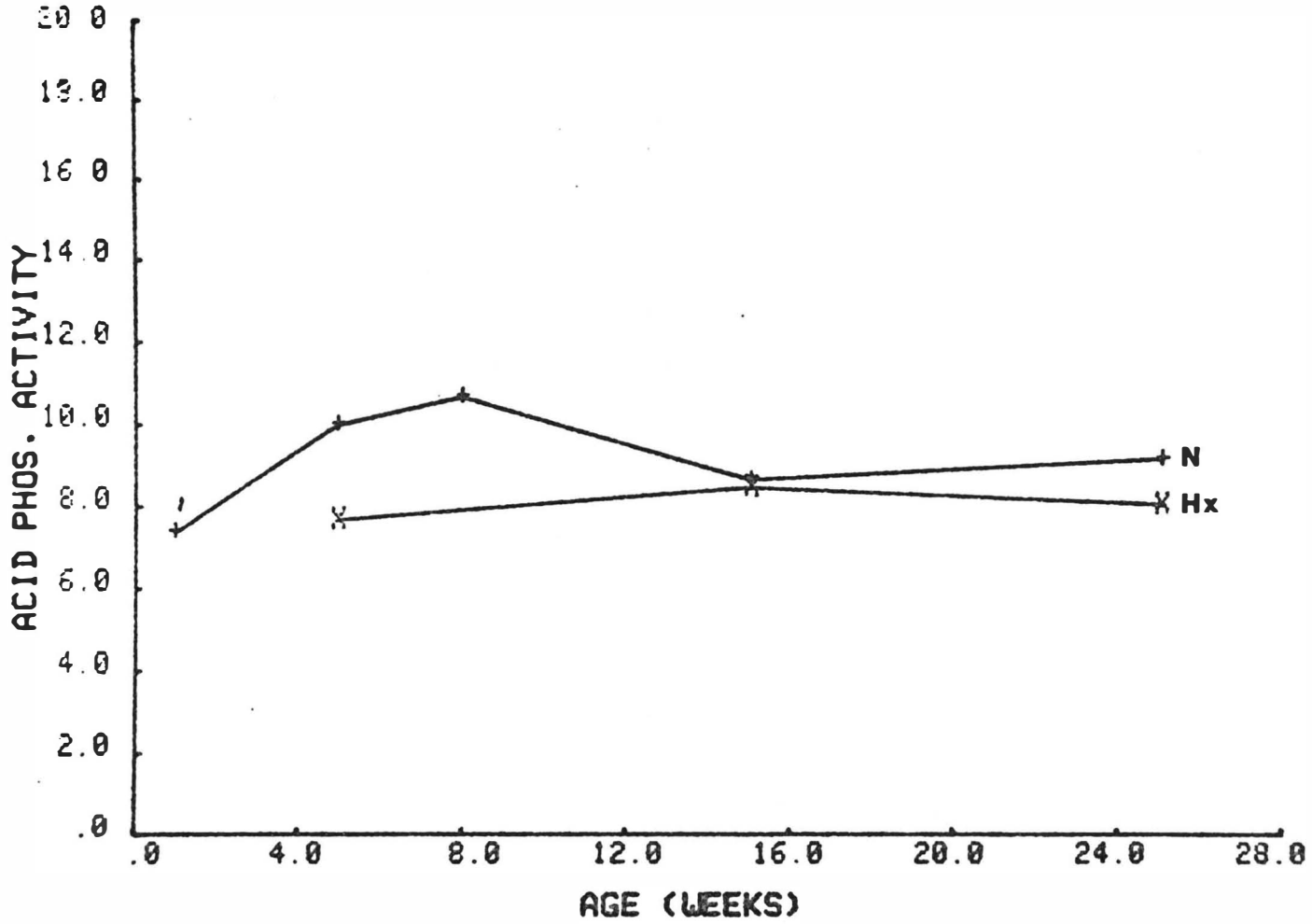
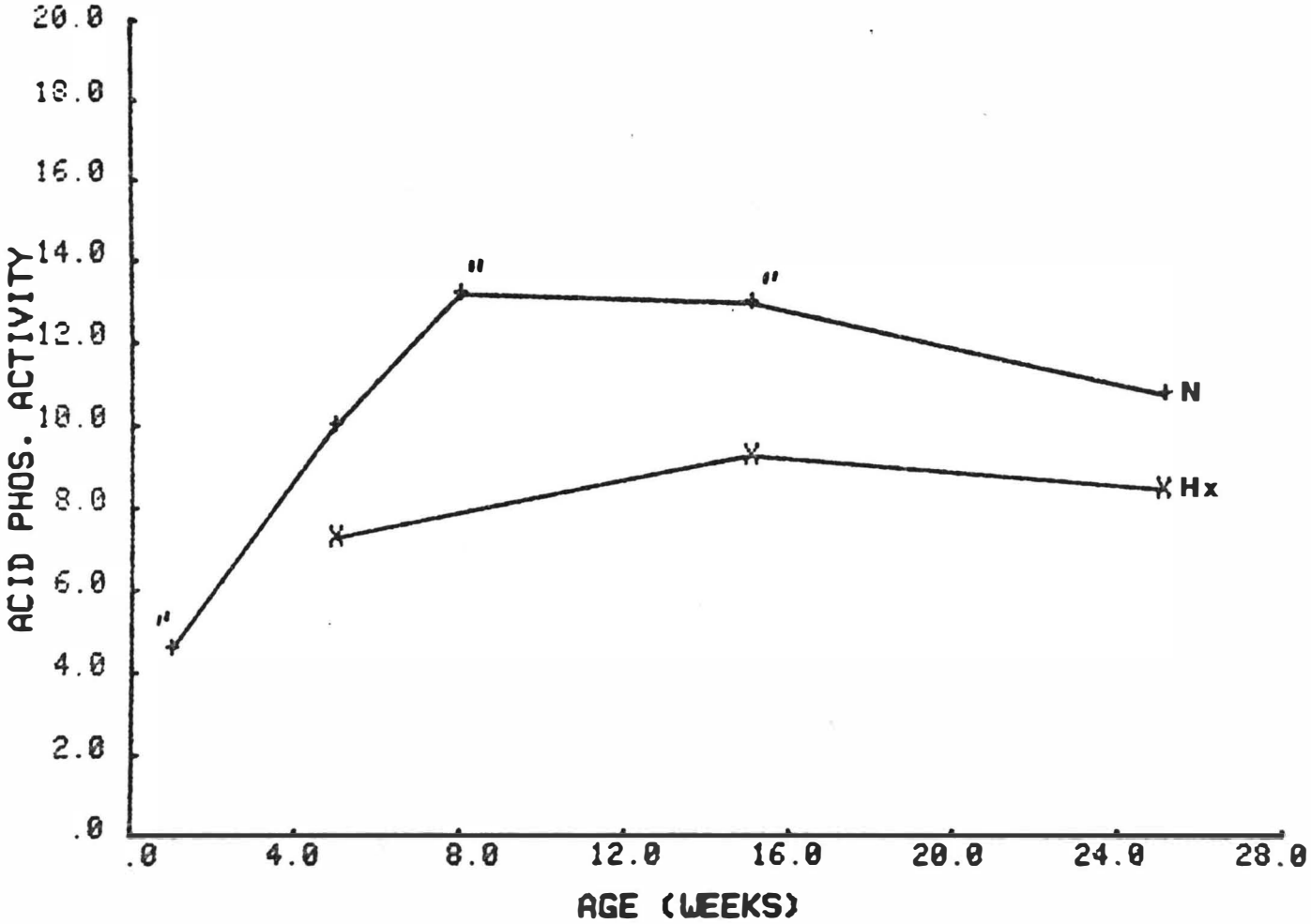

ACID PHOS. ACTIVITY
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)
N
Hx

Figure 37. Age-dependence of liver acid phosphatase activity (per DNA content) of normal and hypophysectomized rats. Enzyme activities were determined from 10% liver homogenates of normal and hypophysectomized rats differing in average age from 1 week to 25 weeks. Results are expressed at percent x 10^{-1} of activity of normal rats averaging 4.5 weeks of age. The SE averaged < 12% of the mean.

[11]Different from values of comparable rats approximately 4.5 weeks old, $P < .01$ by Dunnett's analysis.

N = Normal rats.

Hx = Hypophysectomized rats.


20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
.0
ACID PHOS. ACTIVITY
.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
AGE (WEEKS)
N
Hx

Chapter V

DISCUSSION

These results clearly show that cartilage cathepsin D and acid phosphatase activities are, at least in part, regulated by GH and parallel skeletal growth rate. These acid hydrolases may be intimately involved in the biochemical mechanism of GH action and skeletal growth in general as based on 1) the direct evidence presented in this thesis that these enzymes closely parallel GH and age-dependent cartilage growth rates, 2) observations by others that certain proteases and lysosomal lysates stimulate cartilage cell proliferation and tissue remodeling (68a, b, c), 3) studies by other groups which showed enhanced activity of these acid hydrolases in transformed tissues, i.e. dividing cells (60a, b, c) and 4) results of preliminary studies by the author (and M. Bembenek of the same laboratory) which indicate that pepstatin inhibits costal cartilage and chondrosarcoma DNA synthesis.

The effect of GH on skeletal growth and overall body growth is well established. However, the effect of GH on growth was investigated in this study so that a direct comparison of enzyme activities and growth rate could be made. GH (500 ug total dose) significantly increased rat body and skeletal growth as based on rat body weight, costal cartilage wet weight, and costal cartilage DNA content (Tables 4 and 5). Only a marginal effect of GH on cartilage protein content (Table 5) and liver growth (Table 6) was found. However, this observation does not necessarily indicate a lack of an anabolic influence by GH on liver growth and

cartilage protein synthesis since 1) the parameters examined represent the net effect of anabolic and catabolic processes, 2) statistically significant increases may require a longer injection period, and 3) more sensitive techniques may be required. For example, incorporation of radioactive precursors into macromolecules has revealed that GH, under similar experimental conditions, increases liver and cartilage anabolic activities (97, 98). Based on the results of this study, the GH dependent enhanced turnover of cartilage protein (as well as its enhanced synthesis) is a particularly enticing explanation of the slow net increase in cartilage protein.

The inverse relationship between normal rat age and normal rat growth rate is clearly shown in Tables 7-10. Rat body weight (Table 7), skeletal growth rate (Tables 8 and 9), and liver growth rate (Tables 8 and 10) all decreased with increasing rat age. Thus rat aging and development could be a useful experimental model of skeletal (or general) growth rate regulation. In addition, the blunted effect of aging on growth in Hx rats (Tables 7-10) provides a model of aging which is largely independent of changes in growth rate.

In a preliminary report, N. Owers (84c) showed that an unknown cartilage protease activity may be regulated by GH. This laboratory, in collaboration with N. Owers, more rigorously investigated and quantified the effect of GH on this cartilage acid protease activity. The assay system utilized had the advantage of high sensitivity and low specificity. Thus, detection of potential changes in one or more acid proteases (even those heretofore unidentified) was optimized.

Hypophysectomy greatly reduced a cartilage acid protease activity as shown in Fig. 20. Since GH is the primary regulator of skeletal growth,

a priori it appeared to be the most likely pituitary hormone to modulate this activity. Treatment of Hx rats with GH enhanced protease activity to about normal levels. This observation suggested that the pituitary-dependent acid protease activity is primarily regulated by GH.

The difference between normal age and normal weight protease specific activities suggested that this activity was age-dependent as well. This relationship was further investigated since it potentially could shed more light on the correlation between protease activity and skeletal growth rate. Younger, more rapidly growing animals were found to have higher protease activities than older, less rapidly growing animals (Table 16).

Thus, both GH- and age-dependent growth were correlated with a cartilage, acid protease activity. This observation suggested that one or more of the lysosomal, acid proteases (called cathepsins) may be related to skeletal growth. Therefore, the relationship of three known lysosomal enzymes (including 2 cathepsins) to rat growth rate was studied.

The enzymes chosen for further investigation included cathepsin D, cathepsin B, and acid phosphatase. Cathepsin D was investigated since 1) it is perhaps the major cartilage lysosomal enzyme, 2) its pH optimum on most substrates approximates that the unknown GH-dependent protease, 3) several studies suggested that it may be involved in cartilage remodeling, and 4) it appears to be enhanced in other tissues during rapid growth. Cathepsin B was studied since 1) its pH optimum on most substrates approximates that the unknown GH-dependent protease, 2) several studies suggested that it could be involved in cartilage remodeling and 3) investigation of a second lysosomal protease may provide information as to whether lysosomal cathepsins are regulated independently or as a group in certain physiological situations. Acid phosphatase was the third enzyme investi-

gated since 1) it is elevated in other tissues during rapid growth, 2) it too may be involved with cartilage remodeling and mineralization and 3) it is a non-cathepsin lysosomal enzyme which could be used to probe the relationship of cathepsins to other lysosomal enzymes during enhanced growth rate.

In this study, GH was shown to regulate cartilage cathepsin D and acid phosphatase activities, but not cathepsin B activity in vivo (Tables 17-19). Thus, GH appeared to have a selective influence on cartilage acid hydrolases. Since pepstatin experiments suggested that there were more enzyme molecules in normal versus Hx rat cartilage, it was possible that GH stimulated induction of cathepsin D (and possibly acid phosphatase).

Supportive evidence that GH regulates these acid hydrolases is shown by 1) a preliminary study by Owers (99) indicating that pepstatin inhibits most of the GH dependent protease activity in the gelatin-membrane assay and 2) Steinetz and Manning's study (83) which showed that a very high dose of GH (3.5 mg per mouse) enhanced Hx mouse pubic cartilage acid phosphatase activity. The effect of GH on these specific lysosomal enzymes may provide insight into which enzymes are most likely to have a major role in skeletal growth, and whether their activities may have a positive or negative effect on the process. Thus, since cathepsin D and acid phosphatase were enhanced by GH-treatment, these enzymes may have a positive effect on cartilage growth and may be intimately involved in the mechanism of GH action. On the other hand, cathepsin B was not affected by GH. Therefore, although this enzyme could still be involved in the mechanism of growth, it is unlikely that it plays a major regulatory role in the process.

T_3, which often potentiates the anabolic effects of GH (100),

was used in this study 1) to show that cartilage cathepsin B was not GH-dependent even in the presence of T_3 (Table 19) and 2) as a positive control in liver studies in which GH did not stimulate lysosomal enzymes.

In liver, hypophysectomy produced a marked decrease in cathepsin B activity, but had little or no effect on cathepsin D and acid phosphatase activities. GH administration had no effect on liver cathepsin D, acid phosphatase, or cathepsin B activities (Tables 21-23). Thus, although liver lysosomal enzymes, particularly cathepsin B, may be partially regulated by one or more pituitary hormones, GH alone does not appear to be the pituitary regulator. Mathies et al. (30), and later DeMartino and Goldberg (78), showed that T_3 greatly enhanced liver lysosomal enzymes. In the present study, thyroxine (alone or in combination with bGH) appeared to increase hepatic cathepsin D and acid phosphatase activities but had only a small effect on cathepsin B activities, particularly when expressed per DNA content. Our results indicate that, unlike cartilage, liver lysosomal enzyme activities are not closely correlated with GH-dependent growth. Therefore, the relationship between GH and these lysosomal enzymes is, at least partially, tissue specific.

In other studies, several hormones, e.g., sex hormones, glucocorticoids and parathyroid hormone, have been shown to influence target tissue lysosomal enzyme activities, lysosomal migration and lysosomal fragility (79-83). Interestingly, Szego (77 and 101) has proposed a model for lysosome-mediated mechanisms of hormone action. Our results support certain features of that general proposal.

Age-dependent changes in cartilage growth rate and enzyme activities supported the positive correlation of cartilage cathepsin D and acid phosphatase activities to skeletal growth rate found in the GH studies. Cartilage growth rate, as well as cathepsin D and acid phosphatase

activities, were inversely proportional to rat age (Fig. 28). Thus, younger, more rapidly growing rats exhibited higher activities of these two enzymes than that of older, less rapidly growing animals, irrespective of the manner in which activities were expressed, i.e., per wet weight tissue, DNA content or protein content. The decrease in cartilage lysosomal enzymes was due apparently to decreases in cellularity and enzyme activity per cell. This was suggested by the less pronounced, but statistically significant, decrease in enzyme activities when expressed per DNA compared with expressing the activities per protein or tissue weight. Interestingly, cartilage cathepsin D and acid phosphatase activities changed with age at about the same rate (Fig. 28). This suggests, albeit indirectly, that the molecular mechanism of their regulation may be similar and perhaps coordinated. It may be noted that the age-dependent decrease of cartilage hydrolases is compatible with Bezkorovain's theory of aging (102) in which predicted decreases in proteolytic activities with increasing age result in higher levels of defective enzymes and thus less efficient cellular function.

Cartilage cathepsin B activity also dramatically decreased with rat age at a slightly more rapid rate than that of cathepsin D or acid phosphatase (Fig. 28). The relationship of cartilage cathepsin B to skeletal growth rate is complex since this enzyme was not related to GH-dependent growth rate but positively correlated with age-dependent growth rate. However, it is apparent that cathepsin B activity did not parallel cathepsin D and acid phosphatase activities in cartilage and possibly liver as well.

Hx rat cartilage lysosomal enzymes exhibited less age-dependency than those of normal rats. Thus, while both lysosomal enzyme activities and cartilage growth rate decreased with age in normal rats, both of these

parameters changed much less in Hx rats. Interestingly, secretion of and tissue responsiveness to GH diminish with age (103a, b). Thus, the age-dependence of cartilage cathepsin D and acid phosphatase in normal animals could be related in part to their GH-dependence.

Consistant patterns of enzyme changes with age are often difficult to observe in liver tissue, possibly owing to the complex regulation and cell population of the tissue. Thus, conflicting reports may be found in the literature (72a, b, c). The primary significance of the results reported in this study was that the variation in liver and cartilage lysosomal enzymes with age was dissimilar. Thus, there appears to be a tissue specificity in the age-dependent regulation of lysosomal enzyme activities. Liver acid hydrolase activities were not clearly related to tissue growth rate in this study. The possibility should not be overlooked that certain hepatic proteases and other hydrolases, not examined in this study are related to liver growth. Interestingly, as found in cartilage tissue, the age-dependent pattern of hepatic lysosomal enzyme activities was altered by hypophysectomy (Figs. 32-37). Thus, in liver and cartilage the age-dependence of lysosomal enzyme activities appears to be related at least in part to pituitary function. However, the specific pituitary hormones related to this phenomenon are possibly quite different in these tissues.

Several observations by other groups may lend insight to the roles of cathepsin D and acid phosphatase in skeletal growth. For example, the proteolytic activity of cathepsin D may have a direct effect on DNA synthesis and cellular mitosis. Injection of certain proteases or lysosomal lysates into animal joints has been shown to enhance cartilage cell proliferation (68a, b, c). Preliminary experiments by the author have indicated that pepstatin, an inhibitor of cathepsin D, in-

hibits cartilage DNA synthesis *in vitro*, as assayed by ^{3}H-thymidine uptake into acid-precipitable material. In addition, other workers in Dr. Liberti's laboratory have shown that pepstatin also inhibits ^{3}H-thymidine uptake into chondrosarcoma cells *in vitro*. Interestingly, in osteoarthritis there is an increase in lysosomal enzyme activities (including cathepsin D, cathepsin B and acid phosphatase) and a transient increase in cell proliferation. The action of cathepsin D and acid phosphatase on surrounding matrix macromolecules could serve several other functions including a) increasing cartilage plasticity for interstitial growth, b) supplying nutrients for new cell synthetic processes needed for growth, and c) preparing the matrix for mineralization. Both cathepsin D and acid phosphatase have been shown to be released by chondrocytes (104a, b), and several studies suggest that they can use matrix macromolecules as substrates (105a, b, c). It should be noted, however, due to pH considerations, that their activities may be limited to microenvironments immediately surrounding the cartilage cells.

Because many of the actions of GH appear to be mediated by somatomedin (13a, b), it is possible that somatomedin, *in lieu* of GH, is directly responsible for stimulation or maintainance of cartilage cathepsin D and acid phosphatase activities.

In summary, the following basic relationships between enzyme activities and growth rate have been demonstrated in this thesis (shown on page 140):

Relative Enzyme Activity

Enzyme	Hx	Hx (old)	GH	N age	N wt	N (old)	Positive Correlation with growth rate
Cartilage:							
unknown protease activity	low	--	high	high	high	low	Yes
Cathepsin D	low	low	high	high	high	low	Yes
Cathepsin B	low	low	low	low	high	low	Uncertain
Acid phosphatase	low	low	high	high	high	low	Yes
Liver:							
Cathepsin D	low	low	low	low	low	low	No
Cathepsin B	low	low	low	high	low	high	No
Acid phosphatase	low	low	low	low	low	low	No

These results show that certain cartilage acid hydrolases, notably cathepsin D and acid phosphatase, are regulated by GH and correlated with skeletal growth rate. Together with other studies relating acid hydrolases to tissue growth, these observations stimulate speculation that the mechanism of GH action and skeletal growth in general may occur via acid hydrolase-mediated processes. Lastly, these findings may also have import regarding the etiology and treatment of certain lysosome-related cartilage pathologies such as osteoarthritis.

Chapter VI

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