ACID PHOSPHATASES AND LYSOSOMES IN DYSTROPHIC AVIAN PECTORALIS MUSCLE

by

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ABSTRACT

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Lysosomes from dystrophic chicken pectoralis muscle homogenates exhibit significantly decreased structure-linked latency of several lysosomal marker enzymes when compared to normal muscle homogenates. This difference is retained when lysosomes from normal and dystrophic muscle are partially purified by differential sedimentation, seemingly indicating a membrane defect. However, no gross abnormalities were detected when fragility was tested by shear stress, sonication, or titration with detergents. Various observations, including lower percent recovery, enrichment factors and percent latency of acid phosphatases compared to N-acetyl-ß-D glucosaminidase and cathepsin D in both normal and dystrophic muscle, led to a detailed study of acid phosphatases in avian pectoralis muscle. Initial data showed that at least three acid phosphatase forms were present in all tissues tested. These isoenzymes differ in molecular weight, subcellular localization, and response to various substrates and inhibitors. Subcellular localization and

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quantitation of the three forms from normal and dystrophic muscle showed that the low molecular weight postmicrosomal supernatant form (presumably cytosolic) accounts for 84% of the four-fold increase in total acid phosphatase activity in dystrophic compared to normal avian pectoralis muscle at 33 days _ex ovo_. No alterations in the lysosomal or microsomal forms were observed. Purification of the low molecular weight activity resolved two isoforms differing in isoelectric point, activation by guanosine, Km for 4-methylumbelliferylphosphate, substrate specificity, and apparent molecular weight. Both enzymes are activated by glycylglycine and neither is inhibited by fluoride nor L-(+)-tartrate. Both forms show possible phosphotransferase activity exhibited by methanol activation of 4-methylumbelliferone release from 4-methylumbelliferylphosphate at pH 7.0, but only minimal phosphotransferase activity at pH 5.0.

These results indicate a lysosomal membrane abnormality in dystrophic muscle, presumably related to membrane function, though in a more subtle way than our tests could detect. The increased acid phosphatase activity in dystrophic muscle is distinct from the general lysosomal acid hydrolase elevation in this diseased tissue.

To

my Father, Kenneth F. Baxter

December 18, 1936 - May 20, 1975

Only now am I aware of how much I lost.

also

To my family, friends and collegues; life has been rich and fulfilling with you all.

ACKNO

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LIST OF ABBREVIATIONS

ATPase - adenosine 5'-triphosphate phosphatase

APase - acid phosphatase

DMD - Duchenne's muscular dystrophy

TX100 - Triton X-100

M+L - mitochondria + lysosomes

EDTA - ethylenediamine tetraacetic acid

EGTA - ethyleneglycol bis-ß-aminoethyl ether

N,N,N',N'-tetraacetic acid

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Ca^{+2}-ATPase - Ca^{+2}-activated ATPase

NAGase - N-acetyl-ß-D-glucosaminidase

TRIS - tris(hydroxymethyl) aminomethane

octyl-glucoside - octyl-ß-D-glucopyranoside

lauryl-maltoside - dodecyl-ß-D-maltopyranoside

INT - p-iodonitrotetrazolium violet

SD - standard deviation

vol - volume

RPM - revolutions per minute

MOPS - 3-(N-morpholino)propanesulfonic acid

FMN - flavin mononucleotide

RSA - relative specific activity

CHAPTER I: LITERATURE REVIEW

LITERATURE REVIEW

Human Muscular Dystrophies

The muscular dystrophies are genetically transmitted diseases which result in progressive loss of muscle function. It is likely that a number of abnormalities result in the dystrophic syndrome since the known muscular dystrophies differ widely in age of onset, severity of dysfunction, muscle group(s) affected and mode of inheritance (Table I (1), for reviews, see 2,3). The primary defects are known in only a few of the neuromuscular diseases. Myasthenia gravis is an autoimmune disorder in which the patient's immune system attacks the acetylcholine receptor (4-6), thus affecting signal transduction from nerve to muscle. The muscle myopathies are generally abnormalities in glycogen metabolism or glycolysis (7,8), resulting in muscle weakness and degeneration. Examples of these myopathies include Pompe disease (acid (lysosomal) α-1,4- and α-1,6- glucosidase deficiency), Cori-Forbes disease (glycogen debrancher enzyme deficiency), McArdle disease (muscle phosphorylase deficiency) and Tauri disease (muscle phosphofructokinase deficiency). Childhood Pompe syndrome simulates Duchenne muscular dystrophy, and the adult syndrome is similar to limb-girdle dystrophy and polymyositis.

...ular Dystrophies

	Inheritance	Age at onset	Major clinical Features	Course
Duchenne Type	X-linked recessive	early childhood	Symmetrical weakness; initially pelvifemoral:weakness shoulder girdle later and then trunk muscles; "pseudohypertrophy" of calves; reduced intelligence	Progressive inability to walk by puberty; death by age 20
Becker Type	X-linked recessive	Second decade	Milder variant of Duchenne type	"benign"; ability to walk into adult life
Facioscapulohumeral	Autosomal dominant	Childhood to late adult life	Usually facial weakness first; scapular weakness; humeral weakness	"Benign" course not progressive
Limb-girdle	Autosomal recessive	Variable onset first to sixth decade	Two variants:(1)Pelvifemoral weakness (2) shoulder girdle weakness	Variable progression; disability within 20 years
Distal myopathy	Autosomal dominant	Middle to late adult life	Weakness small muscles hands;weakness tibialis anterior; weakness gastrocnemius	Slow progression
Ocular myopathy	?Autosomal dominant	Variable	Group of syndromes, all having: weakness extraocular muscles initially; sometimes involvement of face, neck, limbs	Rarely progressive

Animal nutritional muscular dystrophies have etiologies similar to the genetic dystrophies; extensive degeneration of muscle tissues and nervous system anomalies are common to both. Deficiencies in vitamin A (9), vitamin E (9-11) or selenium (12) result in neuromuscular disorders in rat, rabbit, chicken, lamb and calf. These disorders are a significant veterinary problem in areas low in natural selenium (13). This effect is not, however, prominant in A- or E-avitaminosis in adult humans, though neuromuscular involvement is noted in children suffering from these avitaminoses (14,15).

Duchenne muscular dystrophy (DMD) is a sex-linked lethal mutation resulting in progressive loss of muscle function and early pseudo-hypertrophy (with late atrophy) of most skeletal muscle groups. This is probably the most debilitating of the muscular dystrophies, normally first appearing as difficulty in sitting, standing or walking; patients are not usually ambulatory after 6-7 years of age and death is generally by age 20, typically due to congestive heart failure. Muscle biopsies reveal intra- and intercellular fatty deposition, extensive necrosis and regenerating fiber bundles of heterogeneous fiber size, as well as multiple lesions at the sarcolemma (16,17). The primary defect resulting in DMD is not known, as is the case with most of the genetically transmitted muscle disorders. However, several theories to account for the pathological

aspects of these diseases have been advanced.

Rowland (2) reviewed the theories dealing with the primary lesion site in the muscular dystrophies. The three major theories are: a) abnormal microvascular supply to muscle (18), b) abnormal neuronal influence on muscle (19,20), and c) an abnormality in the surface membranes (2,3). However, a detailed analysis of the vascularization of muscle showed that blood flow (21) and numbers of capillaries per unit muscle (17) were normal. A comparative analysis of spinal cord motor neurons (22), end plates, and motor nerve terminals (23) in normal and dystrophic tissues shows no abnormalities. Because of these and other observations, the vascular and nerve theories have lost support in recent years. The membrane defect hypothesis is still consistent with much of the work in the field to date.

One of the earlier observations in support of the membrane defect hypothesis was the abnormally high leakage of enzymes from isolated dystrophic muscle (24). This agreed with elevated creatine kinase activity observed in the serum of dystrophic patients which is used as a diagnostic test for the disease (25). Other muscle enzymes (aldolase, pyruvate kinase, etc.) are also elevated in the serum of patients with muscle diseases (26). Several lines of evidence, i.e. enzyme release from isolated muscle and isozyme composition of the elevated activities, point to muscle as the source of these enzymes. The fact that only

some and not all soluble muscle enzymes are elevated in serum from dystrophic organisms, which has been a major point against a simple membrane defect, has been tentatively explained for the chicken model as being due to 1) rapid inactivation of the enzyme by serum factors or 2) rapid clearance from the blood relative to enzymes whose activity is elevated in dystrophic serum (27,28). In view of these observations it seems likely that the elevated serum enzymes are due to a faulty sarcolemmal membrane. Other data in support of abnormalities in membranes from dystrophic tissues are: alterations in intramembraneous particle density in dystrophic muscle plasma membrane, (29), though this has been disputed (30), defective calcium accumulation by sarcoplasmic reticulum (which is presumably dependant on membrane properties) (31-37), and decreased $(Na^{+},K^{+})Mg^{2+}$-ATPase (38) in DMD. Evidence that the suggested membrane defect is more general includes studies on fibroblasts, where increased rates of cell-substratum detachment (39), decreased structure-linked latency of lysosomal dipeptidyl-aminopeptidase I, (40) as well as apparently defective fusion (41), (though previous reports indicated normal fusion (42)) are found in dystrophic cells.

The development and function of muscle are highly dependent on normal membrane structure. One characteristic of myogenic cells is the fusion process to form myotubules. Using microscopic fluorescence relaxation measurements,

Alterman, et al. (43) observed generalized increases in myoblast fluidity, particularly at regions of cell-cell contact just prior to fusion, indicating a distinct, active role of membrane structure in the fusion process.

Many authors believe that the observed defects in dystrophic muscle are indicative of a general failure in at least some aspects of muscle maturation (e.g. 44-46), Vrbova (47) suggested that a disturbance in nerve-muscle interactions may result from certain muscle fiber maturation processes lagging behind motor neuron maturation. Indeed, Karpati, et al. (48) demonstrated that denervation of dystrophic muscle in hampsters delays onset of the histopathological lesions observed in normally innervated dystrophic muscle. Other abnormalities in DMD muscle are: a) abnormal growth kinetics of DMD fibroblasts (49), b) the presence of embryonic type myosins (50), c) increased turnover of contractile proteins (51,52), d) acetylcholine esterase activity in plasma (53), e) reduced function of acetylcholine receptor (54), f) increased levels of lysosomal acid hydrolases in affected muscle (55-57), g) elevated neutral proteinase activity in affected muscle (58) and, h) some alterations in muscle lipids (e.g. increased sphingomyelin, decreased phosphatidylcholine, and increased cholesterol, (59)). It is reasonable to assert that a membrane abnormality exists in DMD, and that such an abnormality could account for a large part of the observed

pathology of the disease.

Studies on human muscle biopsies are subject to question, since only small amounts of tissue are available, these tissues are from patients of varying age, varying stages of the disease, differing chemo- and physical therapy, and suitable controls are almost always lacking. Also, there are ethical and moral obligations involved in the use of human subjects, which severely limit the types of experiments possible. Animal models of the dystrophies, while having definite problems of their own, are useful in studying the onset and progression of the disease.

Avian Muscular Dystrophy

Genetically transmitted muscular dystrophies exist in the mouse (60), hamster (61), chicken (62), sheep (63), mink (64), duck (65), and turkey (66). In our laboratory, the chicken model is used for studies on the etiology of the dystrophic syndrome.

Avian dystrophy was discovered in commercial New Hampshire flocks by Asmundson and Julian (62) in 1956. The disease affected primarily the white, fast-twitch, glycolytic pectoral muscle, resulting in impaired ability to recover from a supine position. The disease is transmitted in a manner consistent with autosomal recessive genetics (67), though the observation of some intermediate symptoms in heterozygotes (68) suggests that co-dominant may be a more appropriate classification. The pectoral muscle of the

chicken is a large, nearly homogeneous white muscle allowing convenient study without the disadvantages of a heterogeneous tissue. The lifespan of the dystrophic chicken is not significantly different from the normal bird. These characteristics, combined with the availability of fertile eggs of known phenotype (allowing study of the embryonic development of the disease) makes the dystrophic chicken a good model for investigation of the dystrophic syndrome.

There are many enzymatic and histopathological similarities between dystrophic chicken and DMD muscle, suggesting that conclusions may have cross-applicability. The chicken line used for our studies was derived by an outcross of the initial dystrophic birds with normals, segregating for early onset of the dystrophic syndrome. This resulted in the closely related lines 412 (normal) and 413 (dystrophic) in use today. A few of the biochemical abnormalities characteristic of these dystrophic birds are early breast muscle hypertrophy, relatively low fat content (though still higher than normal control line 412), high serum creatine kinase, decreased muscle lactate dehydrogenase, and increased muscle cathepsins and other acid hydrolases. Histological observations include abnormal distribution of acetylcholine esterase, enlarged sarcotubular system and signs of fiber necrosis and regeneration. High mitochondrial enzyme levels, and abnormal isozyme levels seem to reflect an anomaly in the

developmental process, and abnormal electromyograms reflect a further, functional abnormality. (See 69-73 for reviews on these and other aspects of avian muscular dystrophy.) As in any comparative study, we must look for similarities between the several model systems available and the human disease of interest. One abnormality observed in all dystrophic muscles, as well as denervated muscle and the nutritional dystrophies is the general elevation of lysosomal acid hydrolases.

The Lysosome System

The lysosome was described in 1955 (74) as a result of a series of studies by DeDuve, et al. (74-79) on subcellular localization of acid phosphatase in rat liver. The somewhat serendipitious events leading to the discovery of this organelle have been described (80,81). The lysosome is a subcellular organelle ubiquitous in eukaryotic cells, usually somewhat smaller than a mitochondrion and having a single bilayer membrane. It is characterized by an acid matrix of pH 4-6.5, depending on the tissue source, isolation procedure, storage medium pH; and method of pH estimation used. The system for maintenance of such a pH gradient is as yet unknown, though two suggestions have been made: a) a Donnan potential caused by a preponderance of fixed anions inside, and a differential cation permeability of the membrane (82-84), or b) a lysosomal membrane proton pump (e.g. 85-90).

Many functions have been directly or indirectly attributed to lysosomes. These organelles contain a wide range of acid hydrolases, and have been characterized as the recycling system of the cell. Their function in normal cytosolic protein turnover is reasonably well established (91-94), and (through autophagy) the lysosome seems to function in the turnover of membraneous elements and even other organelles as well. (95,96). Indeed, treatment of cells with agents causing lysosomal dysfunction (e.g. chloroquine, ammonia, 3-methyl adenine, chymostatin, leucine methyl ester, etc.) results in a significant decrease in the ability of these cells to degrade proteins (97-102). Lysosomes play critical roles in such diverse areas as a) killer cell function in the immune system (96), b) protein regain in livers of starved-refed mice (103), c) liver regeneration after partial hepatectomy (104), d) increases in proteolysis as fibroblasts reach confluence (105), e) morphogenesis of coronaviruses (106), f) loss of muscle mass during ageing (107), g) muscle wasting in hypothyroidism (108) and vitamin A deficiency syndrome (109), h) synthesis and secretion of procollagen in tendon (110), and i) recycling or degradation of certain types of receptors (111,112). The elucidation of a wide range of lysosomal storage disorders graphically illustrates the importance of lysosomes in oligosaccharide, glycoprotein, and lipopolysaccharide catabolism, (113,114).

The lysosomal apparatus seems to be involved in the function (in skeletal muscle, heart, and liver) of a variety of hormones: progesterone (115-117), estrogen (115-117), glucagon (95,118-120), insulin (95,118,121), testosterone (122,123), prostaglandins (95,124), glucocorticoids (95), and thyroid hormones (95,108,125,126). Some of these hormones also have a direct affect on lysosomes in vitro (127).

Lysosomes have only recently been unequivocally demonstrated in skeletal muscle by Bird and his colleagues (128). Differences in lysosomal enzyme activity in muscle cell cultures have been reported depending on (1) the fusion state (129), and (2) treatment of muscle cultures with lysosomotropic amines (which results in inhibition of myoblast fusion (130)), both suggesting a function for the lysosomal system in the fusion process in muscle. Leupeptin and chloroquine cause gross ultrastructural changes in cultured myotubes (131) suggesting a significant function for lysosomes after fusion, and treatment of chickens with Triton WR-1339, a detergent known to accumulate in lysosomes, is reported to cause a skeletal muscle myopathy (132). Evidence for (133,134) and against (135) the importance of lysosomes in myofibrillar protein turnover has been presented.

The involvement of lysosomes in the pathogenesis of numerous diseases affecting muscle has been known for some

time. There is evidence for lysosomal involvement in ischemic heart damage (136), denervation atrophy (137), hyperthermia (138), the inflamatory response (139,140), acute myocardial infarction (141), disuse atrophy (142) and the cardiomyopathy in diabetic mice (143), as well as the muscular dystrophies mentioned earlier in this review. These and other observations suggest a very significant role for lysosomes in both normal and degenerating tissues, particularly muscle.

Based on the nature of lysosomal function, membrane alterations (suggested as the primary lesion site in muscular dystrophies) would have a significant affect on normal lysosome related processes. Some evidence for the putative membrane defect affecting lysosomes includes: a) the structure-linked latency of dipeptidyl aminopeptidase I is reduced in DMD fibroblasts (40), and b) a link between increased endocytosis and lysosomal activation has been suggested in dystrophic (144) and protamine treated mice (145). The observation that most lysosomal membrane proteins are exposed to the cytosol (146) suggested the possibility that these proteins play a significant role in recognition of material destined for lysosomal processing, which provides a further possible site for the effects of a general membrane alteration on lysosomal function in dystrophic muscle.

Acid Phosphatases

One of the many elevated lysosomal enzyme activities consistently reported for muscle dysfunction (including the dystrophies) is acid phosphatase (APase) (e.g. 56,57,72,142). Acid phosphatase alterations also occur as part of the etiology of various other degenerative states such as invasive tumors (147), epidermal chemical irritation (148), the effect of Gaucher disease on spleen (149), Xenopus laevis tadpole tail regression (150,151), response to bacterial endotoxins (152), and prostatic tumors (153,154). Most of these observations were believed to reflect a general lysosomal involvement in the pathology of the malady, since a lysosomal APase was well known and other lysosomal acid hydrolases were affected. However, the acid phosphatase literature shows several forms of this activity in virtually all tissues and organisms. Documented differences between these forms of APase include molecular weight (150,151,155-161), subcellular distribution (155-157,162-164), sensitivity to inhibitors (155,157,158,165), substrate specificity (158,159,164,165), heat (156,158) and sulfhydryl reagent (157) inactivation, and tissue distribution (159,165). A partial listing of reports of multiple acid phosphatase activities observed in a variety of tissues is given in Table II, further supporting the ubiquitous nature of this activity (166). These APases seem to fall into four classes based on molecular weight- a) class I, larger than 200,000 , b) class

Table II: Reports of Multiple Acid Phosphatases Based on Molecular Weight

Tissue Source	No. Forms	Molecular weights	Reference No.
Tadpole Tail	4	>200K,109K,40.9K,18.7K	150
Yeast	2	N.R.	156
Rat Kidney	2	>100K,20K	157
Human Placenta	3	>200K,105K,<35K	158
Bovine Kidney	3	N.R.	159
Bovine Liver	3	N.R.	159
		Small form=14.4-16.5K	168
		medium form=93K	167
Bovine Pancreas	3	N.R.	159
Bovine Spleen	2	Large and small, no med.	159
Porcine Kidney	3	N.R.	159
Porcine Liver	2	Large and small, no med.	159
Rat Kidney	3	N.R.	159
Rat Liver	2	Large and small, no med.	159
Rat Spleen	2	large and small, no med.	159
Human Liver	3	>200K,107K,13.4K	161
		small form=14.4K	169
Horse Liver	2	Large and Small, no med.	227
Wheat Germ	3	All medium, 55K	228
Human Brain*	4	>200K,100-130K, 30-60K,13-18K	175

*Other tissues showing similar elution profiles (Sephadex G-100) were Kidney, Liver, Prostate gland, Placenta, Red cells and Seminal plasma.

N.R. = not reported.
K = x1000
>= Greater than
<= Less than

II, roughly 100-130,000, c) class III, 30-60,000 and d) class IV, 8-18,000, though it is quite probable that much heterogeneity exists within these classes from different tissues and organisms. A number of the APases have been partially purified and characterized (Table III). Several general differences emerge from these studies. The higher molecular weight forms (Class I and II) are generally susceptable to L-(+)-tartrate and fluoride inhibition (154,155,157,158,167), whereas the smaller ones are not (154,155,157,158). The smaller forms are generally sensitive to sulfhydryl reagents (154,155,157,158,168-170,171-173) and are sometimes activated by purines (e.g. 174). Alcohols and glycerols appear to activate the low molecular weight APases, suggesting a phosphotransferase activity (169,172,174), which has not been examined in detail. Sensabaugh (175) notes that most of the low molecular weight APases prefer flavin mononucleotide to other naturally occuring substrates, suggesting a role in the metabolism of flavins. One acid phosphatase, purified as acyl phosphatase from several sources (176-179), is now classed as 1,3-diphosphoglycerate phosphatase (180), acting as a 'safety valve' preventing the intracellular accumulation of 1,3-diphosphoglycerate (181). Accumulation of 1,3-diphosphoglycerate can increase the rate of yeast fermentation (182), and retina glycolysis (183). Ramponi and Grisolia (184) have shown that 1,3- diphosphoglycerate can

Table III: Acid Phosphatases Purified From Various Sources-Partial Listing

Organism-Tissue	Mol. Wt.	pI	Subunits (weights)	pH opt.	Ref.#
Human-Gaucher Spleen	34.5K	8.5	2(16.3K,20K)	5.5	149
Human Prostate	102K	4.05-5.2	2(50K)	4.8-6.0	153
Human Liver I	14.4K	N.R.	N.R.	N.R.	169,170
Human Liver II	93K	N.R.	2(50-52K)	N.R.	161,167
Bovine Liver	16.4K	N.R.	N.R.	5.5	159
	14-16K	N.R.	N.R.	N.R.	168
Bovine Brain	13K	N.R.	N.R.	4.8-5.8	171-174
	12.1K	N.R.	N.R.	N.R.	178
Horse Muscle	9750	11.4	N.R.	N.R.	176
Horse Liver	8.3-9.2K	N.R.	N.R.	N.R.	177
Rat Liver II	100K	7.7,4.5	N.R.	4.5	229
Rat Liver I	33K	7.8,8.0, 8.3,8.5	N.R.	5.0-5.8	185
Rat Spleen	41K	N.R.	2(N.R.)	N.R.	230
Pork Heart	11K	7.24	N.R.	5.4-5.6	178
Rabbit Muscle	23.5K	N.R.	N.R.	N.R.	179
E. Coli	13-14K	N.R.	N.R.	4	160
Potato Tuber	96K	N.R.	2(46K)	N.R.	231
Wheat Germ	55K	N.R.	N.R.	3 Forms(5.3, 4.8 and 4.2)	182

N.R. = Not reported.

acylate histones, particularly lysine rich ones. These results suggest a need for regulation of 1,3-diphosphoglycerate concentration, though the ultimate result of the accumulation of this compound is not known.

The common substrates used for assays of APases include α-napthol phosphate (150,154,158,167,185), p-nitrophenyl phosphate (150,154,158,159,160,167,169,171), and ß-glycerophosphate (150,154,167). Other substrates include riboflavin 5'-phosphate (150,159,169), 4-methylumbelliferylphosphate (185), adenosine 5'-diphosphate (150), uridine 5'-diphosphate (150), adenosine 5'-triphosphate (150,158,185), phospho-enol-pyruvate (150), adenosine 5'-monophosphate (150,167), phenyl phosphate (169,185), p-nitrobenzylphosphate (176,177), and acetylphosphate (176,177,179). It is interesting to note that the higher molecular weight acid phosphatases generally have broad substrate specificity, hydrolyzing the majority of phosphate esters tested, but the low molecular weight forms have relatively restricted specificcity. Their activity against riboflavin 5'-phosphate (150,153,169) and 17-ß-estradiol 3-phosphate (186) is consistent with their involvement in the metabolism of these compounds, though definitive evidence is lacking.

The function of an acid phosphatase in lysosomes is obvious, but those of microsomal or cytosolic origin are

another matter. There are, however, many known systems regulated by phosphorylation. Though a phosphoprotein phosphatase activity has not yet been demonstrated for these enzymes, these phosphorylation-state dependent systems provide many likely substrate possibilities and suggest much further work is needed.

The control of various enzymes and enzyme systems by phosphorylation/dephosphorylation has been reviewed recently by Krebs and Beavo (187) and Cohen (188). A partial listing of these enzymes appears in Table IV. Systems which seem to be under extensive control of this type include glycogen metabolism, cholesterol metabolism, glycolysis and aromatic amino acid hydroxylases (see Table IV). Other systems under phosphorylation state control include: a) receptors for insulin (189), progesterone (190) and somatomedin C (189), b) protein synthesis at eukaryotic initiation factor 2 (191), ribosomal protein S6 (192,193) and histone H1 (194), c) cytoskeleton assembly at myosin (195), actin binding protein (196) and microtubule associated protein 2 and tau factor (197) and d) differential localization of some enzymes (i.e. aryl sulfatase A in lysosomes (198)). Phosphorylation events are also involved in control of the lateral distribution of light harvesting chlorophyll a/b-protein complexes (199), control of bacterial sugar transport (200), membrane changes in transformed cells (201,202) and insulin action (203,204). With regard to

Table IV: Enzymes Exhibiting Phosphorylation State Control

Enzyme	Reference No.
Glycogen Phosphorylase	232
NAD-Dependant Glutamate Dehydrogenase (Yeast)	233,234
Phosphorylase Kinase	235,236
Glycerophosphate Acyltransferase	237
Glycogen Synthase	236,238-241
Hormone-sensitive Lipase	242
Fructose 1,6 Biphosphatase	243
Pyruvate Dehydrogenase	244,245
Hydroxymethylglutaryl CoA Reductase	246-248
Hydroxymethylglutaryl CoA Reductase Kinase	249
Acetyl CoA Carboxylase	250,251
DNA-Dependant RNA Polymerase	252
Liver Pyruvate Kinase	253,254
Cholesterol Ester Hydrolase	255
Type II cAMP Dependant Protein Kinase (R Subunit)	256
Reverse Transcriptase	257
Liver Phosphofructokinase	258,259
Muscle Phosphofructokinase	260
Tyrosine Hydroxylase	261,262
Phenylalanine Hydroxylase	263
Eukaryotic Initiation Factor-2 Kinase (eIF-2 Kinase)	264
cGMP-Dependant Protein Kinase	265
Tryptophan Hydroxylase	266

muscle function, certain types of calcium transport (205-210), acetylcholine receptors (211), myosin light chain (212-217), myosin (218-222), microtubule- associated proteins (223) and actin binding proteins (224) are all subject to function-altering phosphorylation/dephosphorylation reactions. Dephosphorylation of myosin inhibits the actin-myosin interaction in aorta (225), but is not involved in the action of vasodilators on arterial smooth muscle (226), where cyclic AMP levels are the purported mediator, indicating several levels of control of actin-myosin interactions.

These and other data point out the central role of phosphorylation dephosphorylation events in the control of a wide variety of cellular and tissue processes. In the muscle contractile process, phosphorylation state regulation may play important roles at virtually every step from reception of the nerve impulse (acetyl choline receptor) to calcium sequestration in the sarcoplasmic reticulum, and may even regulate the assembly of the contractile apparatus by modification of the physical properties of the proteins involved (actin, myosin and actin binding proteins). Because of the widespread occurrence of regulation through phosphorylation, other control systems must be present (and, in many cases, have been reported) and the impact of a general defect in such regulation is difficult to predict.

Statement of the Problem

Lysosomal enzymes are elevated in dystrophic muscle, and the lysosomal apparatus seems to play a significant role in this and other tissues. Acid phosphatase activity is also elevated in dystrophic muscle, and has always been linked to the general lysosomal activation. This dissertation presents evidence for a lysosomal abnormality in dystrophic muscle, presumably related to lysosomal membrane structure (Chapter II). Acid phosphatases in avian skeletal muscle were also investigated (Chapter III), and those enzymes accounting for the increased APase activity in dystrophic muscle were purified and partially characterized (Chapter IV). Finally, a low molecular weight (Mr 500-1500) endogenous inhibitor of these cytosolic acid phosphatases was detected in avian skeletal muscle. This inhibitor was enriched, and some of its properties were ascertained (Chapter V).

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CHAPTER II: SKELETAL MUSCLE LYSOSOMES: COMPARISON OF LYSOSOMES FROM NORMAL AND DYSTROPHIC AVIAN PECTORALIS MUSCLE AS A FUNCTION OF AGE

SKELETAL MUSCLE LYSOSOMES: COMPARISON OF LYSOSOMES FROM NORMAL AND DYSTROPHIC AVIAN PECTORALIS MUSCLE AS A FUNCTION OF AGE

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SKELETAL MUSCLE LYSOSOMES: COMPARISON OF LYSOSOMES FROM NORMAL AND DYSTROPHIC AVIAN PECTORALIS MUSCLE AS A FUNCTION OF AGE

ABSTRACT

The properties of skeletal muscle lysosomes from normal and dystrophic chickens were studied in order to ascess their involvement in the dystrophic process. A method is described for isolation of a 3-7 fold purified lysosome fraction with 29-33% yield. Lysosomal enzymes in crude homogenates and isolated lysosome-enriched fractions from dystrophic muscle exhibit decreased latency for N-acetyl-ß-D-glucosaminidase, acid phosphatase and cathepsin D. However, no differences in the fragility of lysosomes in isolated lysosome-enriched fractions were observed using shear, sonication and detergent stress. Lower percent recovery, enrichment factor and percent latency of acid phosphatase compared to N-acetyl-ß-D-glucosaminidase and cathepsin D were observed from both normal and dystrophic muscle. These results are consistent with the presence of a significant amount of non-lysosomal acid phosphatase activity in skeletal muscle.

INTRODUCTION

Muscular dystrophy is a genetically transmitted disease affecting muscle development. As the disease progresses, muscle function is lost and in its most severe human form (Duchenne's muscular dystrophy-DMD) death results at or near 20 years of age (17). Many biochemical changes occur in the muscle. In the chicken model, muscle lipid content increases (27), some sarcoplasmic enzymes are elevated in the serum (4), and acid hydrolases generally associated with lysosomes are increased (9,14,22). As reviewed by Strickland, et al., (21) these changes are also observed in the dystrophic mouse. Muscle from DMD patients has many of the same characteristics (19).

The lysosome is a subcellular organelle that functions in the breakdown of biological macromolecules-polysaccharides, proteins, and nucleic acids. Since the lysosome contains an array of hydrolases capable of degrading virtually all macromolecules, it is isolated from the rest of the cell by a membrane.

Marcomolecules are introduced into the lysosomal matrix by two general mechanisms: phagocytosis (or engulfment) and fusion. Both processes are dependant on the functional integrity of the lysosomal membrane. An indirect measure of the integrity of a lysosomal membrane is given by latency of

various lysosomal enzymes. A lysosomal enzyme is said to be latent if the lysosomal membrane must be disrupted (sonication, detergent, etc.) before its enzymatic activity is observed with an exogenously added substrate.

Of the various theories for the molecular basis of muscular dystrophy, that involving a membrane defect is the most widely accepted (18). Abnormal loss of muscle proteins, presumably as a result of increased membrane permeability, and altered properties of membrane-bound enzymes are used to support this model (see Rowland (20) for a review of the membrane defect theory). We reasoned that if the primary defect in muscular dystrophy is related to membrane function in general, an affect on lysosomal latency might be expected.

This study of skeletal muscle lysosomes was initiated for the following reasons: (a) lysosomal enzyme activities are elevated in dystrophic muscle, (b) the lysosomal membrane performs an important role in the organelle's function, (c) muscular dystrophy is accompanied by extensive muscle degeneration and (d) Gelman, et al. (7) reported a difference in the latency of diaminopeptidase I in cultured fibroblasts from patients with DMD. A small, persistent difference in the latency of enzymes associated with lysosomes of normal and dystrophic avian pectoralis muscle is reported in this study. No differences between these two populations were detected by stressing with sonication,

shear, or detergent.

MATERIALS AND METHODS

Chemicals - 4-Methylumbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyranoside and 4-methylumbelliferylphosphate (both analytical reagent grade) and Triton X-100 (TX100) (scintillation grade) were obtained from Research Products International Corporation (Mount Prospect, IL). Octyl-ß-D glucopyranoside (octyl-glucoside) and dodecyl-ß-D-maltopyranoside (lauryl-maltoside) were generous gifts from Dr. S. Ferguson-Miller (Michigan State University, E. Lansing, MI). Deoxycholic acid (Sigma Chemical Company, St. Louis, MO) was passed over charcoal in hot 80% acetone and then recrystallized twice from 80% acetone. It was subsequently adjusted to pH 7.4 with NaOH and lyophylized for storage. p-Iodonitrotetrazolium violet (grade I) and beef blood hemoglobin (type I) were from Sigma Chemical Company. Succinic acid (analytical reagent grade) was from Mallinckrodt (St. Louis, MO). Folin`s phenol reagent was from Harleco (Gibbston, NJ). All other reagents were analytical reagent grade or better.

Animal Model - Normal (line 412) and dystrophic (line 413) eggs were obtained from the Department of Avian Sciences (University of California, Davis, CA) and hatched in facilities provided by the Poultry Science Department (Michigan State University). Chicks were fed chick starter (Kent) and water ad libitum.

Enzyme Assays - Acid phosphatase (APase), N-acetyl-ß-D-glucosaminidase (NAGase) and cathepsin D were assayed as suggested by Barrett (1), using 1 mM 4-methylumbelliferylphosphate, 4 mM 4-methylumbelliferyl N-acetyl-ß-D-glucosaminide and 1.6% (wt/vol) acid-denatured hemoglobin, respectively, as substrates. For APase and NAGase, free activity was the activity expressed in the presence of 0.21 M sucrose in the absence of detergent, or that remaining in solution after sedimentation (130,000 g, 3 minutes, Beckman Airfuge; Beckman Instruments, Inc., Fullerton, CA). Total activity, and the free activity after sedimentation were assayed in the presence of 0.025% (wt/vol) TX100 (no sucrose). This concentration of TX100 was found to be optimal (data not shown). The free activity for cathepsin D was always determined in the presence of TX100, after sedimentation of the particulate material. Percent latency is the percent increase in the activity after membrane disruption, and was calculated using the equation:

$$L = \frac{(At - Af)}{At} \times 100 ,$$

where Af is the free activity and At is the total activity. Succinate:INT reductase was assayed by the method of Pennington (16), using 10 mM succinate and 0.1% (wt/vol) p-iodonitrotetrazolium violet (succinate concentration was optimized for the crude avian breast muscle enzyme). Most

protein concentrations were determined by the Lowry, et al. (10) method using bovine serum albumin as a standard. The protein concentrations of some M+L fractions were estimated from A_{280} measurements in 1% (wt/vol) sodium deoxycholate using $A = 1.54$ ml $mg^{-1}cm^{-1}$, the average extinction coefficient for three M+L fractions assayed by the Lowry procedure as described. All data are expressed as mean $\pm$ SD.

Lysosome-enriched Fraction - Birds were killed by decapitation, all breast muscle was removed and immediatly placed in cold relaxing buffer (0.25 M sucrose, 10 mM EGTA, 60 mM KCl, 40 mM imidazole propionate, pH 7.0; modified from reference 2). After collection, the muscle was blotted dry, trimmed of excess fat and connective tissue, weighed, and finely minced into 10 vol of fresh cold relaxing buffer. It was homogenized by five strokes with a Duall 23 conical ground glass homogenizer (300 rpm: Kontes Glass Co., Vineland, NJ) and five strokes with a Ten-Broeck homogenizer (hand-held: Wheaton Scientific, Millville, NJ). The resulting crude homogenate was sedimented at 600 g, 20 min. The pellet was resuspended into 10 vol of cold relaxing buffer per gram of original tissue and reextracted by homogenization (ten strokes, Potter-Elvejham homogenizer, 1000 rpm: Arthur H. Thomas Co., Philidelphia, PA) and sedimented as before. Supernatants were combined and sedimented (20,000 g, 20 min). The resulting M+L pellet was carefully resuspended into relaxing buffer, diluted to the

appropriate protein concentration, and stored on ice until use. M+L fractions were always used within 4 hours of preparation.

Membrane Fragility Tests - The fragility of lysosomal membranes was examined by both sonication and shear. Lysosome-enriched material (5 mg/ml) in relaxing buffer was sonicated at low power using a Branson sonifier, model 23 equipped with a microtip (Branson Instrument Co., Danburry, CT). Shearing stress was applied with a Potter-Elvehjam homogenizer (0.0019 $\pm$ 0.0002 inches clearance) at 1000 rpm. After stress was applied, the fractions were either sedimented with an airfuge (130,000 g, 3 min, sonication experiment) or the $\pm$ TX100 assay (shear experiment) was used for the determination of free and total NAGase activity. The shear data were corrected for loss of total enzyme activity. The data were normalized to the average initial latency for graphical presentation.

Detergent Stress Tests - The lysis of lysosomes by various detergents was examined as a function of detergent concentration. The M+L lysosomes at 10 mg/ml protein were diluted twofold by addition of detergents in relaxing buffer. The fractions were then incubated for 15 min at 37° C, then assayed for NAGase latency ($\pm$ TX100), as described above.

RESULTS

Organelle Recovery and Enrichment - Skeletal muscle lysosomes were first enriched from the breast muscle of 15 day *ex ovo* chickens to avoid the extensive connective tissue in older muscle. The procedure developed for this study gave 29-33% recovery and a three- to sevenfold purification of NAGase and cathepsin D activities (Table 1). The results with APase were anomalous; only a 12% recovery and a twofold purification were observed. Approximately 45% of the mitochondrial succinate:INT reductase was recovered with nearly sixfold purification. There were no specific differences in the percent recovery of each of the enzymes in the M+L fraction of normal and dystrophic muscle if we discount the APase results as anomalous. However, the enrichment factor (specific activity in M+L/specific activity in crude) for lysosomal enzymes differed for normal and dystrophic muscle; lower values were associated with the dystrophic tissues.

The percent recoveries of NAGase and succinate:INT reductase in the M+L fraction of normal and dystrophic muscle were nearly identical (Fig. 1 A and B). In fact, essentially identical results were obtained when the percent recovery data from normal and dystrophic birds aged 12- to 50-days *ex ovo* was averaged (Table 1). This is true, even though the specific activity of NAGase in normal muscle

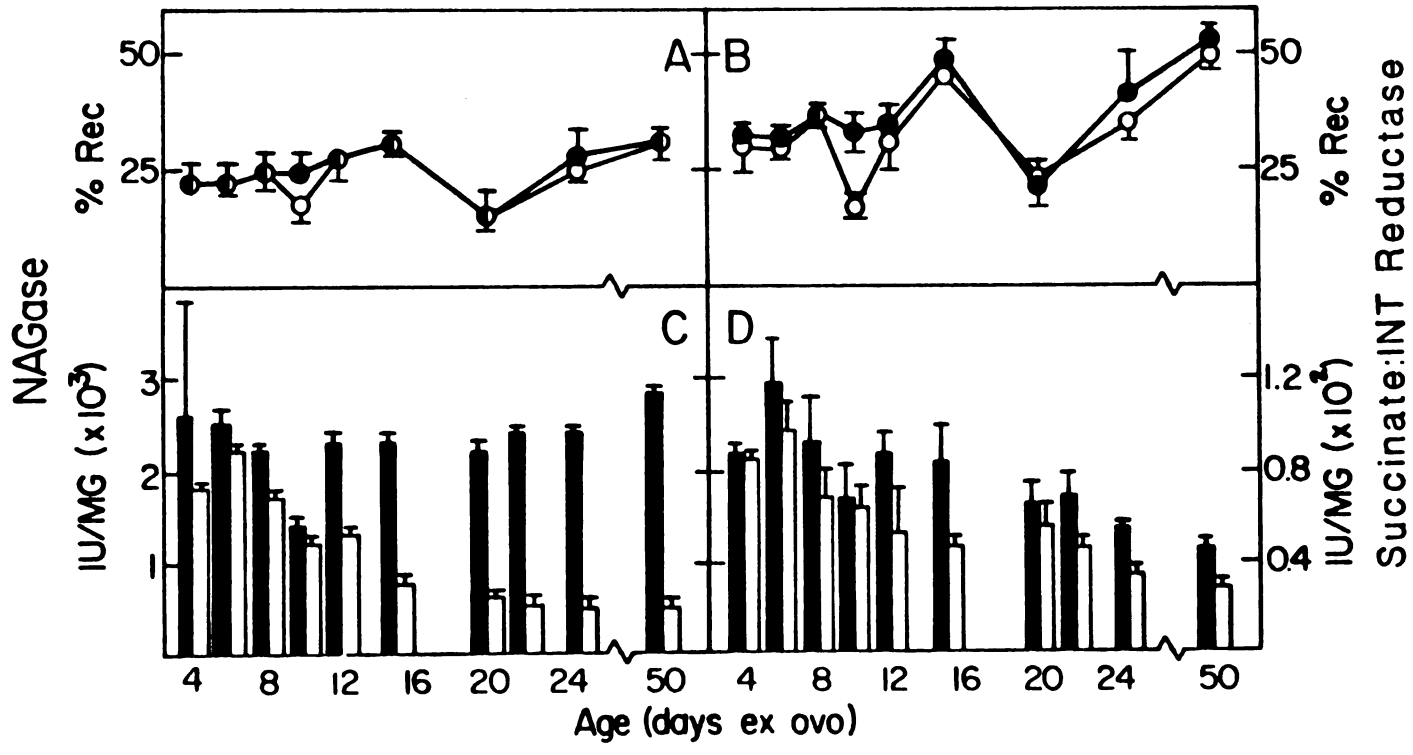
Table 1: Percent Recovery and Enrichment Factors* for Various Enzymes in the M+L Fractions From Normal (N) and Dystrophic (D) Muscle.

		Percent Recovery +			Enrichment Factor		
	Age(s)	Normal	Dystrophic	P++	Normal	Dystrophic	P
				N vs.D			N vs.D
NAGase	15	29(2)[4]	30(4)[4]	NS	4.2(1.0)[4]	2.6(0.4)[4]	0.025
	12-50	24(7)[17]	22(10)[18]	NS	6.0(3.5)[18]	4.0(1.6)[18]	0.05
APase	15	16.3(-)[2]	10.3(-)[2]	--	1.8(---)[2]	0.95(--)[2]	---
	12-50	12(4)[16]	8.5(3.9)[16]	0.05	3.3(2.1)[16]	1.5(0.4)[16]	0.01
Cathepsin D	15	33(3)[3]	30(7)[3]	NS	5.1(1.3)[3]	3.2(0.4)[3]	NS
	12-50	26(12)[17]	21(9)[17]	NS	7.3(5.9)[17]	3.5(1.3)[17]	0.05
Succ.:INT	15	42(4)[4]	44(4)[4]	NS	6.3(2.0)[4]	4.5(1.4)[4]	NS
reductase	12-50	34(10)[18]	36(12)[18]	NS	8.4(5.0)[18]	6.0(1.7)[18]	NS

*Enrichment factors are defined as (specific activity in M+L)/(specific activity in the crude).
+ Data are means, 1 standard deviation in parenthesis and number of birds used in brackets.
++ P-value derived using the Students T-test. NS, not significant (P>0.05).
P-values are the maximum value of a range, i.e. 0.5= 0.5>P>0.25.

Figure 1: Specific Activities in the Crude Homogenate (1C and 1D) and Percent Recovery (1A and 1B) in the M+L Fraction for NAGase (1A and 1C) and Succinate:INT reductase (1B and 1D) as a Function of Age.

Crude homogenates and M+L fractions were prepared and enzyme activities and protein were assayed as described in Materials and Methods. Data are from 3-8 birds. Closed symbols are for data from dystrophic muscle, open symbols are from normal muscle.


NAGase
% Rec
IU/MG (x10³)
Succinate:INT Reductase
% Rec
IU/MG (x10²)
A
B
C
D
50
25
3
2
1
50
25
1.2
0.8
0.4
4
8
12
16
20
24
50
Age (days ex ovo)

decreased fivefold from age 4- to 50-days ex ovo, while it remained nearly constant in the dystrophic muscle (Fig. 1 C).

We confirm the results of Owens (14) showing nearly constant NAGase specific activity from age 4- to 50-days ex ovo in dystrophic muscle and a decreasing specific activity in normal muscle, reaching a plateau level at 3 weeks ex ovo (Fig. 1 C). Nearly identical results were obtained with APase (data not shown). Succinate:INT reductase specific activities decrease with age in both chicken lines, with slightly higher values at all ages in dystrophic breast muscle compared with normal breast muscle (Fig. 1 D).

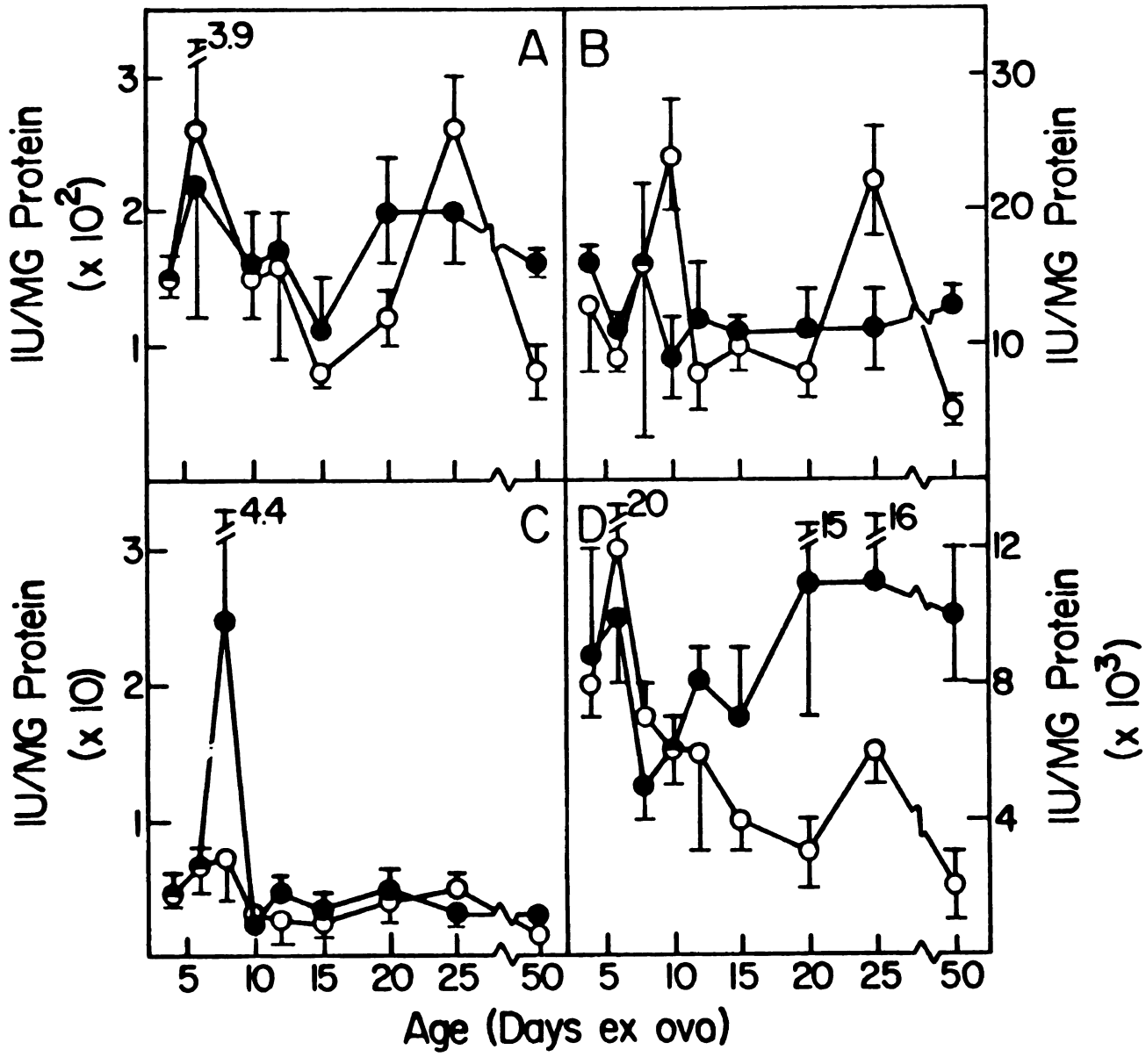
The specific activities of APase, cathepsin D, and succinate:INT reductase in the M+L fraction from normal and dystrophic muscle were not significantly different. NAGase specific activity, however, was significantly higher in the M+L fraction from dystrophic muscle, especially at ages more than 12-days ex ovo (Fig. 2).

On the average, 3-9% of the total protein was recovered in the M+L fraction from all birds aged 4- to 50-days ex ovo. No trend with age was observed, but fractions from dystrophic tissue had significantly higher ($P < 0.05$) protein content than those from normal tissues (normal 4.8 $\pm$ 3.2%, dystrophic 6.6 $\pm$ 3.1% of total protein isolated in the M+L fraction, n=37)

Latency - We next looked for possible differences in

Figure 2: Specific Activities of Various Enzymes in the M+L Fractions from Normal and Dystrophic Muscle as a Function of Age.

M+L fractions were prepared from normal (o) and dystrophic (●) chicken breast muscle, and enzyme activities and protein were assayed as described in Materials and Methods. Data are from 3-8 birds for each point. A- APase, B- cathepsin D, C- succinate:INT reductase and D- NAGase.


A
B
C
D
3.9
4.4
20
15
16
IU/MG Protein (x 10²)
IU/MG Protein
IU/MG Protein (x 10)
IU/MG Protein (x 10³)
Age (Days ex ovo)

the membranes of lysosomes from dystrophic muscle by examining the latency of NAGase, cathepsin D and APase in both crude homogenates and M+L fractions (Table 2). The latencies of all lysosomal enzymes tested are 6-20% lower in crude homogenates and M+L fractions from dystrophic muscle when compared to those from normal muscle. This behavior is noted in all ages from 4- to 50-days ex ovo, though there is a significant overlap at 5-10 days of age (Fig 3, data for APase and cathepsin D not shown). When the latencies of all three enzymes in the crude homogenates from normal and dystrophic birds ranging in age from 12- to 50-days ex ovo are averaged, the differences were significant at the 99.9% confidence level ($P<0.001$, Student's t-test). The differences in the latencies of NAGase and cathepsin D between M+L fractions isolated from normal and dystrophic muscle are also significant at the 99.9% confidence level ($P<0.001$) when data from birds aged 12- to 50-days ex ovo are averaged. The data for APase were significant at the 99.5% confidence level.

The same small differences in NAGase and cathepsin D latency in normal and dystrophic crude homogenates were observed when sedimentation of activity (Beckmen Airfuge; 130,000 g, 3 min) rather than detergent activation of activity was used as the criterion for latency. Latency values obtained by sedimentation were approximately 10% higher than those obtained by detergent activation (data not

Table 2: Percent Latency of Various Enzymes in Normal and Dystrophic Muscle as a Function of Age.

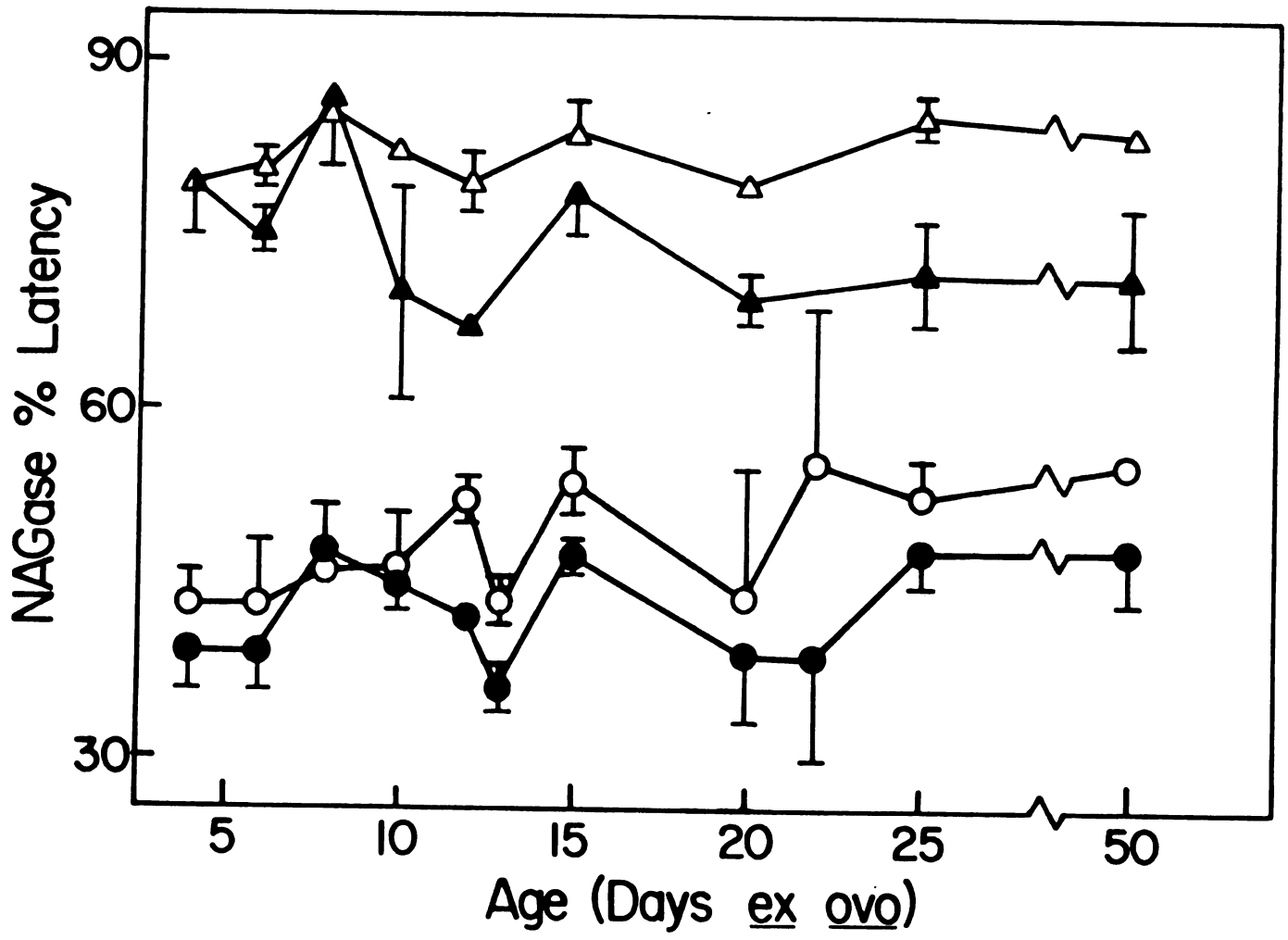
		Percent Latency*					
		Crude			M + L		
Enzyme	Age	Normal (412)	Dystrophic (413)	P-value+	Normal (412)	Dystrophic (413)	P-value
	5	29(5)[5]	26(3)[5]	NS	68(5)[3]	72(1)[3]	NS
NAGase	15	49(6)[8]	45(4)[8]	NS	82(3)[8]	75(5)[8]	0.005
	25	53(4)[4]	48(3)[4]	NS	83(4)[4]	75(10)[4]	NS
	ave.12-50	50(7)[28]	42(6)[28]	0.001	82(3)[24]	72(5)[24]	0.001
	5	27(11)[3]	16(10)[3]	NS	76(2)[3]	70(1)[3]	0.01
Cathepsin	15	49(2)[8]	38(10)[8]	0.01	77(4)[7]	62(11)[7]	0.01
D	25	59(-)[2]	46(--)[2]	--	79(-)[2]	59(--)[2]	--
	ave.12-50	58(12)[18]	38(13)[20]	0.001	79(5)[13]	62(10)[11]	0.001
	5	12(5)[5]	12(8)[5]	NS	44(4)[3]	38(4)[3]	NS
APase	15	19(5)[6]	9(3)[5]	0.005	44(4)[4]	35(6)[6]	0.05
	25	18(5)[4]	18(2)[4]	NS	49(1)[4]	43(4)[4]	0.05
	ave.12-50	21(10)[26]	11(4)[25]	0.001	46(6)[21]	40(6)[21]	0.005

*Values are means, standard deviations are in parenthesis, and number of birds are in brackets

+The Student's t-test was used to determine the P-values. NS, not significant $P>0.05$. P-values given are the maximum values of a range, i.e., 0.01 = $0.01>P>0.005$.

Figure 3: NAGase Percent Latency in Crude Homogenates and M+L Fractions From Normal and Dystrophic Muscle as a Function of Age.

Crude homogenates and M+L fractions were prepared from normal and dystrophic chicken breast muscle, and NAGase percent latency was determined using the detergent activation assay as described in Materials and Methods. Data are from 3-8 birds for each point. (o,●) = Crude, (Δ,▲) = M+L, closed symbols - Dystrophic, open symbols - Normal.

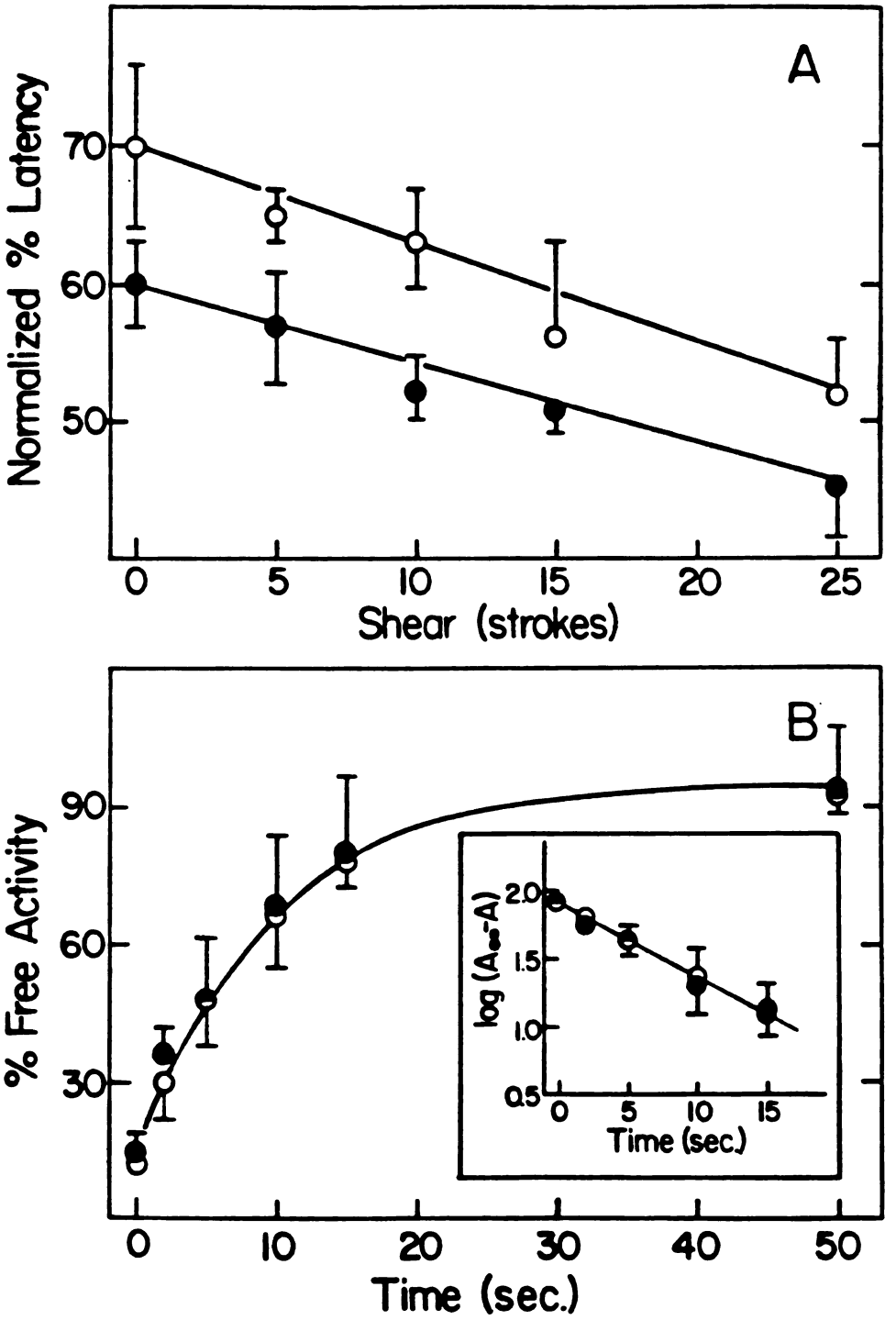

90
60
30
NAGase % Latency
5
10
15
20
25
50
Age (Days ex ovo)

shown). Again, the data for APase were anomalous. NAGase and cathepsin D had the same latency in normal muscle crude homogenates (50 and 58%, respectively) while APase latency was much lower (21%). This discrepency was retained in the M+L fraction, where NAGase and cathepsin D had 82 and 79% latency, respectively, and APase was 46% latent. Using sedimentation criteria, however, the differences in normal muscle crude homogenate latency remained (NAGase, 54%, cathepsin D, 58%; APase 33%) but latencies of the M+L fraction were the same (NAGase, 90%, cathepsin D, 79%; APase 91%).

Membrane Fragility Study - A variety of tests of lysosomal membrane fragility were designed to determine if lysosomes from normal and dystrophic muscle respond differently to stress. Lysosomes subjected to a shear stress lose latency linearly with time, at least for the first 15% of loss. Lysosomes from normal and dystrophic muscle lose latency at essentially the same rate: normal $-0.71 \pm 0.19\%$ latency/stroke, dystrophic $-0.57 \pm 0.16\%$ latency/stroke (Fig. 4 A)($P>0.1$, Student's t-test). Likewise, particles from normal and dystrophic muscle subjected to sonication (low power) for various times and subsequently sedimented to remove latent NAGase activity show identical responses to the stress (Fig. 4 B). Analysis of the individual data, based on an exponential decay model ($A=Ao\ e^{-kt}$) gave rate constants (k) of $-0.052 \pm 0.012\ sec^{-1}$ (normal) and $-0.053 \pm$

Figure 4: Membrane Fragility Tests: (A) Shear and (B) Sonication.

M+L fractions from normal (o) and dystrophic (●) chicken breast muscle were prepared, subjected to stress and assayed as described in Materials and Methods. Data are from 6 birds each line, aged 15 - 19 days *ex ovo*.

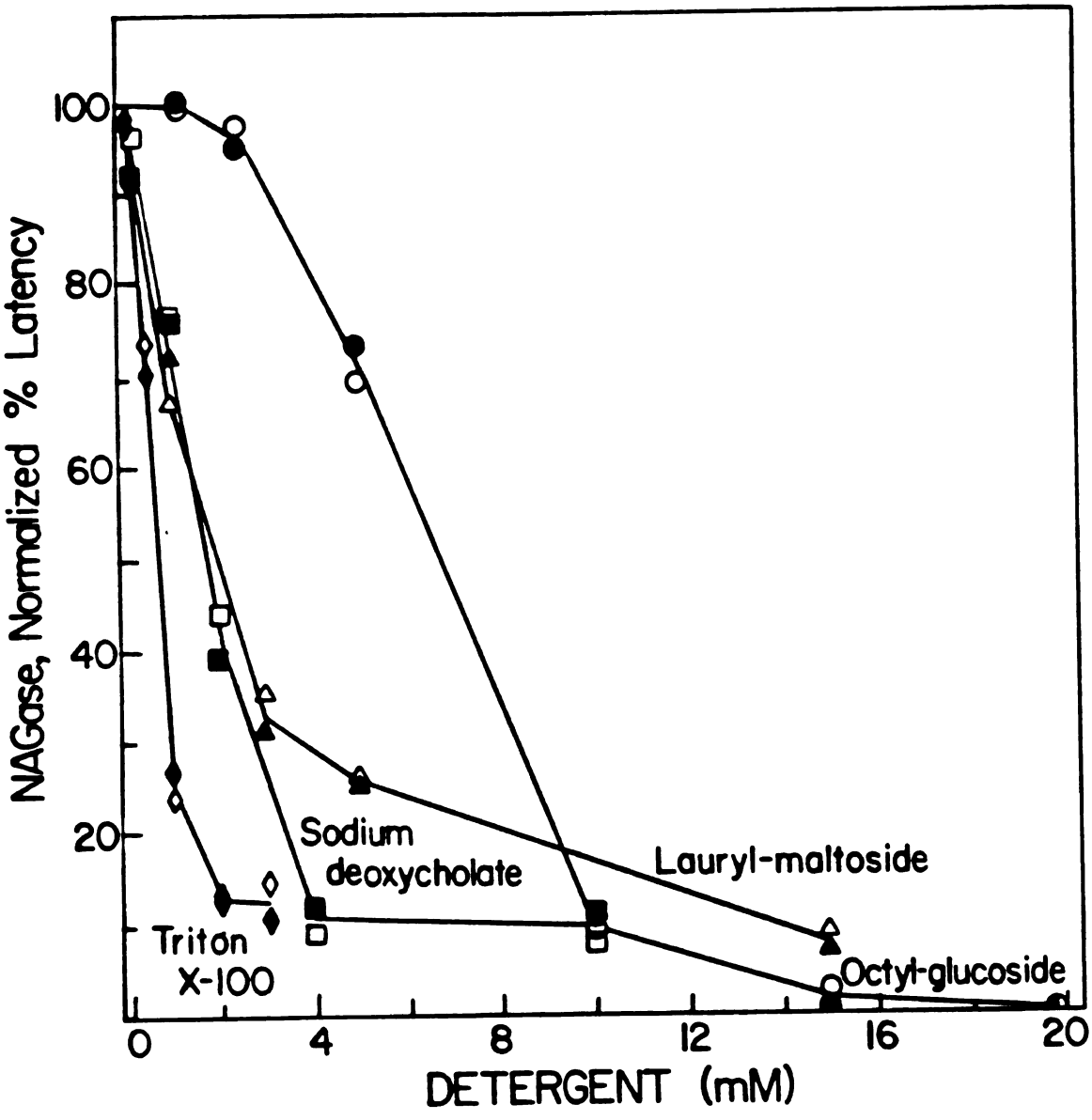

A
Normalized % Latency
70
60
50
0
5
10
15
20
25
Shear (strokes)
B
% Free Activity
90
60
30
0
10
20
30
40
50
Time (sec.)
log (A∞-A)
2.0
1.5
1.0
0.5
0
5
10
15
Time (sec.)

0.014 sec^{-1} (dystrophic) ($P>0.5$, Student's t-test). No rebinding of NAGase to membranes (as observed in kidney cells (11)) was indicated, since long sonication times resulted in release of greater than 90% of the total activity to the supernatant.

Since the physical stress tests do not indicate any gross differences in fragility, the lysosomes were subjected to a somewhat more sensitive test. M+L fractions from normal and dystrophic muscle were titrated with various detergents and NAGase latency was monitored using the detergent-based assay. The results (fig. 5) again show no differences between lysosomes from normal and dystrophic muscle. If we define $C_{1/2}$ as the concentration of each detergent required to decrease NAGase latency by 50%, TX100 is the most effective detergent with $C_{1/2}$ = 0.64mM (assuming a molecular weight of 643), sodium deoxycholate and lauryl maltoside are intermediate in effectiveness with $C_{1/2}$ = 1.6 and 1.8 mM, respectively, and octyl-glucoside is the least effective, $C_{1/2}$ = 6.7 mM.

Figure 5: Detergent Stress.

M+L fractions (birds 14 - 19 days *ex ovo*, 10 mg/ml protein) from normal (open symbols) and dystrophic (closed symbols) muscle were stressed with various detergents and assayed as described in Materials and Methods. The data are normalized to 100% initial latency. Numbers in parentheses represent the number of birds used for that data set. Due to solubility problems, sodium deoxycholate solutions were adjusted to pH = 8.0 with KOH prior to use.


NAGase, Normalized % Latency
100
80
60
40
20
0
4
8
12
16
20
DETERGENT (mM)
Sodium
deoxycholate
Lauryl-maltoside
Triton
X-100
Octyl-glucoside

DISCUSSION

Visual examination of the breast muscle of normal and dystrophic chickens reveals a significant difference in muscle texture, even at relatively early ages (differences are readily visible at 8- to 10-days *ex ovo*). Histochemical and biochemical examinations show increased fatty deposits (27), increased collagen fibers (5) and decreased myotubule content (3,20) in the dystrophic muscle. Yet nearly identical percent recoveries of lysosomal (except APase), and mitochondrial enzymes are obtained in the M+L fractions from normal and dystrophic muscle up to 50-days *ex ovo* (Fig. 1 A and C, and Table 1). Thus, differences in tissue texture and structure do not affect the homogenization process, and are not likely responsible for the differences between normal and dystrophic muscle lysosomes.

The major differences between normal and dystrophic muscle noted in this study are in the enrichment factors (Table 1) and in the percent latencies of lysosomal enzymes from 12- to 50-days *ex ovo* (Table 2 and Fig. 3). There are no significant differences in the enrichment factors for the mitochondrial marker succinate:INT reductase from normal and dystrophic muscle. Enrichment factors for all lysosomal enzymes are lower from the dystrophic muscle than from normal muscle (of the lysosomal enzymes tested, NAGase shows the most significant difference). These lower enrichment

factors are due to an increase in sedimented proteins (i.e., fiberous, organellar, etc.) in the dystrophic muscle, reflected in the increased percentage of total protein isolated in the M+L fractions from dystrophic muscle.

The differences in percent latency of lysosomal enzymes in both the crude and the M+L fraction from normal and dystrophic muscle are apparent when either detergent activation or sedimentation criteria are used. Efforts to ascertain the origin of the differences in latency of lysosomes from normal and dystrophic muscle using an isolated lysosome fraction were not successful. Shear tests by sonication or by homogenization in a Potter-Elvejham homogenizer failed to show differences. Attempts to ascertain differences in the lysosomal membrane by noting the effects of increasing concentrations of a variety of detergents on latency also failed. The titration experiments appeared to be partially related to the critical micelle concentration (CMC) of the detergents rather than to possible differences in the lysosomal membranes (compare the breaks in the curves of Fig. 4 with the detergent CMC values: octylglucoside, 23.4 mM (26); sodium deoxycholate, 14 mM (8); TX100, 0.24 mM (8); and lauryl maltoside, 0.2 mM (6)). These contradictory results (decreased latency, but no difference in fragility) suggest that decreased latency reflects a change in lysosomes other than in fragility, perhaps permeability. The observed identical percent yields

of lysosomal enzymes from normal and dystrophic muscle (Table 1) also indicate similar fragility.

The anomalous results with APase support the observations of Trout, et al. (23-25) indicating more than one site of localization of this enzyme in skeletal muscle. In the crude homogenate, APase latency was half that of either NAGase or cathepsin D, using sedimentation or detergent activation as the latency criterion (Table 2, sedimentation data not shown). This result suggests the presence of a non-lysosomal, cytoplasmic APase activity, since the activity was neither latent (in proportion to other lysosomal enzymes) nor sedimentable. When the latency of the M+L fraction was determined by detergent activation, we still observed significantly lower latency for APase as opposed to NAGase or cathepsin D. However, when the latency of the M+L fraction was determined by sedimentation, all three enzymes (APase, NAGase, and cathepsin D) showed comparable latency in normal and dystrophic M+L fractions. These results suggest the presence of two additional APase activities, one sedimentable but nonlatent (perhaps microsomal), and one lysosomal. These results are consistent with the existence of at least three APase activities in avian dystrophic muscle (lysosomal, microsomal, and cytoplasmic).

Muscle dysfunction results in myotube degeneration and death. This is evident in the neuromuscular diseases, as

well as muscular atrophies caused by dietary deficiency and denervation. The data reported here could reflect a general response to muscle dysfunction, or a general defect in membrane structure of dystrophic tissues. It should be noted that decreased lysosomal latency in skeletal muscle has been reported for muscle dysfunction caused by denervation (15), vitamine E deficiency (13), and starvation (12). Increased acid hydrolase activity is also reported for these dysfunctions. Thus, increased lysosomal acid hydrolase activity and decreased latency may be a general muscle response to dysfunction. The question of the significance of these data in the etiology of muscular dystrophy remains unanswered. Are the lysosomal membranes permeable to certain substrates, or even to the lysosomal enzymes? Does the data reflect a general membrane deficiency, assumed for the sarcolemma as measured by the increase of muscle enzymes in the blood serum? Is the decreased latency a secondary reaction to the disease, whose primary defect lies in membrane structure in general? The specific cause of, and the signal generating the observed decrease in lysosomal latency remains unknown.

In conclusion, we have shown a small decrease in the latency of lysosomal enzymes in dystrophic muscle crude homogenates which persists when lysosomes are partially purified. This decreased latency is not due to gross differences in lysosomal fragility. An increase in

sedimentable protein results in decreased enrichment factors for the lysosomal enzymes in lysosome-enriched fractions from dystrophic muscle. Finally we report anomalous latency, percent recovery and enrichment data for APase when compared to other lysosomal marker enzymes (NAGase and cathepsin D) in both normal and dystrophic avian pectoral muscle. These anomalies are consistent with the presence of several APase species in skeletal muscle as suggested by Trout, et al. (23-25), and suggest that the suitability of APase as a lysosomal marker enzyme should be reevaluated.

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CHAPTER III: MULTIPLE ACID PHOSPHATASES IN AVIAN PECTORALIS MUSCLE - THE POSTMICROSOMAL SUPERNATANT ACID PHOSPHATASE IS ELEVATED IN AVIAN DYSTROPHIC MUSCLE

MULTIPLE ACID PHOSPHATASES IN AVIAN PECTORALIS MUSCLE - THE POSTMICROSOMAL SUPERNATANT ACID PHOSPHATASE IS ELEVATED IN AVIAN DYSTROPHIC MUSCLE

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ABSTRACT

There are at least three forms of acid phosphatase in avian pectoralis muscle differing in molecular weight, subcellular location and response to various substrates and inhibitors. These enzymes are separated by differential sedimentation into postmicrosomal supernatant, lysosomal and microsomal activities with apparent molecular weights in Triton X-100 of 68,000, 198,000 and 365,000 respectively. All of the enzymes show acid pH optima (pH 5), but the postmicrosomal supernatant form is distinctly different from the other two forms in its resistance to most common phosphatase inhibitors and in its reduced activity against several organic phosphates. Quantitation of these three forms of acid phosphatase in normal and dystrophic avian pectoralis muscle shows that the postmicrosomal supernatant form is significantly elevated in dystrophic muscle; at 33 days *ex ovo*, 84% of the increased acid phosphatase activity in dystrophic muscle can be attributed to the postmicrosomal supernatant form. The microsomal form is only slightly elevated; the level of the lysosomal form is not altered.

FOOTNOTES

1) The abreviations used are: M+L, mitochondria + Lysosomes; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis-(ß-aminoethylether) N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ca^{2+}-ATPase, calcium activated Adenosine triphosphatase; APase, acid phosphatase; NAGase, N-acetyl-ß-D-glucosaminidase; TRIS, tris(hydroxymethyl)aminomethane; and RSA, relative specific activity.

INTRODUCTION

Muscular dystrophy is characterized by progressive muscle atrophy, loss of muscle function and numerous alterations in muscle enzyme activities (1). As part of our continuing interest in the disease, we have examined characteristics of several enzymes in normal and dystrophic chicken pectoralis muscle (2). Of recent interest to us (3) are the increased specific activities of lysosomal acid hydrolases, including acid phosphatase, in dystrophic muscle (4). Histochemical evidence supports the existence of acid phosphatase activity in the t-tubule network, as well as in the lysosomes of skeletal muscle (5-7). Soluble and membrane-bound forms of acid phosphatase in lysosomes have been suggested (8), and it is known that carbohydrate processing produces heterogeneity in the isoelectric forms of acid hydrolases (cf 9). Since the specific activity of acid phosphatase is increased in dystrophic muscle, and since recent findings suggest multiple acid phosphatase activities in chicken pectoralis muscle (3, 5-7), the heterogeneous nature of this enzyme prompted several questions. How many different forms of acid phosphatase are in skeletal muscle? What is/are the subcellular location(s) of these forms? Which form is elevated in the dystrophic muscle? What is the physiological significance of multiple acid phosphatases in muscle tissue?

In this paper, we present evidence for at least three acid phosphatases in avian pectoralis muscle, differing in apparent molecular weight, reactivity towards various substrates and inhibitors, and subcellular location. They all have acid pH optima (pH 5) and can hydrolyze a broad variety of organic phosphate esters. The increased acid phosphatase activity in dystrophic avian pectoralis muscle is primarily due to the postmicrosomal supernatant form; the microsomal form is only slightly elevated whereas the lysosomal form is not affected.

EXPERIMENTAL PROCEDURES

Animal Model - Single comb white leghorn fertile eggs were obtained locally; dystrophic (line 413) and control (line 412) fertile eggs were obtained from the Department of Avian Sciences (University of California, Davis, CA). Eggs were hatched in facilities kindly provided by the Department of Animal Science (Michigan State University, E. Lansing, MI). Chicks were fed Chick-GO 125 (Med) feed from Kent Feeds, Inc. (Muscatine, IA), and water *ad libitum*.

Materials - Aquacide III was from Calbiochem-Behring, (La Jolla, CA). Imidazole (grade I) from Sigma Chemical Co., (St. Louis, MO), was recrystallized from $CHCl_3$/petrolium ether prior to use. Triton X-100 (scintillation grade) and 4-methylumbelliferylphosphate (analytical reagent grade) were from Research Products International (Elk Grove, IL). Casein was a gift from Dr. W.W. Wells (Michigan State University). Sephadex G-100 (40-120u), Sephadex G-200 (40-120u) and blue dextran 2000 were from Pharmacia Fine Chemicals (Piscataway, NJ). Protein molecular weight standards and fluorescamine were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or better.

Subcellular Fractionations - The crude homogenate and M+L[1] fraction were prepared from normal white leghorns aged 15-20 days *ex ovo* as previously described (3). The M+L

pellet was either solubilized directly with Triton X-100 (M+L extract), or suspended into cold distilled water and centrifuged at 30,000 x g for 30 min to produce the M+L lysate (supernatant liquid) which was concentrated 5-fold against Aquacide III prior to use.

Microsomes were prepared from the crude homogenate by sedimentation at 30,000 x g for 30 min, discarding the pellet and then sedimenting the supernatant at 75,000 x g for 90 min. The high-speed pellet (microsomes) was resuspended into cold buffer (0.25 M sucrose, 10 mM EGTA, 40 mM imidazole propionate pH 7.0); the postmicrosomal supernatant from this preparation was concentrated 5-fold against Aquacide III before use.

Subcellular fractions were solubilized by addition of 10% (w/v) Triton X-100 to a final concentration of 0.1% (w/v) and sedimented at 30,000 x g for 30 min to remove insoluble debris before application to the Sephadex G-200 gel permeation column.

Separation and Localization of APases - A one ml sample of each solublized fraction was placed on a Sephadex G-200 column (2 x 75 cm) and then eluted with 0.1 M NaCl, 0.1% (w/v) Triton X-100, 10 mM EDTA, pH 7.5; flow rates were 15-20 ml/hr. Eluate fractions were assayed for APase activity using 4-methylumbelliferylphosphate as described below. The column was calibrated for molecular weight estimates with blue dextran 2000 (Mr=2 x 10^6), ferritin

(Mr=450,000), catalase (Mr=247,000), rabbit gamma - globulins (Mr=160,000), bovine serum albumin (Mr=68,000), myoglobin (Mr=17,000) and glycylglycine (Mr=132).

A one ml sample of the postmicrosomal supernatant (not treated with Triton X-100) was also placed on a Sephadex G-100 column (2 x 95 cm) and eluted with 0.1 M NaCl, 10 mM EDTA, pH 7.5, at flow rates of 35-40 ml/hr. Eluate fractions were assayed for APase using 4-methylumbelliferylphosphate as described below. The column was calibrated for molecular weight estimation using blue dextran 2000 (Mr=$2x10^6$), bovine serum albumin (Mr=68,000), ovalbumin (Mr=45,000), chymotrypsinogen A (Mr=25,000), soybean trypsin inhibitor (Mr=17,000), cytochrome c (Mr=12,500), bovine insulin (Mr=6,000) and N-acetyl-L-tyrosine (Mr=223).

Quantification of APases - APases were extracted from normal (line 412) and dystrophic (line 413) avian pectoralis muscle using 4 volumes of 1% (w/v) Triton X-100, 10 mM EGTA, 20 mM HEPES, pH 7.5, homogenizing (5 strokes, 300 rpm) with a Duall homogenizer, and sedimenting at 30,000 x g for 30 min. Pelletable material was re-extracted, and the supernatants were combined. This extract was diluted in 0.1 M NaCl, 10 mM EGTA, 20 mM HEPES, pH 7.5, to reduce the Triton X-100 concentration to 0.1% (w/v), and again centrifuged. Four ml samples were loaded onto a 4 x 75 cm Sephadex G-200 column and eluted with 0.1% (w/v) Triton X-100, 10 mM EGTA, 0.1 M NaCl, 20 mM HEPES, pH 7.5, flow

rates were 40-50 ml/hr, and 8 ml fractions were collected. Fractions were assayed for APase activity using 4-methylumbelliferylphosphate as described below. The relative percent of each enzyme form in total extracts was estimated by cutting out and weighing peaks from elution profiles. These data were used to estimate the total units of each enzyme in the normal and dystrophic muscle tissue.

Enzyme Assays, Inhibitors and Substrate Specificity - APase activity was measured under a variety of conditions with different substrates. Assays with 4-methylumbelliferylphosphate were completed essentially as described by Barrett (10), using 50 mM sodium citrate buffer, pH 4.3, 0.1% (w/v) Triton X-100 and 1.34 mM 4-methylumbelliferylphosphate, measuring the release of fluorescent 4-methylumbelliferone. For assays at pH 7.0, the reaction mixture pH was adjusted with NaOH. Assays using ß-glycerophosphate as a substrate were completed as described by Mak and Wells (11), except that pH 4.3 was used and inorganic phosphate was measured using the modified Fiske-Subbarow reaction as described by Baginski, et al. (12). Samples assayed with ß-glycerophosphate at pH 7.0 used 50 mM HEPES, pH 7.0, instead of 50 mM acetate, pH 4.3. Activity against other substrates was determined by monitoring inorganic phosphate release as detailed above; reaction conditions were 5 mM substrate, 50 mM sodium citrate pH 4.3 and 0.1% (w/v) Triton X-100. The percent

inhibition by several phosphatase inhibitors was examined by assaying APase with 4-methylumbelliferylphosphate at pH 4.3 as described above in the presence and absence of the inhibitor.

Assays for pyruvate kinase (cytosolic marker enzyme), used the method of Bucher and Pfleiderer (13). NAGase (lysosomal marker) was assayed using 4-methylumbelliferyl-N-acetyl-ß-D-glucosaminide as substrate, essentially as suggested by Barrett (10). Ca^{2+}-ATPase (sarcoplasmic reticulum marker) was measured with the method of Meissner (14).

All assays were at 37°C, and activities are reported as international units (IU), defined as 1 umole product formed per minute.

pH Optima - The pH optimum for each APase was determined using 1 mM 4-methylumbelliferylphosphate, 0.1% (w/v) Triton X-100, and 0.14 M buffer (buffers used were: pH2 and 2.5, glycine; pH3-6.5, citrate; pH7-8, HEPES; and pH8.5 and 9, TRIS).

Protein Assay - Protein was assayed as suggested by Udenfriend, et al. (15), adding 0.5 ml diluted protein to 1.0 ml 0.2 M sodium borate buffer, pH 9.0, than adding, with rapid mixing, 0.5 ml 0.2 mg/ml fluorescamine (in acetonitrile). Fluorescence was determined using the Aminco Fluorocolorimeter; bovine serum albumin was used as a standard.

RESULTS

Subcellular Fractionation - Percent recoveries, specific activities and relative specific activities (RSA) of the various marker enzymes for each subcellular fraction and for APase are given in Table I. Each subcellular fraction is preferentially enriched in its respective marker enzyme. The postmicrosomal supernatant preferentially enriches pyruvate kinase (RSA=2.5), the lysosome fraction enriches NAGase (RSA=6.4), and the microsomal fraction enriches Ca^{2+}-ATPase (RSA=10.5). Note a greater than 97% recovery of the cytosolic marker enzyme pyruvate kinase; as expected, a substantial fraction of the lysosomal and microsomal marker enzymes is lost in the pellets from the crude homogenate and lysosomal lysate.

Microsomes are defined operationally as a membraneous fraction sedimentable from a post-mitochondrial supernatant using high speeds for extended times (in this case 75,000 x g for 90 min). From liver they typically contain endoplasmic reticulum and membranes from the Golgi complex, plasma membrane, fragmented mitochondria, lysosomes and peroxisomes (16). Because of the extensive plasma membrane and sarcoplasmic reticular network found in muscle, our microsomal preparation is most likely primarily composed of these two membrane components (14). Table I shows a 10.7 fold enrichment of Ca^{2+}-ATPase from the sarcoplasmic

Table 1: Characterization of Subcellular Fractions.

Enzyme	Fraction			
	Crude	Lysosome Lysate	Microsomes	PMSN
Pyruvate Kinase				
Sp act	5.8 ± 0.7	1.13 ± 0.25	4.27 ± 0.63	14.6 ± 1.2
% Rec[b]	100	0.32	2.04	95.0
RSA[a]	1.000	0.20 ± 0.07	0.75 ± 0.22	2.5 ± 0.4
NAGase				
Sp act	$(7.6 \pm 0.8) \times 10^{-4}$	$(4.8 \pm 0.6) \times 10^{-3}$	$(1.2 \pm 0.2) \times 10^{-3}$	$(6.9 \pm 0.4) \times 10^{-4}$
% Rec[b]	100	10.7	4.3	34.0
RSA	1.000	6.4 ± 1.2	1.6 ± 1.2	0.91 ± 0.05
Ca^{+2}-ATPase				
Sp act	$(6.5 \pm 0.9) \times 10^{-2}$ U	$(0.8 + 1.4) \times 10^{-3}$	0.69 ± 0.11	$(6.4 + 7.6) \times 10^{-3}$
% Rec[b]	100	0.03	30.7	4.03
RSA	1.000	0.014 ± 0.024	10.7 ± 1.7	0.10 ± 0.13
APase				
Sp act	$(4.2 \pm 0.6) \times 10^{-3}$	$(1.8 \pm 0.3) \times 10^{-2}$	$(1.5 \pm 0.5) \times 10^{-2}$	$(6.6 \pm 0.2) \times 10^{-3}$
% Rec[b]	100	7.4	8.4	59.2
RSA	1.000	4.3 ± 0.1	3.5 ± 1.2	1.6 ± 0.1

Note. Breast muscle from 3 chickens (19 days *ex ovo*) was fractionated into lysosomal lysate, microsome, and postmicrosomal supernatant (PMSN) fractions, and assayed for marker enzymes and acid phosphatase (APase) as detailed in *Experimental Procedures*. Data are expressed as means ± 1 SD; percentage recoveries (% Rec) are means. Specific activities are umol min^{-1} mg^{-1} protein.

a RSA (relative specific activity) - specific activity of subcellular fraction/specific activity of the crude; gives a measure of enrichment.

b The difference between the sum of the percentage recoveries of each enzyme in each fraction and 100% is due to activity in pellets from the crude homogenate and lysosomal lysate.

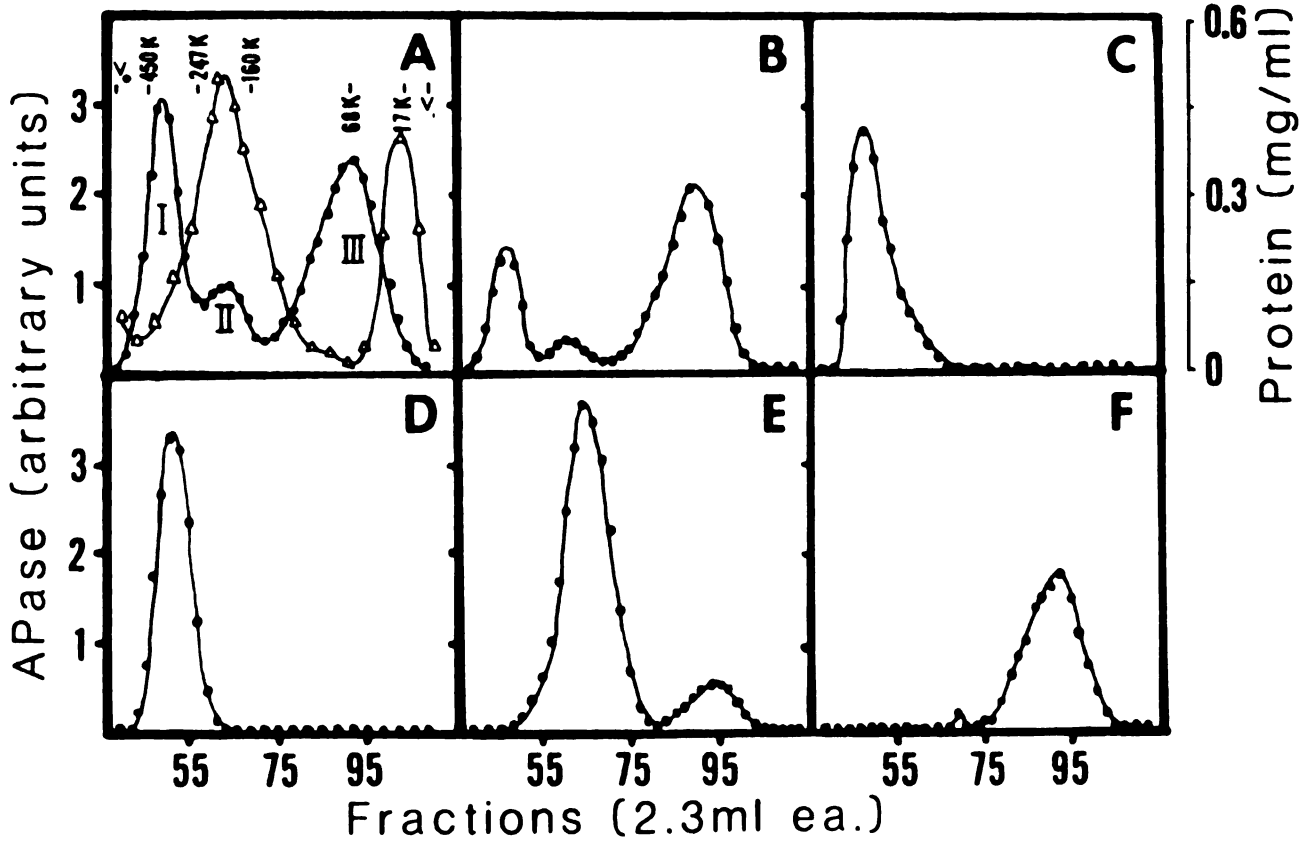
reticulum.

Of particular interest for this study is the enrichment of APase in each of the subcellular fractions (Table I). However, as detailed later, the APase activity in each subcellular fraction is due principally to different isoforms and thus the enrichment factors are somewhat misleading.

Column Chromatography - Chromatography of the crude muscle extract on Sephadex G-200 reveals three peaks of APase activity (Figure 1A). Ten mM mannose-6-phosphate does not significantly alter this elution profile (Figure 1B); alterations would be expected if any of the observed forms represented complexes of the enzyme with a mannose-6-phosphate receptor (e.g.,(17)). Rechromatography of peak I does not yield any of the other forms (Figure 1C), eliminating the possibility that the different peaks represent associated forms of the enzyme. The solubilized microsome fraction contains peak I (Figure 1D), lysosomal lysates are highly enriched in peak II but also contain peak III (Figure 1E) and the postmicrosomal supernatant (cytosol) contains peak III (Figure 1F). Based on the above observations, peak I is tentatively identified as a microsomal integral membrane enzyme; detergent was required to release the enzyme from the microsome fractions since neither sonication nor osmotic lysis was sufficient (data not shown). Peaks II and III are relatively soluble enzymes;

Figure 1: Sephadex G-200 Chromatography.

Fractions were eluted from a 2 x 75 cm Sephadex G-200 column with 0.1 M NaCl, 0.1% (w/v) Triton X-100, 10 mM EDTA, pH 7.5, at a flow rate of 15-20 ml/hr. Acid phosphatase (APase) was assayed with 4-methylumbelliferylphosphate at pH 4.3, and protein was determined as described in Experimental Procedures. (A) solubilized crude homogenate, (B) solubilized crude homogenate + 10 mM mannose-6-phosphate, (C) rechromatography of peak I from (A), (D) solubilized microsomes, (E) solubilized lysosomal lysate, and (F) post-microsomal supernatant. Peaks were labeled I, II, and III, in descending order of molecular weight. (o, acid phosphatase activity; Δ , protein concentration). Molecular weight markers are indicated, and proteins used are described in Experimental Procedures.

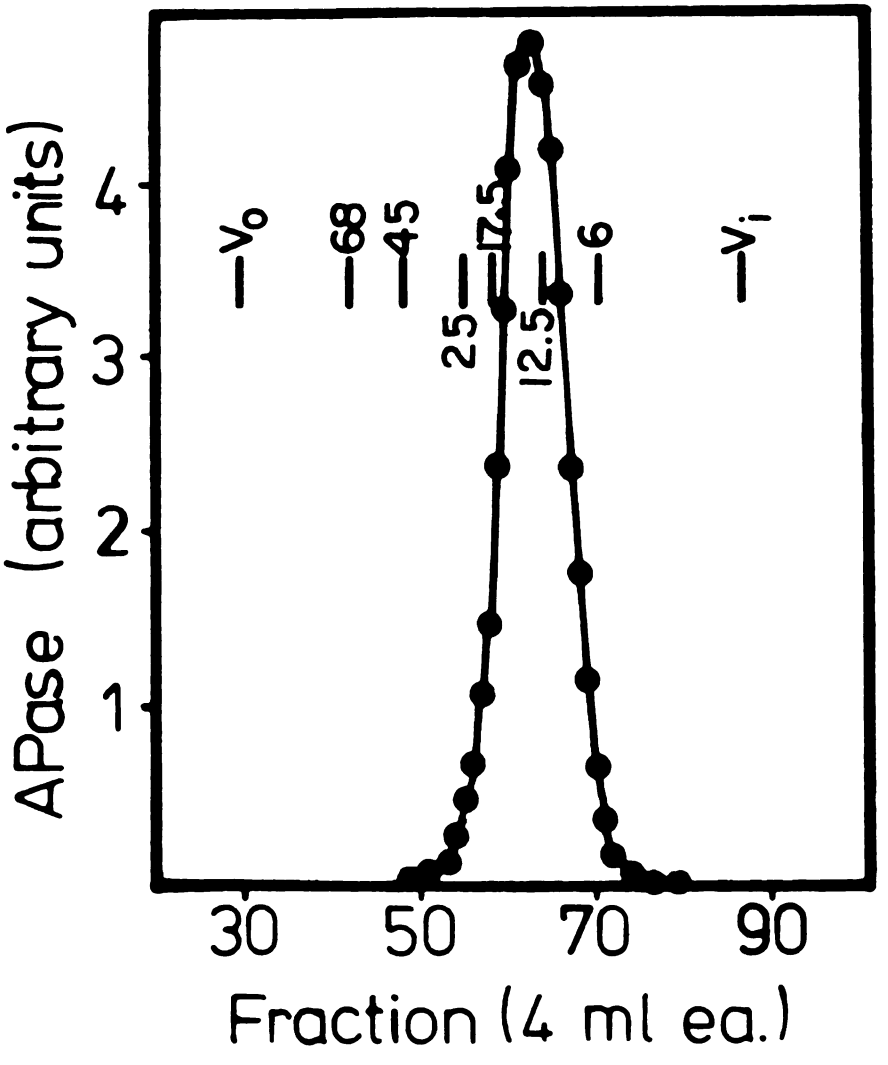

A
B
C
D
E
F
-450K
-247K
-160K
68K-
17K-
I
II
III
APase (arbitrary units)
Protein (mg/ml)
0.6
0.3
0
3
2
1
55
75
95
Fractions (2.3ml ea.)

neither required detergent for solublization (peak II is released by osmotic or sonic lysis of the M+L fraction and peak III is highly enriched in the postmicrosomal supernatant). These enzymes are tentatively assigned lysosomal (peak II) and cytosolic (peak III) subcellular locations.

The apparent molecular weights were obtained by comparative elution of the enzyme and standards from Sephadex G-200 (Figure 1A). The enzyme in the microsomal fraction is the largest (Mr=365,000), followed by the lysosomal (Mr=198,000) and the postmicrosomal supernatant Mr=68,000). These molecular weights are, no doubt, high estimates reflecting the association of Triton X-100. The molecular weight of the enzyme in the microsomal fraction is particularly suspect since the solubilization data are consistent with a strong association with membranes, suggesting a large hydrophobic region which would be expected to form mixed micelles with the detergent. Subsequent chromatography of the postmicrosomal supernatant fraction on a Sephadex G-100 column in the absence of Triton X-100 revealed a single peak, Mr=11,900 (Figure 2) thus eliminating the possibility of unresolved multiple forms in the low molecular weight peak from the Sephadex G-200 elution profile, and suggesting that this enzyme also has a relatively large hydrophobic region. Chromatography of a postmicrosomal supernatant fraction from dystrophic

Figure 2: Sephadex G-100 Chromatography.

The postmicrosomal fraction was eluted from a 2 x 95 cm Sephadex G-100 column, with 0.1 M NaCl, 10 mM EGTA, pH 7.5, at a flow rate of 35-40 ml/hr. Acid phosphatase was assayed as described in Experimental Procedures with 4-methylumbelliferylphosphate at pH 4.3. Molecular weight markers are indicated, and proteins used are described in Experimental Procedures.


APase (arbitrary units)
4
3
2
1
Vo
68
45
25
17.5
12.5
6
Vi
30
50
70
90
Fraction (4 ml ea.)

pectoralis muscle shows a similar profile, indicating that no unique low molecular weight enzyme is present in the diseased muscle (data not shown).

Substrate and pH Differences - APases were also differentiated by their differential activity against two phosphatase substrates, at pH 4.3 and 7.0 (Table II). Each fraction is capable of hydrolyzing 4-methylumbelliferyl phosphate and ß-glycerophosphate, however, they are more effective in hydrolyzing the artificial substrate (compare the ratios of the activity with 4-methylumbelliferyl phosphate to that of ß-glycerophosphate at pH 4.3 or 7.0). While all forms hydrolyze 4-methylumbelliferylphosphate more rapidly than ß-glycerophosphate, the lysosomal enzyme is the most efficient form for hydrolysis of ß-glycerophosphate relative to 4-methylumbelliferylphosphate at either pH.

Hydrolysis of various other substrates at pH 4.3 confirms the differences between the postmicrosomal supernatant form and the other forms of the enzyme (Table III). The postmicrosomal supernatant APase hydrolyzes ATP and glucose 6-phosphate more slowly than the lysosomal and microsome-associated forms. Both the microsome-associated enzyme and the lysosomal enzyme can be classified as ATPases, since they hydrolyze ATP at about twice the rate of any other substrate (except 4-methylumbelliferylphosphate). These two enzymes also hydrolyze other substrates with similar rates; however, the hydrolysis of ß-glycerophosphate

Table 2: Activity of Various Subcellular Fractions with Different Substrates at pH 4.3 and pH 7.0.

	Specific Activities (IU/mg Protein)				Ratios	
	pH 4.3		pH 7.0		4-MUP/ß-GP	
Enzyme Source	4-MUP	ß-GP	4-MUP	ß-GP	pH 4.3	pH 7.0
M+L Lysate	0.196±0.042	0.018±0.004	0.081±0.014	0.017±0.003	10.9	4.8
Microsome Extract	0.130±0.016	0.009±0.001	0.075±0.018	0.006±0.001	14.4	12.5
Postmicrosomal supernatant	0.087±0.004	0.002±0.001	0.076±0.005	0.003±0.001	43.5	25.3

Lysosomal lysate, microsomal extract, and postmicrosomal supernatant were prepared separately from five birds (18 days *ex ovo*), as detailed in *Experimental Procedures*. The specific activity of each fraction against ß-glycerophosphate (ß-GP) and 4-methylumbelliferylphosphate (4-MUP) was determined at pH 4.3 and 7.0. Ratios of activities against 4-methylumbelliferylphosphate versus ß-glycerophosphate were calculated. Data are expressed as means ± standard deviation.

Table 3: Activity with Various Substrates Relative to the Activity With 4-methylumbelliferylphosphate.

Enzyme \ Substrate	Relative Activity						
	ß-GP	ATP	AMP	IMP	G-6P	Fl,6P	Casein
Microsomal	0.152± 0.009	0.354± 0.015	0.052± 0.005	0.055± 0.005	0.135± 0.026	0.165± 0.029	0.008± 0.001
Lysosomal lysate	0.218± 0.016	0.370± 0.018	0.056± 0.013	0.081± 0.002	0.141± 0.027	0.177± 0.005	0.028± 0.014
Postmicrosomal supernatant	0.122± 0.026	0.086± 0.071	0.055± 0.011	0.058± 0.005	0.040± 0.015	0.125± 0.024	0.009± 0.007

Microsomes, lysosomal lysates, and postmicrosomal supernatants (cytosol) were prepared as detailed in Experimental Procedures from normal white leghorn chickens, (21 days *ex ovo*), and stored at -60° C prior to use. Release of inorganic phosphate was monitored as detailed in Experimental Procedures. All substrates were at 5 mM with the exception of casein which was at 0.1 mg/ml. Activities are presented relative to the activity against 4-methylumbelliferryl-phosphate. Values are means ± 1 standard deviation for three separate preparations of each enzyme fraction. ß-GP - ß-glycerophosphate, ATP - adenosine 5'- triphosphate, AMP - adenosine 5'- monophosphate, IMP - inosine 5'- monophosphate, G-6P - glucose 6- phosphate, Fl,6P - fructose 1,6 diphosphate.

and IMP is slightly but significantly different. The lysosomal enzyme is the best general phosphatase; it hydrolyzes every substrate tested at least as fast as the other enzymes. It is the only form to hydrolyze casein (a protein phosphate) at a significant rate.

pH Optima - Each enzyme has a pH optimum between pH 5-5.5 with the substrate 4-methylumbelliferylphosphate. None of the enzymes had significant activity above pH 8.0, and activity at pH 7.5 (cytosolic pH) was approximately 10% of that at optimal pH (data not shown).

Inhibitors - The postmicrosomal supernatant enzyme is distinctly different from the other two in its response to various phosphatase inhibitors (Table IV). The microsome-associated enzyme and the lysosomal enzyme show similar inhibition by NaF (80%), L-(+)-tartaric acid (63%), Na_2MoO_4 (71%) and Na_2HPO_4 (54%), whereas the postmicrosomal supernatant form is not significantly inhibited (<12%) by these compounds. $HgCl_2$ (a sulfhydryl reagent) inhibited all the forms of acid phosphatase, but the postmicrosomal supernatant form is most sensitive.

APase Quantitation in Normal and Dystrophic Muscle - APase was extracted from muscle homogenates with 1% Triton X-100 as detailed in <u>Experimental Procedures</u>, with 99 $\pm$ 4% (N=3) recovery of total activity; dilution of the extract to reduce Triton X-100 to 0.1% resulted in no observable loss of enzyme activity (recovery 103 $\pm$ 2% (N=3)). Chromatography

Table 4: Effect of Various Inhibitors on Activity of Acid Phosphatase in Various Fractions.

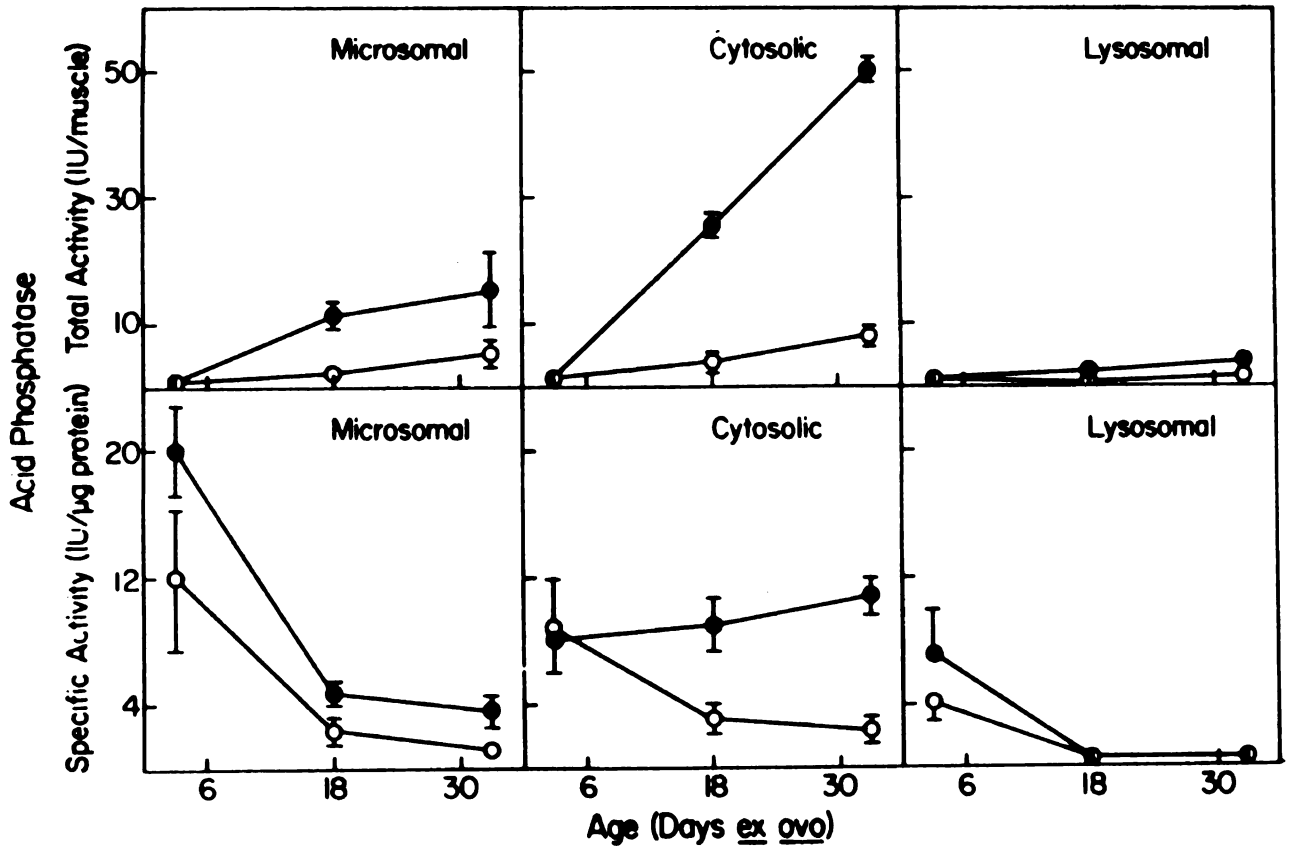
	Percent Inhibition By				
Inhibitor / Enzyme	10 mM NaF	20 mM Tartrate	2 mM $HgCl_2$	20 mM Na_2MoO_4	20 mM Na_2HPO_4
Microsomal	79	59	66	68	55
Lysosomal Lysate	80	67	88	75	54
Postmicrosomal supernatant	7	12	100	8	12

Pectoralis muscle from three birds (normal white leghorns, 18 days *ex ovo*) was separated by differential sedimentation into M+L, microsomal, and postmicrosomal supernatant fractions; the lysosomal lysate was prepared from the M+L fraction as detailed in Experimental Procedures. Each fraction was assayed at pH 4.3 using 4-methylumbelliferylphosphate monitoring the release of fluorescent 4-methylumbelliferone, in the presence and absence of inhibitor, as detailed in Experimental Procedures.

on Sephadex G-200 gave 97 ± 3% (N=3) recovery of loaded activity. Quantitation of the various forms from normal (line 412) and dystrophic (line 413) avian pectoralis muscle shows that the lysosomal form of APase is not significantly elevated in the dystrophic muscle at any age between 3 and 33 days *ex ovo* (Figure 3). The microsome-associated enzyme is slightly elevated (2x), but the postmicrosomal supernatant form is highly elevated, accounting for 84% of the increased APase total activity observed in dystrophic muscle at 33 days *ex ovo*. Comparison of specific activities of the various types of APase in normal and dystrophic muscle as a function of age shows that only the postmicrosomal supernatant enzyme is significantly affected (Figure 3). The specific activity of the postmicrosomal supernatant form remains high in dystrophic muscle with increasing age.

Figure 3: Total and Specific Activity of Acid Phosphatases in Normal and Dystrophic Muscle.

Acid phosphatase from normal (o) and dystrophic (●) avian pectoralis muscle from chickens of various ages was quantified as detailed in Experimental Procedures. Values are means ± 1 standard deviation for 3 birds per point.


Acid Phosphatase
Total Activity (IU/muscle)
Specific Activity (IU/μg protein)
Microsomal
Cytosolic
Lysosomal
50
30
10
20
12
4
6
18
30
Age (Days ex ovo)

DISCUSSION

Multiple APase Forms - Our data supports the existance of at least three distinct APases in avian pectoralis muscle (Figure 1). The breast muscle enzymes differ in subcellular location and have differing responses to various substrates and inhibitors. Though the multiplicity of APases found in most tissues has been the subject of extensive research (see 18, 19 for reviews), the percentage distribution amongst the various tissues is generally not known; either complete extraction of APase activity was not demonstrated or the separation of each form was not complete. Our procedure yields virtually complete extraction of activity and the gel permeation chromatography shows three molecular weight forms; chromatography of the low molecular weight activity from either normal or dystrophic muscle on Sephadex G-100 shows only one peak, eliminating the possibility of heterogeneity in the low molecular weight fraction from the Sephadex G-200 columns. Since the enzyme fractions are virtually free of contamination by the other forms, differential characterization of these enzymes was possible.

Acid Phosphatase in the Microsomal Fraction - The microsomal fraction is a high speed particulate fraction containing the sarcoplasmic reticulum (Ca^{2+}-ATPase) and other membranous components. Analysis of marker enzymes shows that it is relatively free of lysosomal and cytosolic

contamination (Table I). Twenty-one percent of the microsomal APase with an RSA = 8.8 was recovered in the microsomal fraction. These values were obtained by correcting the corresponding APase data in Table I to account for the fraction (40%, Figure 3) of the total APase activity in the muscle that is of the high molecular weight form. These corrected data are in good agreement with the 30.7% recovery and RSA = 10.7 for Ca^{2+}-ATPase, the sarcoplasmic reticulum marker enzyme. Histochemical data (5-7) supports a t-tubule localization for acid phosphatase in skeletal muscle; histochemical data also localizes ß-glucuronidase (another acid hydrolase) in the endoplasmic reticulum of mouse liver, rat preputial gland and rat cartilage (20,21). All of these observations are consistant with a microsomal acid hydrolase system; the function of such a system is unknown.

The exact localization of APase in the microsomal fraction cannot be identified. Previous reports (8) have assigned this enzyme to the lysosomal membrane, suggesting "free" and "membrane bound" forms of lysosomal APase. Our data are consistant with this interpretation, since lysosomal membrane fragments, generated by the homogenization procedure, would be expected to pellet with the microsomes. Also, we observe the "microsomal" enzyme form in our M+L preparation, where it makes up 40-50% of the total activity (data not shown).

The APase associated with the microsomal fraction may be an ATPase, though ATP is only a slightly better substrate than others tested (Table III). This enzyme form is very similar to the lysosomal form in its enzymatic properties (Tables II-IV), but the relationship, if any, between these two enzymes is not clear. The total activity of the microsome-associated APase is elevated 2-fold in dystrophic muscle; the specific activity is the same in both normal and dystrophic muscle (Figure 3).

Since we observe substantial amounts of the microsome-associated form of APase in skeletal muscle, previous studies not showing this form, or reporting low levels of the enzyme may be erroneous due to incomplete extraction (e.g. 24). Heinrickson (23) notes differences in total activities and in relative amounts of each form extracted when different extraction times were used. The low molecular weight enzyme is extracted most readily and the lysosomal enzyme shows enhanced extraction with incubation time; low levels of the microsomal form were extracted, which is to be expected since detergent was not used in the extraction buffer. Thus previous reports of the levels of these enzyme forms from various tissues must be re-evaluated.

Lysosomal APase - The lysosomal enzyme is of intermediate molecular weight and appears to be a non-specific phosphatase: it hydrolyzes all of the

substrates at least as well as the other two forms and is the only form to show measurable activity with casein, a phosphorylated protein. The enzyme is localized within the lysosomes, as evidenced by co-purification with NAGase in the lysosomal lysate fraction, which is relatively free of contamination by cytosol and sarcoplasmic reticulum (Table I). The lysosomal APase is a water soluble protein, probably not tightly associated with membranes since it is released upon sonication or other non-detergent lytic procedures. As noted above, its enzymatic properties are similar to those of the enzyme in the microsomal fraction. This form is also not affected in dystrophic muscle, as is evidenced by similar total and specific activities at various ages in normal and dystrophic pectoralis muscle (Figure 3). Such behavior is inconsistant with the data for other lysosomal enzymes, which are elevated in dystrophic muscle (cf 24). This "abnormal" behavior for APases versus other "lysosomal" acid hydrolases is not an isolated phenomenon. Examples where APases are not affected when most lysosomal enzymes show abnormal levels include I-cell disease (Mucolipidosis II) (25), pseudo-Hurler's syndrome (Mucolipidosis III) (26), and the toxic response to rapeseed oil (27). In all these cases, lysosomal enzymes as a group were dramatically affected whereas APase showed normal behavior. These observations suggest a regulatory mechanism for the expression of lysosomal APase activity which is distinct

from the general response of lysosomal acid hydrolases to some physiological stimuli.

Cytosolic APase - The cytosolic enzyme has a low molecular weight- 11,900 by Sephadex G-100 chromatography, and is distinctly different from the other two forms in its enzymological properties. The enzyme is similar to the red cell APase previously reported (cf 28), in that it has a low molecular weight, is insensitive to tartrate and F^-, and is completely inhibited by 2 mM Hg^{2+}. Since the red cell enzyme seems to have a wide tissue distribution (18,19), we suspect that these enzymes are similar. This enzyme form is distinctly elevated in dystrophic muscle, and accounts for the previously reported elevation (4) of APase specific activity in this diseased tissue.

The cytosolic assignment for this enzyme is justified since it is enriched in the postmicrosomal supernatant fraction (Figure 1) (which is primarily cytosolic), and does not show the same behavior (% recovery, RSA) as the contaminating lysosomal NAGase (Table I). Recovery of the cytosolic APase activity in the postmicrosomal supernatant fraction was 108% with RSA=2.9. These results were obtained from the data in Table I by correcting for contaminating lysosomal APase (assuming recovery of lysosomal APase and lysosomal NAGase are identical (34%)) and noting that lysosomal APase is 10% and cytosolic APase is 50% of the total APase activity in the muscle (Figure 3). Since the

postmicrosomal supernatant fraction is essentially free of sarcoplasmic reticulum (shown by Ca^{2+}-ATPase recovery data, Table I), we do not need to correct for microsome-associated APase contamination. These corrected data are in excellent agreement with the data for pyruvate kinase (95% recovery, RSA=2.5).

Summary - To conclude, our results and the literature are consistant with the following: (1) Non-lysosomal acid hydrolases (particularly APases) are widely distributed and sometimes account for quite substantial proportions of the total acid hydrolase activity. (2) The levels of lysosomal APases (and perhaps some other lysosomal enzymes, e.g. ß-glucuronidase (20)), do not respond to stimuli in the same manner as other lysosomal enzymes. (3) The low molecular weight postmicrosomal supernatant acid phosphatase activity accounts for over 80% of the elevation in acid phosphatase activity in dystrophic muscle at 33 days _ex ovo_. Thus the elevation of acid phosphatase activity previously reported in dystrophic muscle is distinct from the general activation of the lysosomal apparatus.

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CHAPTER 4: PURIFICATION AND CHARACTERIZATION OF TWO LOW MOLECULAR WEIGHT ACID PHOSPHATASES FROM AVIAN PECTORALIS MUSCLE.

ABBREVIATIONS USED

MOPS - 3-(N-Morpholino)propanesulfonic acid

EGTA - ethylene glycol-bis-(ß-aminoethylether)-N,N,N',N'-tetraacetic acid

APase - acid phosphatase

TX100 - Triton X-100

FMN - flavin mononucleotide

4-MUP - 4-methylumbelliferylphosphate

INTRODUCTION

Several acid phosphatases (APases) exist in most tissues (1). They have different molecular weights, and are associated with different cellular compartments. The higher molecular weight APases are associated with microsomes (Class I, Mr>200K) and lysosomes (Class II, Mr 90-120K). At least two low molecular weight forms exist called Class III (Mr 20-40K) and Class IV (Mr 8-18K) (2,3). Despite a considerable literature regarding the various APases, there is little agreement regarding their role in metabolism. Increased APase activity in dystrophic compared to normal muscle has been known for some time (eg. 4-7), but was always associated with the general increase in lysosomal acid hydrolases observed in this diseased tissue. However, we recently reported (8) that the increased APase activity observed in dystrophic avian pectoralis muscle is due to a postmicrosomal supernatant, presumably cytosolic, low molecular weight enzyme; the lysosomal APase is not significantly affected. This paper describes the purification of two different APases, both in Class IV, from avian pectoralis muscle. These two forms differ in isoelectric point, substrate specificity, guanosine activation, and kinetic parameters. Neither of the two are inhibited significantly by L-(+)-tartrate or fluoride.

MATERIALS AND METHODS

Materials- Compounds used as phosphatase substrates are 4-methylumbelliferylphosphate from Research Products International (Elk Grove, IL), and ß-glycerophosphate, α-naptholphosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, fructose 1,6-diphosphate, O-phospho-L-tyrosine, O-phospho-L-serine, O-phospho-D,L-threonine, flavin mononucleotide, and phosvitin from Sigma Chemical Company (St. Louis, MO), and ^{32}P-myosin light chain (a gift from Dr. R.S. Adelstein, National Institutes of Health, Bethesda, MD). Sephadex G-75 (superfine), Sephadex G-100 (40-120 u), Sephadex G-200 (40-120 u), sulfopropyl Sephadex (C50, 40-120 u), polybuffer exchanger (PBE94) and polybuffers (PB96) were from Sigma Chemical Company. Pronase was from Calbiochem-Behring (LaJolla, CA), and neuraminidase (Type VI) was from Sigma Chemical Company. All other chemicals were analytical reagent grade or better.

APase was isolated from frozen chicken breast muscle from Pel-Freez Biologicals (Rodgers, AR) and stored at -20°C until use.

Methods

Enzyme Assays- APase was typically assayed using the

method suggested by Barrett (9), with 4-methylumbelliferylphosphate at pH 4.3 as previously described (8). When release of inorganic phosphate was monitored, reaction conditions were 5 mM substrate, 0.25 mM Triton X-100 (TX100), and either 0.125 M MOPS (pH 7.0) or 0.125 M citrate (pH 5.0). Inorganic phosphate was assayed by the modified Fiske-Subbarow method suggested by Baginski (10), as previously described (8). Assays with ^{32}P-myosin light chain were completed using the reaction conditions cited above for pH 5.0 and 7.0, except each reaction mixture contained approximately 0.02 uCi ^{32}P-myosin light chain instead of 5 mM substrate. Reactions were incubated for 30 minutes, and inorganic ^{32}P release was measured using the Berenblum and Chain assay (11); inorganic ^{32}P was counted using the Cherenkov procedure (12) in a liquid scintillation counter. The total units of each of the three major types of APase (microsomal, lysosomal, and cytosolic) were quantified by Sephadex G-200 chromatography as previously described (8).

Kinetic experiments were completed by monitoring release of 4-methylumbelliferone from 4-methylumbelliferylphosphate as described above, at various concentrations of the substrate. Effector compounds were tested at fixed substrate concentration, (5 mM), varying the effector concentration. In the case of guanosine activation, several substrate concentrations were used. All assays were

completed at 37° C; each unit of activity is defined as 1 umole product formed per minute. Km and Vmax values were calculated by a weighted least squares procedure described by Wilkinson (13).

Purification of APases-

Preparation of Crude Extract- Pel-Freez frozen white chicken muscle (500 g) was minced into 3 volumes of cold buffer I (40 mM MOPS, 10 mM EGTA, 20 mM 2-mercaptoethanol, pH 7.0), homogenized with a Waring blender (Waring Products Corporation, N.Y.) for 4 x 30 seconds at high speed in the cold. Particulate matter was sedimented at 8,000g for 30 min.; the pellet was reextracted (as above) and sedimented. The two supernatants were combined to give the crude extract.

Ammonium Sulfate Fractionation- Solid $(NH_4)_2SO_4$ (32.6 g/100ml giving 55% saturation) was added to the crude extract, stirred at 4° C for 30 minutes, sedimented at 8,000g for 30 minutes; the pellet was discarded. The supernatant was brought to 80% saturation by addition of 16.1 g/100 ml of solid $(NH_4)_2SO_4$, stirred for 30 minutes at 4° C, and sedimented as above, discarding the supernatant liquid. The pellets were suspended into a minimal volume of buffer II (0.25 mM TX100, 5 mM NaH_2PO_4, 5 mM EGTA, 25 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, pH

7.0), and dialyzed against 2 x 20 volumes of 0.25 mM TX100, 25 mM 2-mercaptoethanol, pH 7.0 (1 hour each), then against 1 x 20 volumes of buffer II, overnite.

Sephadex G-100 Chromatography- The dialysate was concentrated twofold against Aquacide II, placed on a Sephadex G-100 column (5 x 95 cm, equilibrated with buffer II), and eluted at 50-70 ml/hr. Fractions (10.5 ml each) were collected and tubes containing APase activity were pooled, adjusted to pH 5.0 by slowly adding 6 N HCL and sedimented at 8000g for 45 minutes to remove precipitated proteins.

Sulfopropyl Sephadex Chromatography- Sulfopropyl Sephadex (200 ml of packed resin) was added to the pooled Sephadex G-100 fractions, and stirred slowly overnight in the cold. After allowing the sulfopropyl Sephadex to settle, the supernatant liquid was decanted, the resin was poured into a column (5 x 10 cm), and washed with 2 column volumes of 10 mM sodium acetate, 0.1 M $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.25 mM TX100, pH 5.0. APase activity was then eluted with 0.3 M NaH_2PO_4, 1 mM EDTA, 0.25 mM TX100, 10 mM 2-mercaptoethanol, pH 5.0, collecting 5.7 ml fractions. The peak activity was pooled, adjusted to pH 7.0 with 6 N NaOH, concentrated to 5-10 ml total volume using immersible CX-10 ultrafilters (Millipore Corp., Bedford, Mass.), and dialyzed against 3 x 100 volumes of buffer II.

Chromatofocusing- The concentrated dialyzate from the

previous step was loaded onto a chromatofocusing column (Polybuffer Exchanger 96, 1 x 27 cm, equilibrated with 25 mM ethanolamine, 20 mM 2-mercaptoethanol, 0.25 mM TX100, pH 9.3), and eluted with 1:10 diluted Polybuffer 96 containing 0.25 mM TX100 and 20 mM 2-mercaptoethanol, pH 7.0. Tubes containing the two peaks of activity were pooled separately and concentrated with CX-10 ultrafilters.

Sephadex G-75 Chromatography- Each pooled concentrated peak of activity from the chromatofocusing column (approx. 4 ml) was loaded onto a Sephadex G-75(superfine) column (2.5 x 65 cm, equilibrated with buffer II), and eluted at 8-12 ml/hr. Fractions containing enzyme activity were pooled, concentrated with CX-10 ultrafilters, dialyzed against 500 volumes of buffer II + 0.1 M NaCl, and stored at 4° C until use.

Isoelectric Focusing- Isoelectric focusing was completed using Servalyt Precotes pH 3-10 (Serva Fine Biochemicals, Inc., Garden City Park, NY), focusing at 1 W constant power for 2 hours at 8° C. APase was visualized after overlaying the precote with Whatman number 1 filter paper soaked in APase assay mix (pH 4.3) by illuminating with a Mineralight UVS-12 (Ultraviolet Products, Inc., San Gabriel, Ca). Gradient pH was monitored using isoelectric point markers pI 5.65 - 8.3 (BDH Chemicals, Ltd., Poole, England) and some markers from Calbiochem-Behring Corp.

(LaJolla, Ca.): acylated horse heart cytochrome c (pI 9.7, 8.3), sperm whale Met myoglobin (pI 8.3), trifluoroacetylated sperm whale Met myoglobin (pI 7.72), equine Met myoglobin (pI 7.30), porcine Met myoglobin (pI 6.45), trifluoroacetylated equine Met myoglobin (pI 6.86), trifluoroacetylated porcine Met myoglobin (pI 5.92), and P. aeruginosa azurin (pI 5.65).

Neuraminidase Treatment- The purified APases were each subjected to neuraminidase treatment in 0.1 M citrate, 0.2 M NaH_2PO_4, 0.25 mM TX100, 10 mM 2-mercaptoethanol, 1 mM $CaCl_2$, pH 5.0 with 1.25 U/ml neuraminidase for 2.5 hours at 20° C (14,15). Controls were incubated as above, except that neuraminidase was not added. Samples were analyzed by isoelectric focusing as described above.

RESULTS

Tissue Distribution of APases- A previous report described the separation of APase in chicken breast muscle into three forms by chromatography on Sephadex G-200 (8). The low molecular weight form was enriched with the cytosol, whereas the two higher molecular weight forms were associated with the microsomes and lysosomes. The distributions of APases amongst other chicken tissues are presented in Table I. Liver and spleen contain the highest concentrations of APase activity. The lysosomal form of the enzyme predominates in the lung, where it comprises 56% of the total activity, whereas spleen, heart, and red muscle have especially high percentages of the cytosolic APase (>58% of total activity). The low molecular weight APase accounts for at least 30% of the total APase activity in all tissues, except the lung. White muscle and brain have a moderately high percentage of microsomal APase, (>42% of total activity).

Purification and Properties of Low Molecular Weight APases- Because the low molecular weight (Class IV) APase is elevated in dystrophic avian breast muscle (8), a purification scheme was developed starting with normal breast muscle (Table II) as described in <u>Materials and</u>

Table I: Tissue Distribution of Acid Phosphatases.

	Acid Phosphatase (IU/gm tissue)			
Tissue	Total Activity	Microsomal	Lysosomal	Cytosolic
Liver	47 ± 3	15 (31)	18 (39)	14 (30)
Spleen	44 ± 4	11 (26)	5.7(13)	27 (61)
Lung	12 ± 1	3.5(30)	6.6(56)	1.6(14)
Brain	12 ± 1	5.2(42)	3.3(27)	3.8(31)
Heart	8.3±1.1	1.5(18)	1.6(20)	5.1(62)
Red Muscle	9.0±0.7	2.3(26)	1.4(16)	5.2(58)
White Muscle	7.5±0.2	3.5(47)	1.0(13)	3.0(40)

Extracts of each tissue were assayed for total acid phosphatase activity using 4-methylumbelliferylphosphate at pH 4.3. The distribution between the three forms of the enzyme was then determined as detailed in Materials and Methods. Total activity values are means ± 1 standard deviation, activities for each type of enzyme are means for three birds aged 26 days *ex ovo*. The percentage distribution among the three forms are given in parenthesis.

Table II: Summary of the Purification of Acid Phosphatases A and B.

Fraction		Volume	APase	Protein*	Spec. Act.	RSA	% Yield	Ave % Yield	N
		ml	units+	mg	units/mg				
Extract		2570	201	25100	8.0×10^{-3}	1.0	100	---	-
$(NH_4)_2SO_4$		164	163	18100	9.0×10^{-3}	1.1	81	69±16	9
Sephadex G-100		1070	140	5400	2.6×10^{-2}	3.2	70	56±15	9
SP-Sephadex		180	29	164	0.12	22	14	28±15	9
Chromato-focus	A	9.0	5.0	2.4	2.0	255	2.5	5.6±2.9	6
	B	16	11	4.0	2.7	335	5.3	3.8±1.5	6
Sephadex G-75	A	31	4.0	0.54	7.5	931	2.0	1.5	2
	B	42	6.4	0.94	6.8	846	3.2	2.4	2

Data are for a single, typical preparation. Ave % yield is the average ± 1 standard deviation for N preparations.
+ Units - 1 umole product formed per minute.
* Protein was determined using BioRad Bradford dye-binding protein assays.

Methods. Two distinct APases, called A and B were resolved by the chromatofocusing column (Figure 1). Several properties of these two enzymes are summarized in Table III. The two enzymes have distinct isoelectric points (A- 7.5, B- 5.9), which are not altered by treatment with neuraminidase (Figure 2). They differ in their Km for 4-methylumbelliferylphosphate (A- 0.18±0.01 mM, B- 0.09±0.02 mM); substrate inhibits form A, but not form B (Figure 3). Mixtures of these two enzymes can be resolved on a Sephadex G-75 (superfine) column (Figure 4) suggesting a small difference in molecular weight.

Activators - Because purines are known to activate low molecular weight APases (16-19), their effect on APases A and B was examined. Guanosine activates APase B but not APase A (Figure 5). The effect of several other purines on the activity of APase B was then examined (Table IV). Guanosine was the most effective activator, followed by adenine and adenosine; hypoxanthine, purine riboside and guanine have little to no effect at 1 or 5 mM. The effect of guanosine on the kinetic parameters of APase B is presented in Figure 6; guanosine increases both Km and Vmax; the ratio of Km/Vmax increases linearly with guanosine concentration.

As indicated in Table III, 2 M methanol and 0.125 M glycylglycine also activate APase A and B. To explore this in more detail, the effect of several compounds on the activity of APase from the Sephadex G-100 column, (Table

Figure 1: Low Molecular Weight APases- Resolved by Chromatofocusing Column.

Pooled fractions from the sulfopropyl-Sephadex column were loaded onto a Polybuffer Exchanger 94 column (1 x 27 cm), eluted and assayed as detailed in Materials and Methods. The pH of several fractions of the elution show the pH gradient (x-x). APase activity (o-o).

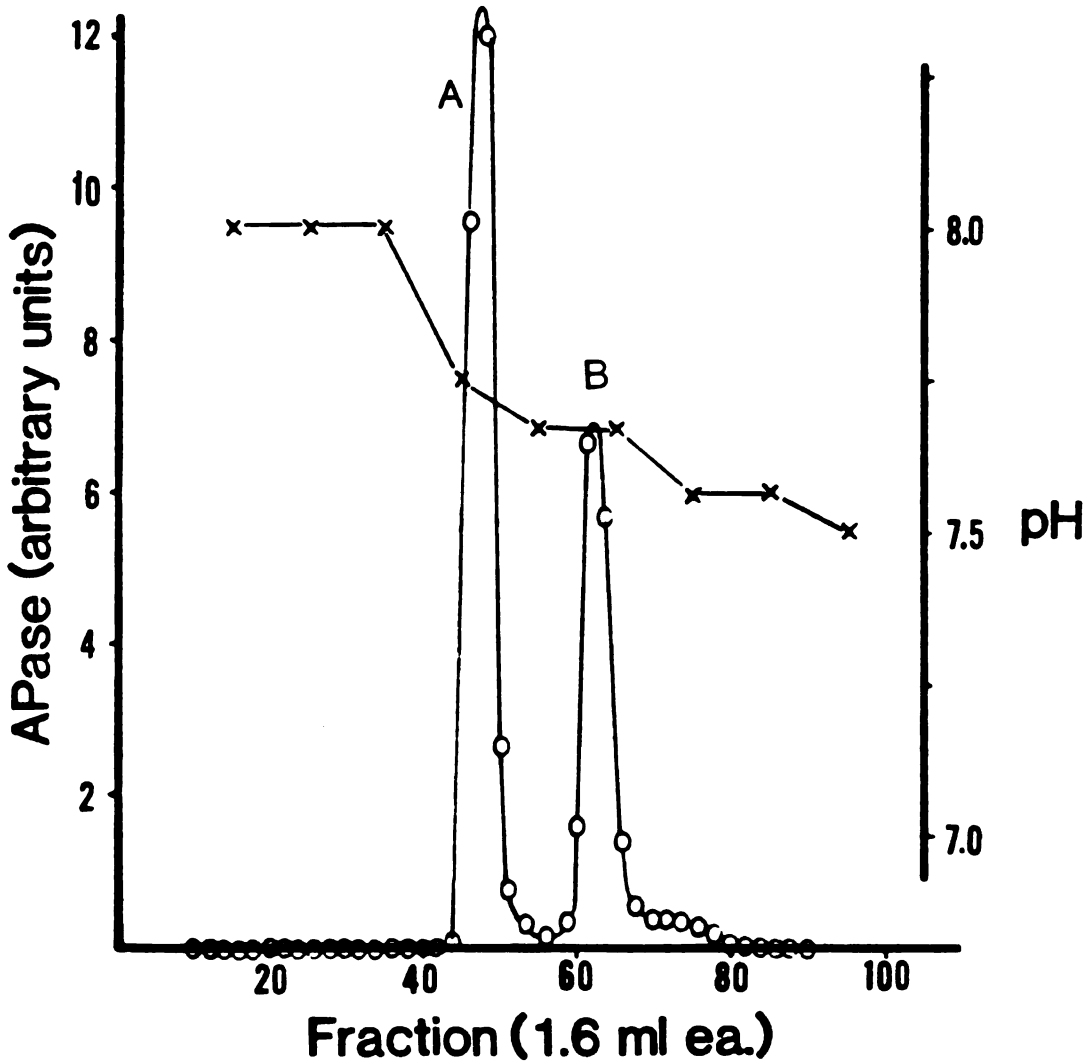

12
10
8
6
4
2
APase (arbitrary units)
A
B
8.0
7.5
7.0
pH
20
40
60
80
100
Fraction (1.6 ml ea.)

Table III: Properties of Low Molecular Weight APases.

Property	APase A	APase B
Isoelectric Point	7.5	5.9
Vmax (IU/mg)	8.85	7.41
Km (4-MUP, pH 5.0)	0.18±0.01 mM	0.090±0.025 mM
Purine Activation	NO	YES
Ka Guanosine	--	2.2±0.2 mM
Methanol Activation (2 M, pH 4.3)	2.3 X	1.4 X
Glycylglycine Activation(0.125 M, pH7)	4.1 X	6.6 X
L-(+)-tartrate inhibition 12.5 mM, pH 4.3	13%	6%
Fluoride inhibition 50 mM, pH 4.3	0%	3%

Figure 2: Neuraminidase Treatment of Low Molecular Weight APases: Effect on the Isoelectric Point.

Samples of APases A and B were treated with neuraminidase, loaded on an isoelectric focusing gel with standards and untreated controls, focused, and stained as detailed in Materials and Methods. Lanes 1 and 6- Isoelectric point markers, Lane 2- neuraminidase treated B, Lane 3- neuraminidase treated A, Lane 4- untreated control B, Lane 5- untreated control A. Isoelectric points of standards are indicated on the photograph.

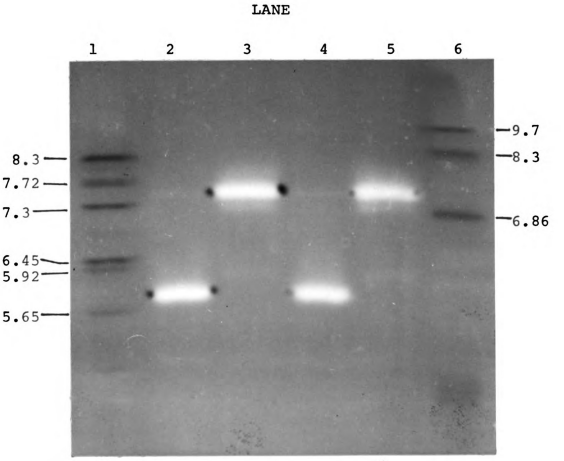

LANE
1
2
3
4
5
6
8.3
7.72
7.3
6.45
5.92
5.65
9.7
8.3
6.86

Figure 3: Lineweaver-Burk Plots For APase A and B with 4-methylumbelliferylphosphate at pH 5.0.

APases A and B were assayed with increasing amounts of 4-methylumbelliferylphosphate at pH 5.0 as detailed in Materials and Methods. The solid lines are theoretical, drawn using Km = 0.12 ± 0.02 mM and Vmax = 1.62 ± 0.13 umole/min/ml for APase A (Panel A) and Km = 0.067 ± 0.003 mM and Vmax = 0.634 ± 0.011 umole/min/ml for APase B (panel B). Dashed curve in panel A indicates deviation from the theoretical line.

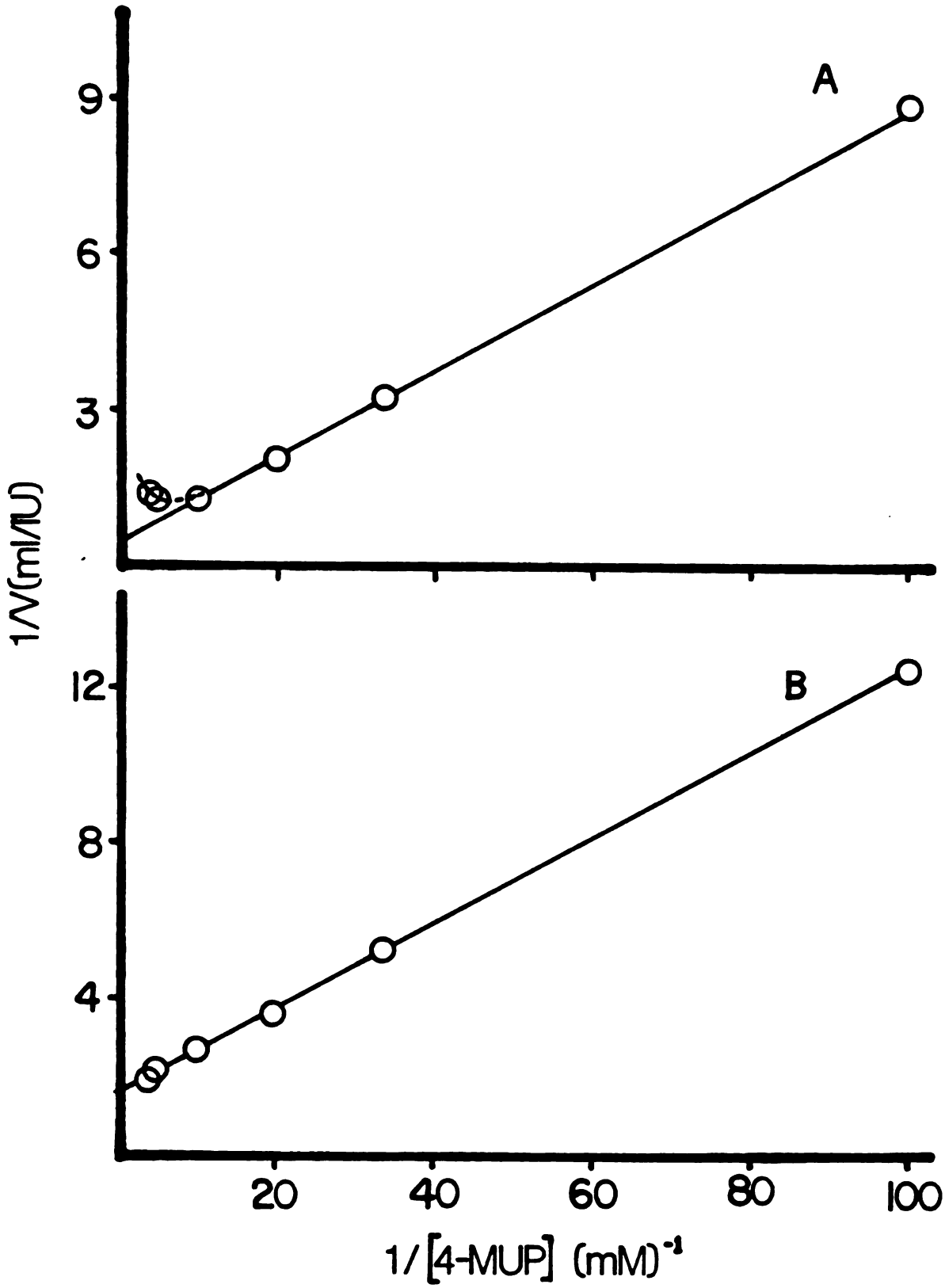

A
B
9
6
3
12
8
4
1/V(ml/IU)
20
40
60
80
100
1/[4-MUP] (mM)$^{-1}$

Figure 4: Sephadex G-75 Elution Profile of a Mixture of APases A and B.

Approximately equal units of APases A and B were mixed, loaded onto, and eluted from a Sephadex G-75 (superfine) column (2.5 x 65 cm), and APase activity was assayed as detailed in Materials and Methods. A = APase A, B = APase B. The enzyme forms in each peak were identified by isoelectric focusing and guanosine activation.

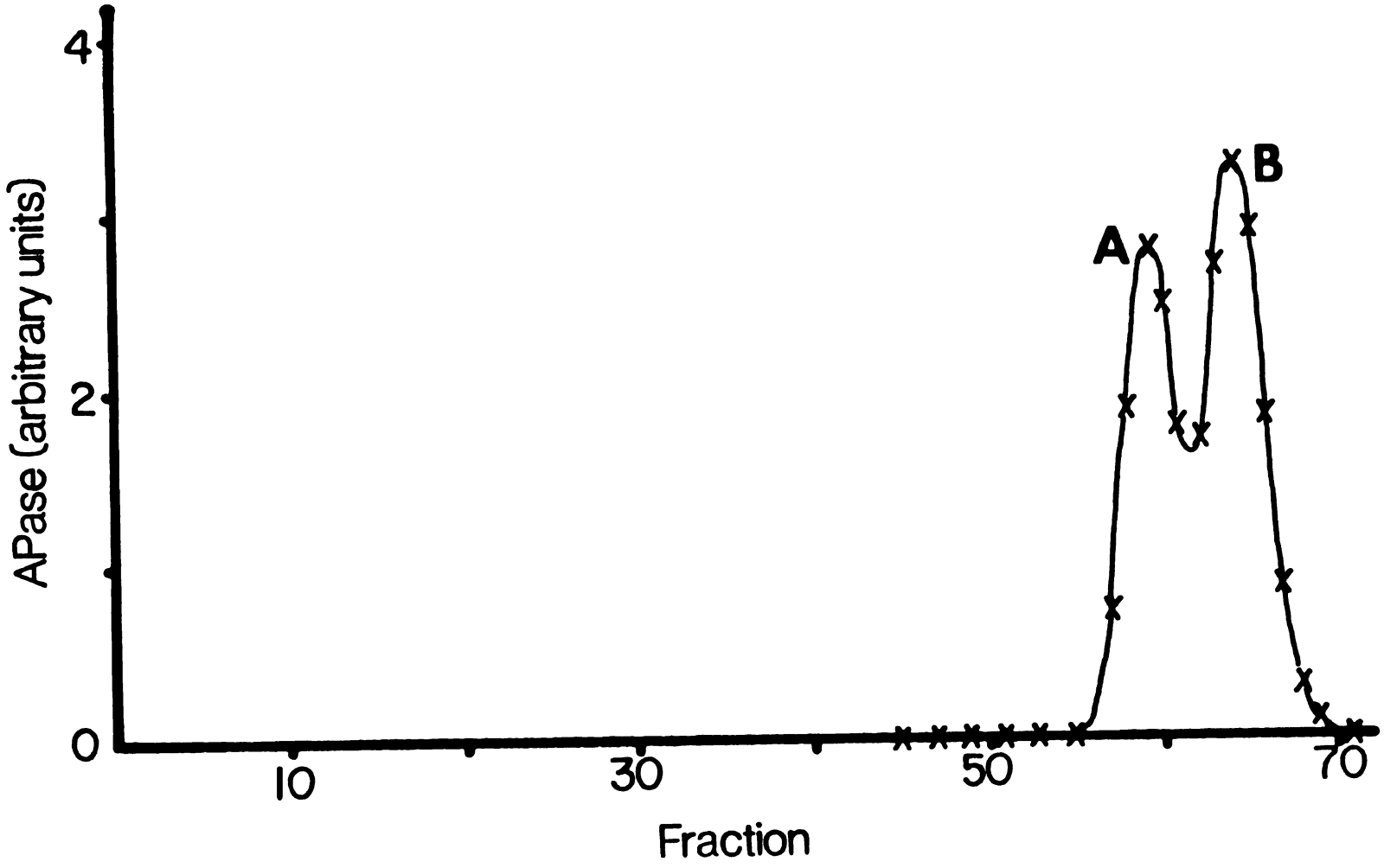

4
2
0
APase (arbitrary units)
10
30
50
70
Fraction
A
B

Figure 5: Guanosine Activation of APases A and B.

APases A and B were assayed in the presence of increasing amounts of guanosine at pH 5.0 with 5 mM 4-methylumbelliferylphosphate as detailed in Materials and Methods. Data are expressed as the ratios of activity in the presence and the absence of guanosine for APase A (x) and B (o), versus the concentration of guanosine.

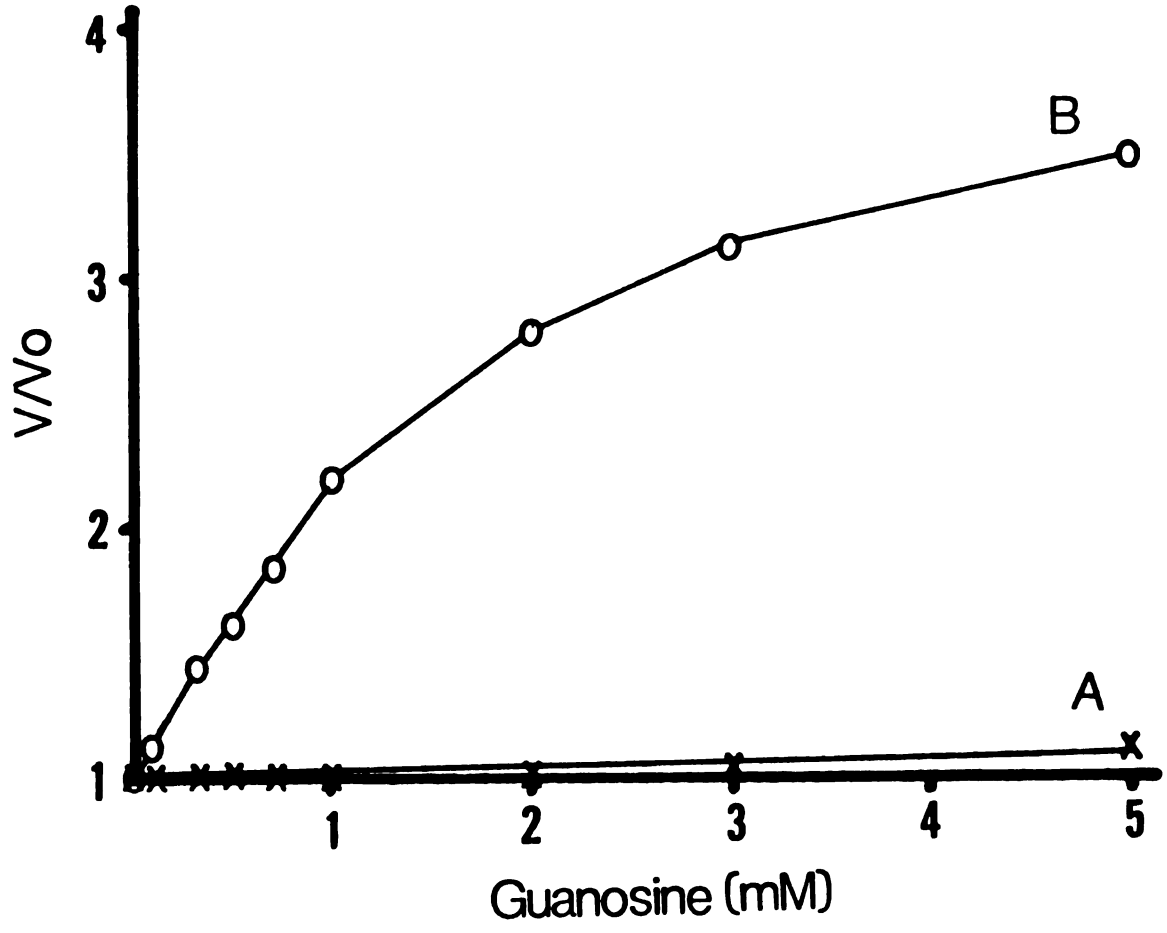

V/Vo
4
3
2
1
B
A
1
2
3
4
5
Guanosine (mM)

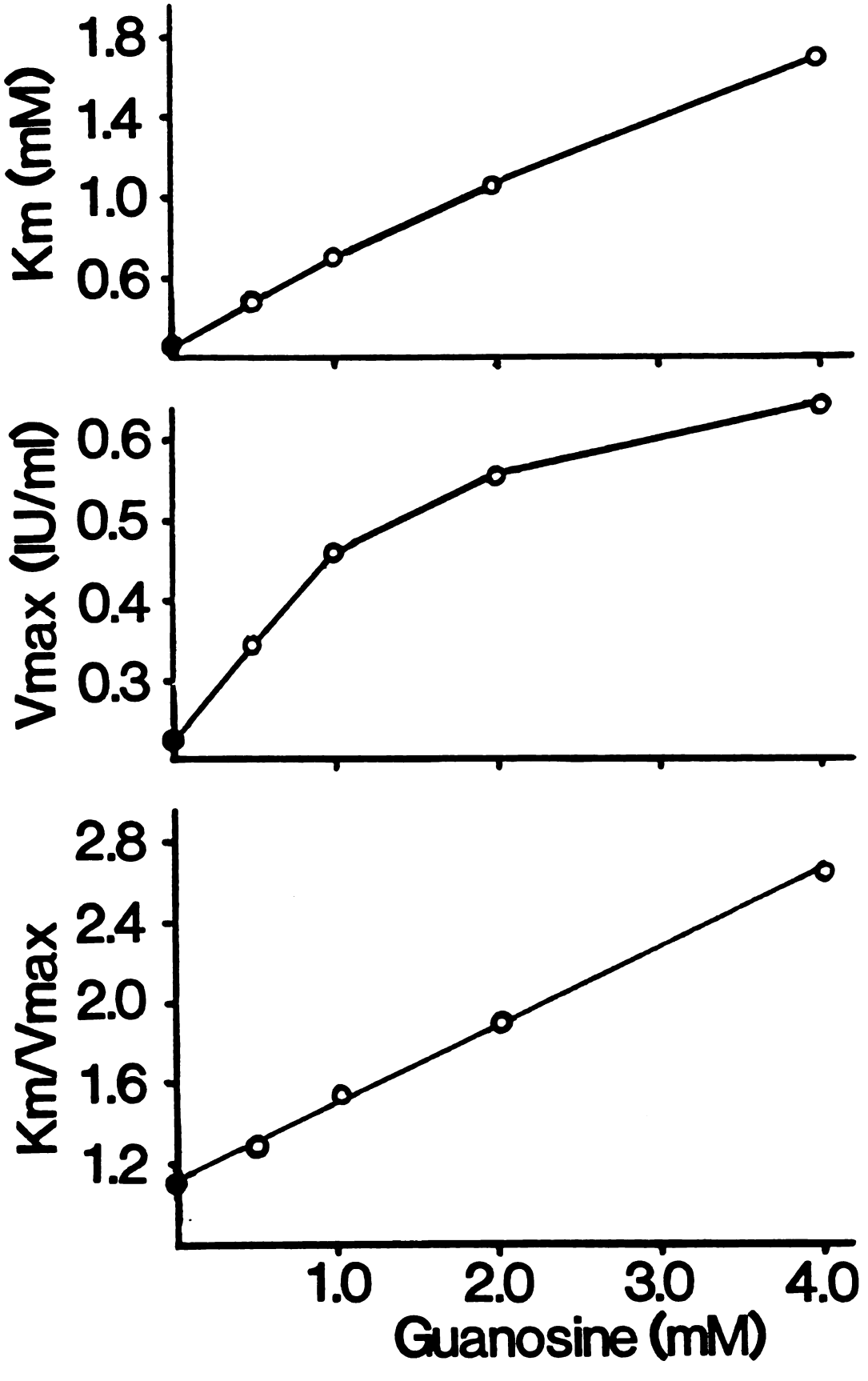
Table IV: Effect of Purines on APase B.

	Purine Concentration Added	
Purine Added	1 mM	5 mM
guanosine	2.46	4.15
adenine	1.61	2.00
adenosine	1.25	1.41
purine riboside	1.17	1.12
hypoxanthine	1.15	1.10
guanine	1.03	1.05

* Ratio of APase B activity in the presence of purine to that in the absence of purine.
Assays of APase B activity were done at pH 4.3 in the presence or absence of purines. Activity ratios reported are means of duplicate assays.

Figure 6: Effect of Guanosine on the Kinetic Parameters of APase B.

Km and Vmax values were determined for APase B as a function of increasing concentrations of guanosine. Activities were measured by monitoring the release of 4-methylumbelliferylphosphate at pH 5.0, as detailed in Materials and Methods. Data were fit by computer analysis (20), using the method described by Wilkinson (13).


Km (mM)
1.8
1.4
1.0
0.6
Vmax (IU/ml)
0.6
0.5
0.4
0.3
Km/Vmax
2.8
2.4
2.0
1.6
1.2
1.0
2.0
3.0
4.0
Guanosine (mM)

II), which is free of the high molecular weight forms as determined by isoelectric focusing, was examined. Methanol activates, whereas ethanol does not; TRIS is an inhibitor (Figure 7A). That the methanol activation reflects phosphotransferase activity was confirmed by assessing 4-methylumbelliferone and inorganic phosphate release in the presence of increasing methanol concentrations (Figure 7B). The data show that the release of inorganic phosphate decreases slightly with increasing concentrations of methanol, whereas release of 4-methylumbelliferone is increased. The ratio of 4-methylumbelliferone release to inorganic phosphate release increases linearly with methanol concentration (Figure 7C).

Substrate Specificity- 4-Methylumbelliferylphosphate is the best substrate for both APase A and B (Table V) at either pH. However, APase A hydrolyzes O-phospho-L-tyrosine at over 90% of the rate with 4-methylumbelliferylphosphate at pH 5.0, though at pH 7.0 flavin mononucleotide (FMN) was more rapidly hydrolyzed. O-phospho-D,L-threonine and FMN are also good substrates at pH 5.0; at pH 7.0, only FMN and 4-methylumbelliferylphosphate are hydrolyzed to any significant extent. Other phosphate esters were poor substrates when compared to 4-methylumbelliferylphosphate, though APase A generally had measurable activity at pH 5.0. Of the compounds examined with APase B, only 4-methylumbelliferylphosphate, FMN, and O-phospho-L-tyrosine

Figure 7: Phosphotransferase Activity in the Low Molecular Weight APases.

APase was assayed in the presence and absence (A/Ao) of various compounds, methanol (Δ), ethanol (o), and TRIS (□) (panel A). Panel B shows the effect of increasing methanol concentrations, monitoring release of both inorganic phosphate (o) and 4-methylumbelliferone (o) at pH 5.0 as described in Materials and Methods. The ratio of the activity monitored by release of 4-methylumbelliferone to that by release of inorganic phosphate (4MU/Pi) is plotted as a function of methanol concentration in panel C.

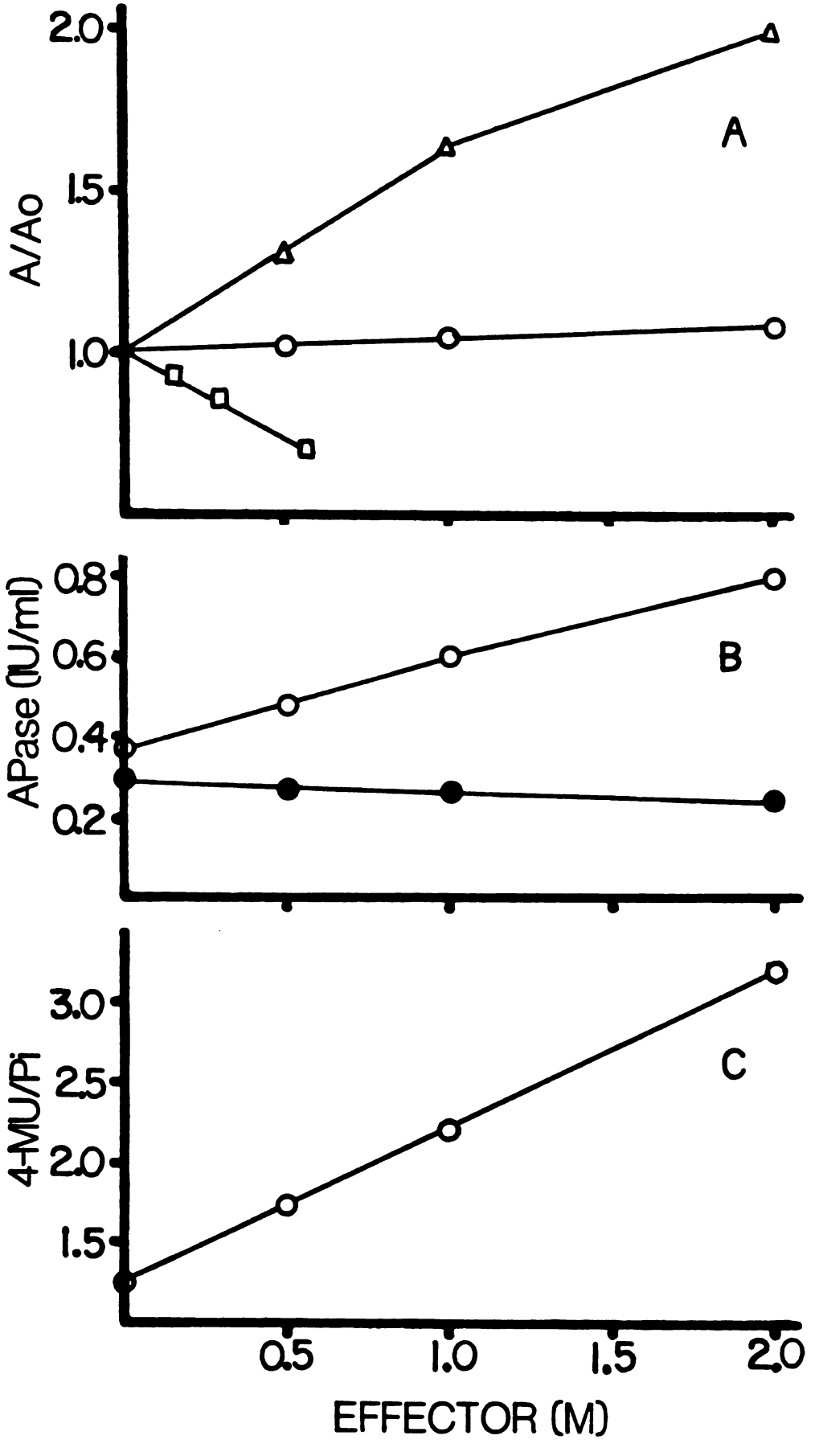

2.0
1.5
1.0
A/Ao
A
0.8
0.6
0.4
0.2
APase (IU/ml)
B
3.0
2.5
2.0
1.5
4-MU/Pi
C
0.5
1.0
1.5
2.0
EFFECTOR (M)

Table V: Substrate Specificity of APases A and B.

Substrate	APase A pH 5	APase A pH 7	APase B pH 5	APase B pH 7
4-methylumbelliferyl-phosphate	1.00	0.77	1.00	0.31
α -napthol phosphate	0.13	0.00	0.04	0.01
adenosine 5'-triphosphate	0.05	0.03	0.04	0.05
guanosine 5'-triphosphate	0.04	0.00	0.00	0.02
fructose 1,6 diphosphate	0.05	0.00	0.02	0.00
ß-glycerophosphate	0.03	0.00	0.02	0.01
O-phospho-L-tyrosine	0.92	0.04	0.26	0.01
O-phospho-L-serine	0.06	0.00	0.04	0.01
O-phospho-D,L-threonine	0.25	<0.01	<0.01	0.00
flavin mononucleotide	0.48	0.22	0.51	0.10
phosvitin	0.03	<0.01	<0.01	0.03

Assays were completed as detailed in Materials and Methods, and normalized to the activity at pH 5.0 against 4-methylumbelliferylphosphate. Reactions were monitored for release of inorganic phosphate. Activity was A- 0.29 IU/ml, B- 0.26 IU/ml at pH 5.0, against 4-methylumbelliferylphosphate. All substrates (except phosvitin) were 5 mM; phosvitin was 7 mg/ml.

were hydrolyzed at reasonable rates at pH 5.0. Moreover, APase B is more stringent in its requirement for an acid pH than enzyme A; using 4-methylumbelliferylphosphate, APase B has only 31% of the rate at pH 5.0, (APase B has 77% of its activity at pH 7.0 compared to pH 5.0). The other substrates show more drastic loss in activity at pH 7.0. Phosphorylated myosin light chain (^{32}P-labeled) was not hydrolyzed by either APase A or B at pH 5.0 or 7.0 (data not shown).

DISCUSSION

Purification of APases A and B - Two postmicrosomal supernatant APases were purified 890-fold with a combined yield of 5.3%; purified enzymes had specific activities of 7.5 IU/mg (A) and 6.8 IU/mg (B) with 4-methylumbelliferylphosphate at pH 4.3 (Table II). Both enzymes were relatively unstable unless kept in 0.1 M salt; inorganic phosphate and 2-mercaptoethanol stabilize the enzyme, and 0.25 mM TX100 was used to eliminate adsorption losses. These enzymes tend to lose activity rapidly at pH < 4.0; pH 7.0 was generally used for storage. Neither APase A or B were significantly inhibited by L-(+)-tartrate or fluoride which are potent inhibitors of Class I and II APases (Table III).

Comparative Properties of APases A and B - The two APases called APase A and APase B purified from avian pectoral muscle cytosol differ in several respects. First, they have differing isoelectric points, as determined by isoelectric focusing gels (APase A pI=7.5 and APase B pI=5.9) allowing their resolution on a chromatofocusing column (Figure 1). Because their isoelectric points were not altered by neuraminidase treatment (Figure 2), this difference in pI cannot be due to differing degrees of sialylation. The apparent isoelectric points determined by elution from chromatofocusing columns (see Figure 1) do not

agree with those determined by isoelectric focusing, probably because of other factors influencing elution from a chromatofocusing column such as molecular mass and size, and partial adsorption to the column matrix.

A mixture of the two APases is resolved on a Sephadex G-75 column (Figure 4), indicating differences in their apparent molecular weight. However, whether these apparent molecular weight differences reflect different gene products, are due to differing molecular shapes, or to differing amounts of associated TX100, resulting in apparent larger molecular size cannot be determined at this time.

APases A and B also have differing catalytic properties. APase A with pI=7.5 has the higher Km for 4-methylumbelliferylphosphate at pH 4.3 (Km = 0.18$\pm$0.01 mM) and also exhibits substrate inhibition (Figure 3); APase B with the lower pI=5.9 has the lower Km for this substrate (Km = 0.09$\pm$0.02 mM) and exhibits normal Michaelis-Menten behavior (Figure 3). Because APase A would have the larger net overall charge at pH 4.3, the higher Km value for negatively charged 4-methylumbelliferylphosphate should not reflect charge repulsion.

These two enzymes are also distinguished by differential purine activation. APase A is not affected by guanosine, whereas APase B is strongly affected by some purines (Table IV), especially guanosine (Figure 5 and Table IV). Guanosine activates APase B at pH 5.0 with a Ka =

2.1±0.2 mM, which is not a physiologically significant concentration but may indicate that a related compound is functional *in vivo*.

APases A and B differ in their activity against various organic phosphate esters (Table V), though these differences are of degree, and do not reflect absolute substrate specificity. APase A appears to be less tightly constrained to acid pH than APase B (see data for 4-methylumbelliferylphosphate and FMN, Table V). O-phospho-L-tyrosine is a good substrate for both APases A and B at pH 5.0, though better for APase A; APase A hydrolyzes O-phospho-L-tyrosine at 92% of the rate for 4-methylumbelliferylphosphate at pH 5.0, whereas enzyme B activity is only 26% of that against 4-methylumbelliferylphosphate at this pH.

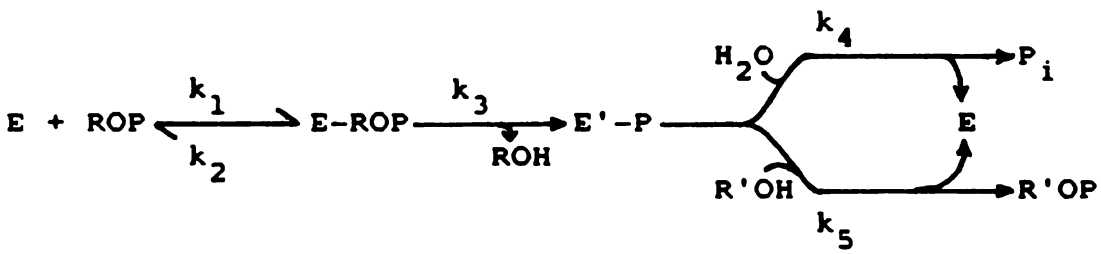
Two low molecular weight APases have also been reported in Xenopus tadpole tail (2,3), human brain, kidney, liver, prostate gland, placenta, red cells, and seminal plasma (21). These enzymes have molecular weights of 40K and 18.7K and, therefore represent class III and class IV APases, respectively (2,3). Both class III and IV APases are resistant to inhibition by fluoride and L-(+)-tartrate, and they appear to require reduced sulfhydryls for activity (2). Class IV APases prefer FMN as a substrate, whereas class III APases show high activity against nucleoside di- and triphosphates (2). APases A and B, however, differ only

slightly in molecular weight (Figure 4), and do not hydrolyze nucleoside triphosphates to any significant extent (Table V). The apparent molecular weight of APases A and B is approximately 11,900 (8). Thus, APases A and B appear to be class IV APases. APases of this type generally exhibit molecular weights of 8 - 18K, pH optima around 5.0, insensitivity to fluoride and L-(+)-tartrate, and efficiently hydrolyze FMN (2), and 17-ß-estradiol 3-phosphate (22). They require free sulfhydryls, and are sometimes activated by purines (2). All of these properties are consistent with those of APases A and B, further supporting their classification in group IV.

APases A and B differ in several respects, as has been previously discussed, and probably represent distinct forms of class IV APases. While our data are consistant with APases A and B being distinctly different enzymes, rigorous proof that they are separate gene products will require additional data.

Aspects of Their Mechanism - Both APase A and B are activated (increased V_{obs}) by methanol when assayed by monitoring release of 4-methylumbelliferone. On the other hand, methanol slightly inhibits (decreased V_{obs}) enzyme activity when release of inorganic phosphate is monitored. The ratio of the rates of release of 4-methylumbelliferone to the release of inorganic phosphate increases linearly with methanol concentration. This same type of activation

was reported for the brain enzyme (Mr 13K (23)). This, coupled with other data, led to the following ping pong reaction mechanism for the brain enzyme (17):



Implicit in this reaction scheme is a phosphorylated enzyme intermediate. Such an intermediate has been demonstrated for the wheat germ APase (Mr 59K (24)), prostatic APase (Mr 120K (25)), and rat liver type II APase (Mr 100K (26)), but has not yet been reported for any of the class IV APases. However, the type IV (14-16K) APase from bovine liver catalyzes phosphate transfer to propane 1,3 diol with retention of configuration of the phosphate oxygens (27), consistent with a two step process involving a phosphorylated enzyme intermediate. No native phosphate acceptors other than water have been demonstrated for any of the APases.

In order to understand the mechanism of guanosine activation, the effect of guanosine on the Km and Vmax of APase B was examined in more detail. Increasing guanosine concentrations increases both Km and Vmax values (Figure 6). However, at low substrate concentrations, guanosine inhibits

enzyme activity, whereas at higher substrate levels activation results. The ratio of Km/Vmax increases linearly with guanosine concentration.

In order to more fully understand these data, one needs to consider the kinetic model. When water is the final phosphate acceptor, its concentration is essentially infinate. The normal BI BI Ping Pong rate equation:

$$\frac{v}{Vmax} = \frac{[A]}{Km_A + [A](1 + Km_B/[B])}$$

where A = ROH and B = H_2O in the reaction mechanism suggested above, is closely approximated by the Michaelis-Menten equation:

$$\frac{v}{Vmax} = \frac{[A]}{Km_A + [A]}$$

since $(1 + Km_B/[B])$ is approximately equal to 1 when H_2O ([B]) is infinite.

Northrop (28) recently emphasized the point that the ratio of Vmax/Km is a measure of the rate constant for binding of substrate to free enzyme (k_1) when Michaelis-Menten kinetics are examined. Thus, the decrease in Vmax/Km values observed with increasing guanosine concentrations (Figure 6) indicate a decrease in k_1; since $Km = (k_2 + k_3)/k_1$, a decrease in k_1 is consistant with the observed increase in Km (the concentration of substrate

required to half saturate the enzyme) in the presence of guanosine. However, Vmax is also increased. Since binding of substrate is less efficient, increased Vmax may be due to (a) increased ability to release either ROH or P_i or, (b) increased rate of formation of E-P once the substrate binds. Which of these possibilities is actually the case cannot be determined from our data. This behavior (increased Km and Vmax) is unusual, generally increased Vmax and constant, or decreased Km values are observed and Km/Vmax decreases. Further studies of this system may lead to new insights into an unusual type of modulation of enzyme activity.

Physiological Role - In normal chickens, spleen, and liver have levels of the low molecular weight APase(s) 3 - 5 times higher than any of the other tissues examined (Table I). Of the total APase activity in red muscle and heart, more than 58% is cytosolic; however, the absolute levels of this activity are only about one third that found in liver.

Liver is involved in detoxification, and has high levels of catabolic activity, as well as a high capacity for regeneration. Very large fluxuations in total liver mass occur as a physiological response to various stress regimines. Spleen is involved in the immune system, and in the catabolism of damaged or ageing red blood cells. As such, it also has high levels of catabolic activity during normal function. Heart is continually functioning in an oxidative environment, and micro regeneration is probably

necessary. Furthermore, damage to the heart muscles must be rapidly repaired for survival, requiring a regenerating capacity. Large losses in red (and white) muscle occur during starvation, as well as many disease states; in fact, if muscle is not regularly used, it degenerates. These tissues are important not only for movement, but also as an emergency source for nutrients during long term starvation. A highly regulated degenerative system is therefore an integral part of muscle. Acid phosphatase activity is also high in embryonic tissues where high rates of degradation are observed concomitent with the high rates of synthesis associated with a rapidly growing/maturing tissue. Regressing Xenopus tadpole tail contains elevated levels of a low molecular weight APase (2,3), and elevated levels of cytosolic APase are observed in dystrophic muscle (8). All of these observations are consistent with a physiological role for the low molecular weight APase(s) related to tissues and physiological states involving massive degeneration and/or regeneration.

The activity of APases A and B against O-phospho-L-tyrosine is consistent with a phosphotyrosyl-protein phosphatase activity, suggesting a possible regulatory role for the enzymes, though protein substrates of this type have not yet been tested. Indeed, phosphorylation at tyrosine is thought to play an important role in cellular transformation (29) and in the regulation

of cell growth (30). However, while O-phospho-L-tyrosine is an excellent substrate at pH 5.0, it is not hydrolyzed rapidly at pH 7.0, whereas FMN is readily hydrolyzed at either pH 5.0 or 7.0 (Table V). This supports a previously suggested role for these enzymes in the metabolism of flavins (2,31), which may result in the indirect regulation of activity of the flavoproteins.

Other substrates for low molecular weight, class IV APases include 1,3 diphosphoglycerate (32), and 17-ß-estradiol 3-phosphate (22), suggesting that, in some tissues, these enzymes have rather specific roles. Both APases A and B show increased activity in the presence of methanol, indicating a phosphotransferase capability. No physiological phosphate acceptors other than water have yet been demonstrated for any of the low molecular weight enzymes.

Both APases A and B are activated by glycylglycine (Table III), though activating levels were not physiological. Some purines activate APase B, but again, effective levels were not physiological. These data are consistent with a rather complex regulation of these enzymes *in vivo*, which may involve peptides or amines, and purine analogues. Very little is known about these regulatory systems.

In conclusion, we have purified two low molecular weight APases from avian pectoralis muscle. These appear to

reflect two different enzymes based on comparison of physical and enzymological properties. Liver and spleen (two tissues with high catabolic activity) are rich in the low molecular weight APase(s), and other systems reflecting large rates of catabolic activity also show high activities of this group of enzymes, suggesting an active role for these enzymes in catabolic processes. APases A and B also show high activity against FMN, consistent with an active role in the metabolism of flavins. The exact role of these enzymes is, however, unknown. The unusual kinetic properties of this enzyme (purine activation increases both Km and Vmax), the lack of a known function, and the observed elevation of the activity in several degenerative states, as well as the suggestion of complex, multi level regulation of activity, all indicate that further work on this system will yield exciting results furthering our understanding in many areas of research.

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CHAPTER V: DETECTION OF AN ENDOGENOUS ACID PHOSPHATASE INHIBITOR - ENRICHMENT AND PROPERTIES.

INTRODUCTION

Low molecular weight acid phosphatases (APase) have been studied for a number of years (1-8), but little is known regarding their physiological function. Because 17-ß-estradiol-3-phosphate (9), riboflavin 5'-monophosphate (4,10-12), and 1,3 diphosphoglycerate (6-8,13,14) are effective substrates, some have suggested a role for the low molecular weight APases in the metabolism of these compounds, though no definitive data demonstrating a physiological role for these enzymes has been presented. On the other hand, because of the wide range of physiological processes and activities that are regulated by phosphorylation (eg., 15,16), acid phosphatases may be involved in regulation of metabolic pathways. However, no regulatory mechanisms have yet been demonstrated for these enzymes.

This paper presents preliminary data demonstrating the presence of an endogenous inhibitor of the low molecular weight APases in avian pectoralis muscle. The inhibitor is stable to boiling, mild acid hydrolysis, charcoal filtration, and pronase digestion; more severe acid hydrolysis destroys its inhibitory properties. It is present at roughly equal concentrations in normal and dystrophic muscle, and is equally effective against both forms of the

low molecular weight APase found in this muscle at pH 7.0, but inhibits neither at pH 4.3. The inhibitor has an apparent molecular weight between 500 and 1500, based on elution between hemoglobin and NaCl on Sephadex G-15 columns.

MATERIALS

Sephadex G-15 (40-120 u) was obtained from Pharmacia Fine Chemicals (Piscataway, N.J.). Pronase was obtained from Calbiochem-Behring (LaJolla, Ca). Activated charcoal was from Will Scientific, Inc. (Rochester, NY). Acid phosphatases A and B were prepared as detailed previously (Chapter IV, this dissertation), form A was used for most experiments. 4-Methylumbelliferylphosphate was from Research Products International (Elk Grove, Il). All other chemicals were analytical reagent grade or better.

METHODS

Preparation of Inhibitor - A low molecular weight fraction was prepared from chicken breast muscle as follows: 100 g frozen muscle was homogenized into 10 volumes of distilled, deionized H_2O and centrifuged at 8,000g for 30 minutes to remove debris. The supernatant was cooled, and concentrated $HClO_4$ (12 M) added slowly to a final concentration of 0.4 M, with stirring. Stirring was continued in the cold for 30 minutes; the precipitated protein was then sedimented at 8,000g, 30 minutes. The supernatant liquid was adjusted to pH 7.0 with 6 N KOH, and sedimented at 8,000g, 30 minutes to remove the resulting precipitate ($KClO_4$). The resulting supernatant was filtered

through activated charcoal, then lyophylized to dryness. The resultant solid was extracted 2 x with 10 ml distilled H_2O, and excess $KClO_4$ removed by sedimentation. This extract was used in all tests of the inhibitor.

Pronase Treatment - The extract was treated with pronase, as suggested by LeDunne, et al. (17), by incubating with 2% (w/v) pronase in 0.1 M TRIS.HCl, 10 mM $CaCl_2$, pH 8.0 at 56° C for 20 hours; toluene was placed on the liquid surface to prevent microbial growth. The control was incubated in an identical manner, replacing pronase with bovine serum albumin. The reaction was terminated by boiling for 10 minutes, and precipitated protein removed by sedimentation. Assays were completed as described below.

Charcoal Treatment - Activated charcoal (0.1g) was added to 0.2 ml of inhibitor solution, incubated for 30 minutes, and sedimented to remove charcoal.

Heat Treatment - The inhibitor solution was heated in a boiling water bath for 5 minutes in a sealed tube. The solution was then sedimented at 20,000g, 30 minutes, and inhibitor assayed as detailed below.

Acid Treatment - The inhibitor was heated with either 6 N HCl for 24 hours, or in 1 N HCl for 3 hours, at 100° C, under an argon atmosphere. Reactions were then cooled, sedimented to remove precipitate, and assayed for inhibitor effect as detailed below.

Assays - The concentration of the inhibitor was

monitored by its effect on the activity of low molecular weight APase A from chicken muscle (See Chapter IV). APase reaction mixtures contained 4-methylumbelliferylphosphate (1 mM), 0.125 M MOPS buffer (pH 7.0), and 0.25 mM Triton X-100. The reaction was monitored by the fluorescence of released 4-methylumbelliferone, using a constant amount of enzyme in each experiment, with and without added inhibitor. Assays at pH 4.3 contained 0.125 M citrate buffer instead of MOPS.

RESULTS

Effect of Various Treatments on Inhibitor - The inhibitor is stable to boiling, mild acid and base hydrolysis, charcoal filtration, and pronase digestion (Table I). Conditions normally used for complete digestion of peptides and proteins (6 N HCl, 100° C, 24 hours) (18), destroy the inhibitor, and result in an activating fraction. Conditions used for complete hydrolysis of oligosaccharides (1 N HCl, 100° C, 3 hours) (19), do not significantly affect the inhibitor activity. The inhibitor is not retained on charcoal, suggesting the absence of aromatic groups.

Miscellaneous Properties of the Inhibitor - The inhibitor is effective at pH 7.0, against both APase A and B ((Table II), but is not effective at pH 4.3 (Data not shown). Elution of the inhibitor from a Sephadex G-15 column in an elution volume intermediate between hemoglobin and NaCl indicates a molecular weight between 500 - 1500 (exclusion limit for Sephadex G-15) (Figure 1). Normal and dystrophic avian pectoralis muscle appear to have identical amounts of the inhibitor, since careful preparation from identical amounts of muscle gave inhibitor fractions of equal concentrations (Table III).

Table I: Effect of Various Treatments on Inhibition.

Treatment	APase A Activity (IU/ml)* With Inhibitor After Treatment	APase A Activity (IU/ml)* With Inhibitor Before Treatment	APase A Activity (IU/ml)* Without Inhibitor	Effect ** of Treatment
Pronase	0.043(-32)	0.042(-33)	0.063	-0.6%
0.2M NaOH 15min, 20°C	0.024(-37)	0.024(-38)	0.038	-0.3%
Charcoal	0.022(-40)	0.021(-43)	0.036	-3.1%
Boiling, 5min	0.020(-44)	0.021(-43)	0.036	+1.1%
6N HCl, 100°C 24 hours	0.108(+44)	0.019(-75)	0.075	-118.9%
1N HCl, 100°C 3 hours	0.006(-89)	0.027(-52)	0.058	+36.7%

* APase A activity- values are IU/ml, averages for duplicate assays, percent affect is in parenthesis, negative values indicate inhibition, positive values indicate activation.
** Effect of treatment on the inhibition by Inhibitor fraction- values are obtained by subtracting the percent inhibition after treatment from that before treatment. Negative values indicate decreased inhibition, positive values indicate increased inhibition.

Table II: Effect of Endogenous Inhibitor Fraction on Acid Phosphatases A and B.

Inhibitor \ Enzyme	A	B
+	0.145	0.170
-	0.292	0.328
%	50.2	48.2

Data are expressed as IU/ml at pH 7.0; values are the means of duplicate assays.

Figure 1: Sephadex G-15 Column Chromatography of Inhibitor-Enriched Fraction.

Inhibitor-enriched fraction was prepared as detailed in Methods, and loaded onto a 1 x 34 cm Sephadex G-15 column equilibrated in distilled water. Eluted fractions were tested for effect against APase A (A); OD_{280} was monitored directly (B). Panel C shows the elution volumes for hemoglobin (o, OD_{280}) and NaCl(Δ ,conductance).

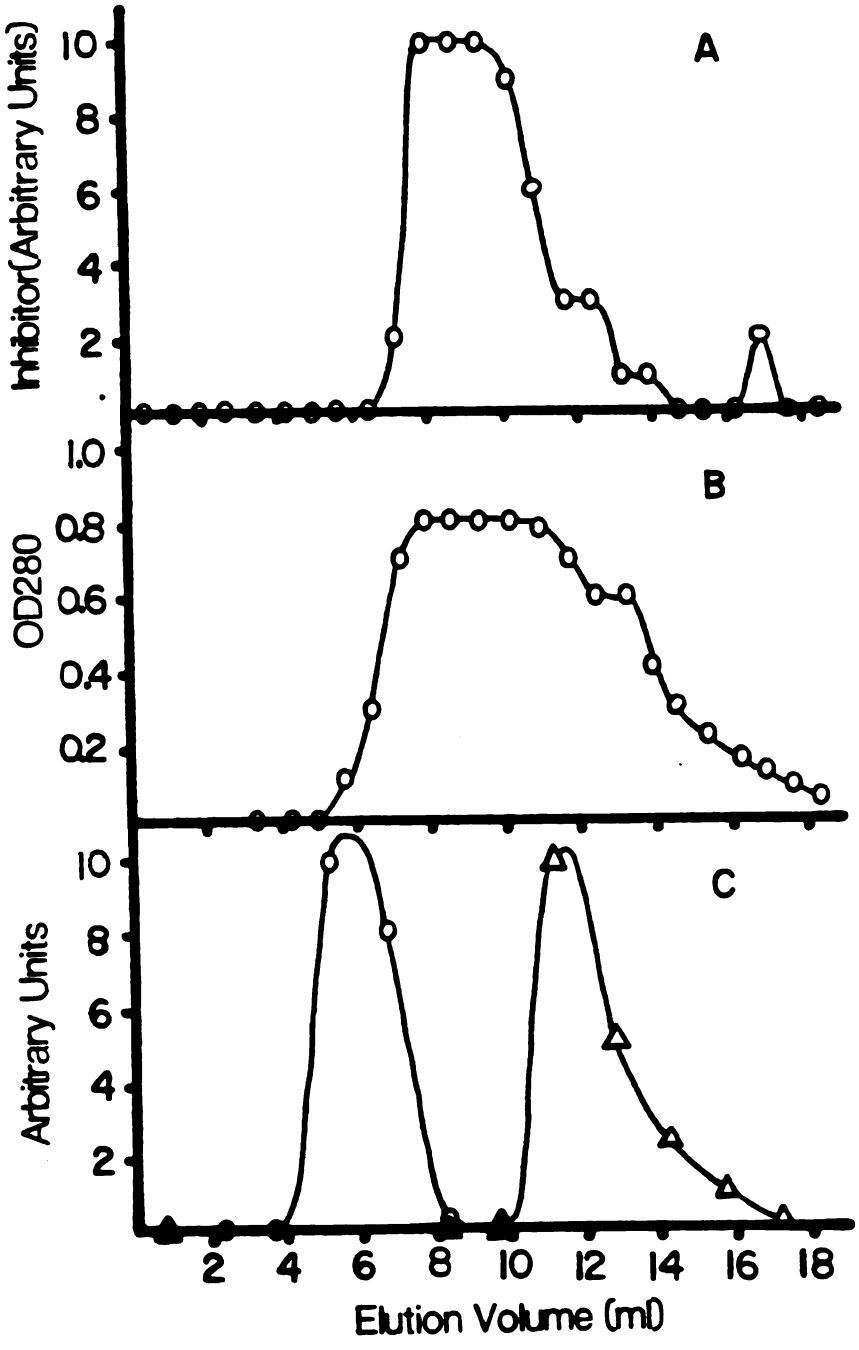
Inhibitor(Arbitrary Units)
10
8
6
4
2
A
1.0
0.8
0.6
0.4
0.2
OD280
B
10
8
6
4
2
Arbitrary Units
C
2
4
6
8
10
12
14
16
18
Elution Volume (ml)

Table III: Relative Inhibitor Concentration in Normal and Dystrophic Pectoralis muscle.

	APase A Activity (IU/ml)	
Addition	Normal	Dystrophic
None	0.015 (0)	0.014 (0)
10ul Inhibitor solution	0.012 (20)	0.012 (16)
25ul Inhibitor solution	0.010 (33)	0.010 (33)

Values are IU/ml APase A, means of duplicate assays. Values in parenthesis are percentage inhibition. Total assay volume was 100ul.

CONCLUSIONS AND DISCUSSION

In conclusion, an endogenous inhibitor of low molecular weight acid phosphatases is present in normal muscle. It is soluble in water, low in aromatic groups, and stable to mild acid and alkaline hydrolysis. Destruction by 6 N HCl, 100° C, 24 hour treatment indicates a peptide-like compound; however, if it is a peptide, it is not hydrolyzed by pronase. Comparison of fluorescence, absorbance, and mass spectral data, (data not shown), argue against the inhibitor being related to flavin mononucleotide, which is also an inhibitor of 4-methylumbelliferone release from 4-methylumbelliferylphosphate (presumably competitive, since FMN is a substrate, See chapter IV, Table V). The exact chemical nature of the inhibitor is not known.

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SUMMARY

SUMMARY

Lysosomes and lysosomal acid hydrolases are elevated in dystrophic muscle. In order to more fully characterize this lysosomal system, we prepared a lysosome - enriched fraction from normal and dystrophic avian pectoralis muscle. Lysosomes from dystrophic muscle exhibit significantly decreased structure - linked latency for a number of acid hydrolases. However, subjecting normal and dystrophic muscle lysosome - enriched fractions to a variety of stress regimines designed to test membrane integrity failed to show any significant differences in stability. Therefore, the decreased structure - linked latency in dystrophic muscle lysosomes is not the result of an alteration in the gross stability of the lysosomal membrane. A more subtle alteration in membrane integrity is not ruled out by these data, but more definitive tests of membrane structure can only be accomplished using a much more highly purified lysosome fraction. Such a preparation has not yet been described.

During these studies, we observed that results obtained by monitoring acid phosphatase activity differed significantly from those obtained by monitoring N-acetyl-ß-D-glucosaminidase or cathepsin D. Lower structure linked latencies in both the crude homogenate and the

enriched fraction, and lower percent recovery and fold - purification were consistently obtained with acid phosphatase when compared to the other marker enzymes in lysosome - enriched fractions. These and other data were consistent with at least three acid phosphatase pools in the muscle: a detergent latent, presumably lysosomal pool, a sedimentable, but not detergent latent pool, presumably microsomal, and a non-latent pool, presumably cytosolic.

Subsequent studies showed at least three different acid phosphatase activities in muscle associated with these different subcellular compartments, and differing in apparent molecular weight, substrate specificity, and response to various phosphatase inhibitors. Quantitation of these three populations of acid phosphatase in normal and dystrophic avian pectoralis muscle resulted in the suprising conclusion that the cytosolic acid phosphatase pool accounts for 84% of the total increase in activity observed in dystrophic muscle at 33 days _ex ovo_. No alterations in lysosomal, and only minimally increased microsomal acid phosphatase pools were observed. Thus, the elevation of the lysosomal enzyme activities does not include lysosomal acid phosphatase; the elevated acid phosphatase activity in dystrophic muscle is phenomenologically distinct from the elevation of several lysosomal enzymes.

Purification of the cytosolic acid phosphatase activity resulted in the resolution of two distinct enzymes,

differing in substrate specificity, isoelectric point, Km for 4-methylumbelliferylphosphate, activation by purines, and in apparent molecular weight. These enzymes were purified approximately 870 - fold, with 5.2% total yield. They show specific activities of approximately 7 IU/mg. These enzymes were not inhibited by either L-(+)-tartrate or fluoride. The isoelectric point difference is not altered by pretreatment with neuraminidase, indicating that sialic acid differences do not account for the differing isoelectric points. The enzymes show strong activity against O-phospho-L-tyrosine and flavin mononucleotide, and exhibit limited phosphotransferase ability. The effect of guanosine is completely selective, activating one of the enzymes but not the other. Careful kinetic studies of the effect of guanosine on this enzyme show increases in both Km and Vmax values. Increasing both kinetic parameters has interesting consequences: in the presence of guanosine, at low substrate concentrations, the enzyme activity is depressed, whereas at high substrate concentrations, it is elevated.

Both enzymes are also activated by glycylglycine indicating the possibility of an endogenous peptide or amine effector as well. Preliminary data indicate the presence of a low molecular weight, heat stable inhibitor of cytosolic acid phosphatases. The inhibitor may be a peptide, though pronase digestion failed to affect the inhibitory properties of an enriched preparation. The possibility that the

"inhibitor" is an endogenous substrate (inhibition was monitored by the effect on activity with an artificial substrate) cannot be ruled out by our data.

These data indicate that the low molecular weight acid phosphatase system holds great promise for future work, since no function for these enzymes is known, and they exhibit a potentially complex regulatory system, as well as unusual kinetics for guanosine activation.

APPENDIX I

APPENDIX I: PAPERS, ABSTRACTS, AND MANUSCRIPTS IN PREPARATION

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