as contrasted with the absence of antibody effect in the postpartum patients and patients with galactorrhea whose growth hormone was not elevated, we interpret as being due to an intrinsic lactogenicity of the circulating growth hormone molecule. The persistence of some prolactin effect after exposure to antiserum, even though the latter was present in amounts sufficient to neutralize very large quantities of growth hormone, suggests that prolactin is released in some individuals concomitantly with growth hormone after induction of hypoglycemia by administration of insulin. This view is strengthened by the fact that the ratio of prolactin to growth hormone in the plasma of these subjects, as indicated in Fig. 2, although quite variable, is in many cases considerably above the highest ratio we have observed for any growth hormone preparation.

The high ratio of prolactin activity to growth hormone encountered in some patients with acromegaly (Fig. 2) similarly leads us to believe that prolactin as well as growth hormone may be secreted in this condition. In the one patient we have so far studied with antiserum, whose growth hormone was 150 ng/ml and whose prolactin activity was 400 ng/ml ovine equivalents, the prolactin activity after neutralization with antiserum to growth hormone was still markedly elevated at 161 ng/ml.

These studies provide evidence that a human prolactin molecule exists which circulates in human blood and is distinct from growth hormone. Ratios of the two hormones may differ widely in different conditions. These studies also indicate that growth hormone as it circulates in blood, like the material extracted from human pituitaries, possesses intrinsic lactogenic activity.

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14. We thank Miss Charity Young for technical assistance, and Dr. A. E. Wilhelmi for gifts of human growth hormone. Supported by PHS grants AM-11294 and AM-5397, and American Heart Association grant 68-111.

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Ribonuclease-Inhibitor System Abnormality in Dystrophic Mouse Skeletal Muscle

Abstract. *Skeletal muscle extracts from mice with muscular dystrophy contain severalfold higher than normal levels of free alkaline ribonuclease II activity and none of the free ribonuclease inhibitor normally present. This abnormal pattern is not seen in heart or liver extracts from dystrophic mice.*

The etiology of the disease or diseases of muscle called "progressive muscular dystrophy" is as much a mystery today as it was when Duchenne described "pseudohypotrophic muscular paralysis" in humans (*1*). A useful animal model for research into this genetic disease is the mouse with dystrophia muscularis, a condition first described in 1955 (*2*). There is considerable biochemical evidence suggesting altered rates of protein and ribonucleic acid (RNA) turnover in skeletal muscle of such mice (*3*).

In studies of the role of several neutral hydrolases in the degradation of protein and RNA in skeletal muscle (*4*), we have discovered large increases of alkaline ribonuclease II activity in dystrophic mice. Alkaline ribonuclease II (*5*) normally is present in mammalian tissue extracts almost completely inhibited by a low molecular weight protein inhibitor which occurs in consider-

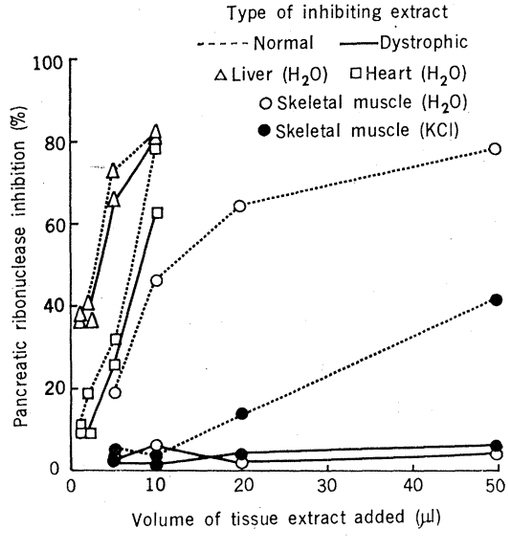

Fig. 1. Inhibition of pancreatic ribonuclease by extracts of normal and dystrophic mouse skeletal muscle, heart, and liver. Inhibitor assay reaction mixtures [modified from those of Shortman (*13*) and based on those for ribonuclease described in the legend to Table 1] consisted of 100 μl of assay buffer, enough water to give a final volume of 500 μl, 5 μl of pancreatic ribonuclease (1 μg/ml in 0.10 percent gelatin), the indicated amounts of tissue extracts, and after a 15-minute incubation at 0°C, 100 μl of RNA solution. Otherwise, assays and calculations were carried out exactly as described in the legend to Table 1. A small correction was made for the ribonuclease activity of added tissue extracts. The activity of pancreatic ribonuclease in an assay mixture containing no tissue extract was the control against which inhibition was measured. Inhibition by tissue extracts was expressed as the percentage of loss from this control value. The added pancreatic ribonuclease had an activity of 400 milliunits. The range of KCl concentrations present in the experiments did not measurably affect the inhibition. Data shown are for liver and skeletal muscle from the same individual animal. Because of their small size, three normal or dystrophic hearts were extracted together for this experiment.

able excess of the available enzyme (*6*). The inhibition can be reversed in extracts by 0.4 m*M* *p*-chloromercuribenzoate (PCMB), a sulfhydryl group complexing agent. The free excess inhibitor inhibits pancreatic ribonuclease (E.C. 2.7.7.16) which can be used in assay of the inhibitor (*7*). We describe here an alteration in the normal pattern of the ribonuclease II–inhibitor system in dystrophic mouse skeletal muscle extracts.

The mice used were males purchased in age-matched pairs of clinically normal (+/+ or dy/+) and dystrophic (dy/dy) animals (of the strain 129 B6F$_1$/J-dy) from the Jackson Laboratories, Bar Harbor, Maine. They were killed by cervical hyperextension at 50 to 80 days of age, and all muscles of the hind limbs were quickly removed, weighed, and placed on ice. Hearts and livers were removed, weighed, and frozen (*8*). Tissues were homogenized at 0°C with a ground glass tube and pestle with 5 volumes (volume/weight) of water. After centrifugation of the homogenate at 37,000*g* and 0°C for 15 minutes, the supernate (H_2O extract) was decanted. The pellet was homogenized with five volumes of 0.30*M* KCl–1 m*M* tetrasodium ethylenediaminetetraacetate(Na_4EDTA). After centrifugation as above, the supernate (KCl extract) was collected. Ribonuclease and inhibitor assays are based on the spectrophotometric measurement of perchloric acid-soluble nucleotides released from RNA.

Typical results for the ribonuclease activity of normal and dystrophic muscle are presented in Table 1. Dystrophic muscle contains much more free (uninhibited) enzyme. Both types of muscle contain considerable latent (inhibited) ribonuclease II activity, although its concentration is again higher in the diseased tissue (*9*).

The presence of large amounts of free ribonuclease II activity in dystrophic skeletal muscle is associated with the absence of measurable pancreatic ribonuclease inhibitor (Fig. 1). Normal muscle extracts, on the other hand, contain easily measurable amounts of ribonuclease inhibitor. This could account for the low amount of free ribonuclease II activity which can be detected therein. Interestingly, the inhibitor, like the latent and unlike the free ribonuclease (Table 1), is readily extracted from the muscle with water.

Evidence that the pancreatic ribonuclease inhibitor of muscle is active as well against muscle ribonuclease II is presented in Table 2. One hundred microliters of normal muscle H_2O extract inhibited about 130 milliunits of dystrophic muscle KCl extract ribonuclease which is comparable in magnitude to the approximately 170 milliunit excess of dystrophic over normal latent ribonuclease activity (Table 1). This suggests that normal and dystrophic muscles have about the same total (free plus complexed) inhibitor concentrations. The difference in free alkaline ribonuclease II activity then could be accounted for by normal muscle having sufficient inhibitor to block almost all ribonuclease II activity and by dystrophic muscle having most of its ribonuclease inhibitor complexed because of a twofold higher than normal concentration of total ribonuclease II with the excess ribonuclease being freely active. If operative in the living cell, this system could result in a severalfold higher than normal free alkaline ribonuclease activity in dystrophic skeletal muscle.

The probable identity of the muscle factors responsible for inhibition of pancreatic ribonuclease and muscle ribonuclease II were demonstrated further in control experiments which showed (i) that dystrophic muscle H_2O extracts did not inhibit muscle ribonuclease II and (ii) that inhibition of both pancreatic ribonuclease and muscle ribonuclease II by normal muscle H_2O extracts was completely reversed by 0.4 m*M* PCMB.

The tissue specificity of the dystrophic alteration was tested in studies of liver and heart, as indicated in Table 1 and Fig. 1. Unlike skeletal muscle, these tissues from dystrophic mice appear to have a nearly normal alkaline ribonuclease II–inhibitor pattern. In other studies of altered protein turnover in muscle (including tissue from the myopathic hamster, denervated rat muscle, vitamin E-deficient rats, and starved and starved-refed rats), we have shown that the normal pattern of inhibitor excess prevails (*10*).

The tissue and disease specificity of the ribonuclease-inhibitor alteration in dystrophic mouse skeletal muscle suggests that it may not be merely a secondary response to wasting, but rather may play some primary role in the etiology of murine dystrophy. Knowledge of this role must await a more complete definition of the function of ribonuclease

Table 1. Free and latent alkaline ribonuclease II activity in skeletal muscle, heart, and liver extracts from normal (N) and dystrophic (D) mice. To insure good reproducibility for the ribonuclease assay (*4*) all manipulations except the incubation and spectrophotometric measurements were performed at 0°C. The assay mixtures consisted of 100 μl of assay buffer [100 m*M* tris(hydroxymethyl)aminomethane–1 m*M* Na_4EDTA, adjusted to *p*H 8.5 with HCl], 100 μl of 20 m*M* Na_4EDTA (*12*), 100 μl of water with or without 2 m*M* PCMB when KCl extracts were assayed or 100 μl of 300 m*M* KCl with or without 2 m*M* PCMB when H_2O extracts were assayed, 100 μl of tissue extract, and after a 15-minute incubation at 0°C, 100 μl of RNA solution (Torula yeast RNA, Calbiochem catalog No. 55711, 30 mg/ml in assay buffer). Duplicate assay mixtures were prepared; one, the control, was maintained at 0°C and the second was incubated at 30°C for 60 minutes and then placed on ice. After addition of 25 ml of ice-cold 0.30*M* perchloric acid, the solutions were mixed and allowed to stand for 15 to 60 minutes. After centrifugation at 37,000*g* for 15 minutes, supernates were collected and diluted threefold with 0.30*M* perchloric acid. The optical density of the diluted solutions was determined at 260 nm with a Beckman DB spectrophotometer. A milliunit of ribonuclease activity was defined as the amount of enzyme which produced an optical density difference of 0.001 between the final diluted samples from incubated and control reaction mixtures. Free ribonuclease II activity was that measured in the absence of added PCMB. Latent ribonuclease II activity was calculated by subtracting the free activity from the total activity measured in the presence of PCMB.

Source of activity		Ribonuclease II (milliunits per 100 μl of extract)	
Mouse	Extract	Free	Latent
		Muscle	
N	Water	24	219
N	KCl	14	66
D	Water	38	380
D	KCl	185	73
		Heart	
N	Water	7	157
N	KCl	9	21
D	Water	11	159
D	KCl	9	21
		Liver	
N	Water	5	362
N	KCl	60	145
D	Water	8	335
D	KCl	23	148

Table 2. Inhibition of dystrophic muscle alkaline ribonuclease II by normal muscle water extract. Inhibition was measured as described in the legend to Fig. 1. Reaction mixtures consisted of 100 μl of assay buffer, enough water to give a final volume of 500 μl, 100 μl of 20 m*M* Na_4EDTA, the indicated volumes of normal muscle water extract, 100 μl of dystrophic muscle KCl extract, and after a 15-minute incubation at 0°C, 100 μl of RNA solution. A small correction was made for the ribonuclease activity of the water extracts.

Normal water extract added (μl)	Ribonuclease II (milliunits per 100 μl of dystrophic KCl extract)
0	202
10	180
50	132
100	76

II in the cell. It has been suggested that alkaline ribonuclease II is involved in the degradation of messenger RNA and ribosomal RNA precursor in liver (*11*). If such is generally the case, it seems quite possible that the large increase in ribonuclease II activity noted here could well account for the increased muscle RNA and protein turnover in murine dystrophy (*3*).

Further study of this model disease holds promise for fresh insight into the elusive causes of muscular dystrophy and into the intriguing problem of the physiological function of the alkaline ribonuclease II inhibitor system.

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9. Control studies have shown that the values for free and latent ribonuclease in water and KCl extracts represent substantially all of the aikaline ribonuclease II activity of the muscle. No free or latent activity can be found in the residual particulate matter from normal muscle, and the small amounts of free activity remaining in the dystrophic pellet can be removed by repetition of the KCl extraction. Thus the data of Table 1 are not the result of mere differences in extractability of enzyme from dystrophic and normal muscle; they represent true differences in tissue content of enzyme activity.
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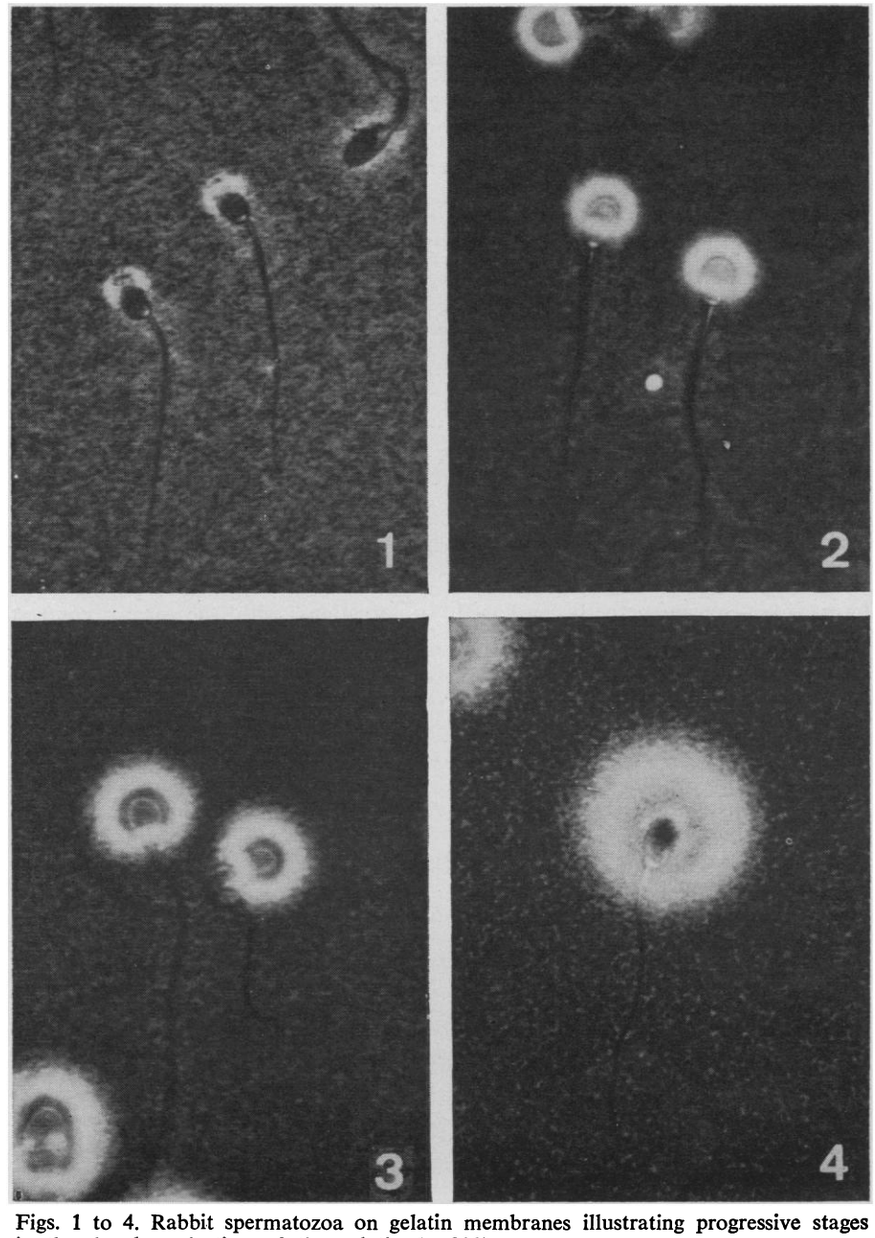
Proteolytic Reaction of Mammalian Spermatozoa on Gelatin Membranes

Abstract. *The acrosomes of spermatozoa of several mammalian species show proteolytic activity when applied to fixed gelatin membranes. The technique permits continuous observation of the enzymatic reaction of an individual spermatozoon. Release of the enzyme occurs solely in the region of the acrosome, in a manner which is species-specific.*

The mechanism by which spermatozoa penetrate the cumulus oophorus and zona pellucida of the mammalian egg to effect fertilization is not fully understood. Extracts prepared from the acrosomes of ram, bull, or rabbit spermatozoa have proteolytic and hyaluronidase activities and can bring about removal of the cumulus oophorus, corona radiata and sometimes the zona pellucida of rabbit eggs (*1*). In addition, Stambaugh and Buckley have isolated hyaluronidase and a "trypsin-like" enzyme from acrosomal extracts of rabbit



Figs. 1 to 4. Rabbit spermatozoa on gelatin membranes illustrating progressive stages in the depolymerization of the gelatin (× 800).