Increased Lysosomal Enzymes in Muscular Dystrophy of Vitamin E-deficient Rabbits*

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The antioxidant function of vitamin E is well established and has received experimental confirmation in several laboratories in recent years (1-7). A central question is how vitamin E, functioning as a lipid antioxidant, prevents the physiological alterations of the deficiency state. Many of the vitamin E deficiency symptoms are histologically degenerative and result in tissue wasting, and biochemical evidence for increased turnover of tissue components as a result of degenerative processes has been reported. In recent years, vitamin E has been shown to be essential for the maintenance of normal tissue function, and this role appears to be mediated by its antioxidant properties. The antioxidant function of vitamin E is well established and has been experimentally confirmed in several laboratories in recent years (1-7). A central question is how vitamin E, functioning as a lipid antioxidant, prevents the physiological alterations of the deficiency state. Many of the vitamin E deficiency symptoms are histologically degenerative and result in tissue wasting, and biochemical evidence for increased turnover of tissue components as a result of degenerative processes has been reported. In recent years, vitamin E has been shown to be essential for the maintenance of normal tissue function, and this role appears to be mediated by its antioxidant properties.

EXPERIMENTAL PROCEDURE

Vitamin E-deficient and control rabbits were raised as previously described (1). They were taken for assay when signs of muscular weakness were exhibited and were used to obtain the data in Tables I, II, and III. The rabbits used for Fig. 1 and Table IV were assayed during the progression of muscular dystrophy after 24-hour urine samples were collected. Creatine and creatinine were determined on frozen urine samples by the Folin method described by Hawk, Oser, and Summerson (20). Urinary allantoin and amino acids were determined by the methods of Young and Conway (21) and Albanese and Irby (22), respectively.

Enzyme assays were on 20% homogenates, prepared by blending for 20 to 30 seconds in a Waring Blender, or on particulate preparations in 0.25 M sucrose containing 1 mM EDTA. Two forms of enzyme activity must be distinguished. Large amounts of the lysosomal enzymes are particle-bound and inaccessible to substrates. Smaller amounts of the enzymes are in solution and termed "free." The free plus bound activity equals the "total" activity. Extreme care must be taken to preserve the bound enzyme during homogenization and assay. The free activity of each enzyme was determined during a 10-minute incubation at 37°. Total activity was determined under identical conditions except for the inclusion of 0.2% Triton X-100 which was rehomogenized into the enzyme preparation with 10 strokes in a rotary homogenizer. In the event of low activities, 30-minute or 1-hour assays for total activity were run.

Arylsulfatase was determined by the method of Roy (23), with 2-hydroxy-5-nitrophenyl sulfate as substrate and 2% phosphotungstic acid in 0.1 M HCl as a protein precipitant. Ribonuclease activity was measured by the method of de Duve et al. (24). Acid phosphatase, β-glucuronidase, and cathepsin were determined according to the method of Gianetti and de Duve (25). The amount of phenolphthalein liberated enzymically by β-glucuronidase was measured by spectral absorbance at 550 mg/ml. β-Galactosidase was assayed according to the method of Sellinger et al. (26).

One hind leg from each of the rabbits used to study the time course of lysosomal enzyme increase in the development of muscular dystrophy was removed at the time of death and preserved in 10% formalin until the termination of the experiment. Specimens of the vastus lateralis, semimembranosus, and soleus muscles from representative animals were removed and embedded in paraffin, and hematoxylin and eosin staining was performed on 7-μ sections.

Subcellular fractions of rabbit leg muscle were prepared from 20% homogenates in 0.25 M sucrose containing 1 mM EDTA and sufficient 5% KOH to obtain a pH of 7 after homogenization in a Waring Blender at full speed for 45 seconds. After the blended tissue was passed through cheesecloth, the debris was removed by centrifugation at 500 x g for 10 minutes, the mitochondrial fraction was sedimented at 13,000 x g for 15 minutes, and a smaller particle fraction was obtained by centrifuging at 78,000 x g for 45 minutes.

RESULTS

Increased Lysosomal Enzymes in Dystrophic Rabbits—Seven- to ten-week-old dystrophic rabbits were assayed for four lysosomal indicator enzymes and compared with suitably supplemented controls. Summarized data of Table I show increases in specific activity from 10-fold for ribonuclease to infinity for aryl sulfatase. Thirty minute assays at 37° failed to detect aryl sulfatase activity in muscle homogenates from control rabbits. Statistical treatment indicates that all increases are highly significant. Results with these indicator enzymes indicate an

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1 Triton X-100 was obtained from Rohm and Haas, Philadelphia 5, Pa.
Ten rabbits, 3 to 4 weeks old, were fed a vitamin E-deficient diet for 4 weeks until dystrophic. Ten vitamin E-supplemented controls were of similar age. Enzyme assays were performed on 0.25 M sucrose homogenates as described in "Experimental Procedure." \( p \) was determined with Student's \( t \) test.

### Table I

**Increased lysosomal enzymes in vitamin E-deficient dystrophic rabbit leg muscle**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Group</th>
<th>Specific activity*</th>
<th>% Free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>Control</td>
<td>3.8 ± 2.4</td>
<td>8.3 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>33.9 ± 13.3</td>
<td>92.4 ± 27.2</td>
</tr>
<tr>
<td>Cathepsin</td>
<td>Control</td>
<td>0.01 ± 0.05</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>1.3 ± 0.4</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>( \beta )-Galactosidase</td>
<td>Control</td>
<td>0.01 ± 0.01</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>4.9 ± 3.8</td>
<td>9.8 ± 3.5</td>
</tr>
<tr>
<td>Aryl sulfatase</td>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>7.0 ± 1.4</td>
<td>10.8 ± 3.5</td>
</tr>
</tbody>
</table>

* Millimicromoles of substrate hydrolyzed per mg of N per minute ± standard deviation.
† Specific activity of deficient group is significantly higher than control group; \( p < 0.001 \).
‡ \( p < 0.01 \).

Increase in the entire lysosomal complement, which has the enzymic capacity for muscle hydrolysis and catabolism.

In the vitamin E-deficient rabbit the liver, although it has no grossly visible pathology, does undergo lipid peroxidation in vivo (4). Assays of four indicator enzymes on the livers from 10 control and 10 dystrophic rabbits are summarized in Table II. Statistically significant increases were found for cathepsin and aryl sulfatase. Data of Tables I and II indicate not only increases in the total activity of each enzyme but also increased relative amounts of free, unbound enzyme for all but ribonuclease in the vitamin E-deficient animals.

**Lysosomal Enzymes in Muscle**—Since the presence of any of the lysosomal enzymes in a tissue may serve as an indicator of the entire complement (17), data of Table III provide evidence for the lysosomal origin of six indicator enzymes in leg muscle of vitamin E-deficient rabbits. Two of the most important criteria for the definition of lysosomes have been met; namely, the activity of the enzymes is concentrated in particulate fractions and the enzymes exhibit structure-linked latency, i.e. detergent activation. These data define subcellular localization for the total distribution of lysosomal enzymes whether they are in invading macrophages and leukocytes or in muscle cells. Because of the low activity there has been no attempt to demonstrate that the enzymes are not localized in mitochondria or microsomes. In addition to these six enzymes it has been shown that phosphoprotein phosphatase (27), deoxyribonuclease (17), \( N \)-acetyl-\( \beta \)-glucosaminidase (26) and probably \( \alpha \)-d-mannosidase (28) and \( \beta \)-d-fucosidase (29) are lysosomal in other tissues.

**Correlation of Enzyme Increases with Metabolic and Histopathological Changes**—A question of great concern is the relevance of increased lysosomal enzymes to muscular dystrophy of the vitamin E-deficient rabbit. Since the possibility existed that increased enzyme activities were a reflection of nonspecific tissue destruction and occurred only as an aftermath following the development of muscular dystrophy, an experiment measuring lysosomal enzymes as a function of the number of days on the diet for 4 weeks until dystrophic. Ten vitamin E-supplemented controls were of similar age. Enzyme assays were performed on 0.25 M sucrose homogenates as described in "Experimental Procedure." \( p \) was determined with Student's \( t \) test.

### Table II

**Lysosomal enzymes in vitamin E-deficient rabbit liver**

Conditions are the same as in Table II.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Group</th>
<th>Specific activity*</th>
<th>% Free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>Control</td>
<td>20.6 ± 0.4</td>
<td>33.3 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>25.2 ± 2.8</td>
<td>36.6 ± 5.3</td>
</tr>
<tr>
<td>Cathepsin</td>
<td>Control</td>
<td>0.6 ± 0.1</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>1.1 ± 0.3</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>( \beta )-Galactosidase</td>
<td>Control</td>
<td>31.3 ± 10.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>30.4 ± 13.0</td>
<td></td>
</tr>
<tr>
<td>Aryl sulfatase</td>
<td>Control</td>
<td>2.8 ± 0.2</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>3.9 ± 0.3</td>
<td>5.8 ± 0.3</td>
</tr>
</tbody>
</table>

* Millimicromoles of substrate hydrolyzed per mg of N per minute ± standard deviation.
† Specific activity of deficient group is significantly higher than control group. For cathepsin free and total, \( p < 0.001 \).
For aryl sulfatase free, \( p < 0.01 \), and for total, \( p < 0.02 \).

### Table III

**Fractionation of vitamin E-deficient rabbit leg muscle**

A pooled sample of 70 g from two vitamin E-deficient rabbits was homogenized 45 seconds in 280 ml of 0.25 M sucrose containing 1 mM EDTA and 1.0 mM of 5 N KOH. Debris was sedimented at 500 X g for 10 minutes, mitochondrial fraction at 13,000 X g for 15 minutes, and small particles at 78,000 X g for 45 minutes. Total activity was determined in the presence of 0.29% Triton X-100.
vitamin E-deficient diet was initiated. Thirty-one rabbits were chosen as experimental animals. Twenty-four-hour urine specimens collected before death were used to determine amounts of creatine, allantoin, and amino acids excreted. Urinary components were expressed as ratios relative to creatinine to minimize variations due to urinary volume differences.

Fig. 1 shows changes in activity of aryl sulfatase, ribonuclease, cathepsin, β-galactosidase, and β-glucuronidase as a function of days the rabbits were on a dystrophic diet. Each bar represents the average of two animals and is shown as percentage of maximal activity found during the experimental period. The arrows indicate the detection of the earliest lesions (10 days) and the appearance of the earliest histopathological changes with many lesions (17 days).

The pathological changes observed in these muscles conformed very closely with those reported by others (9). The sequence of occurrence of these changes, as determined by examination of sections made from animals killed at various times during the course of the experiment, appears to be: (a) changes in staining reaction of muscle fibers and central rowing of muscle nuclei; (b) hyalinization of contents of muscle fibers, retraction clumping, and actual coagulation of the contents resulting in formation of granular masses within fibers; (c) polymorphonuclear neutrophils and mononuclear cells including macrophages in many affected areas. In some cases successful regeneration of muscle fibers occurred. It should be noted that these events seemed to occur in rapid succession so that, even in sections made from rabbits killed as early as the 16th day, small foci may be seen which exhibit nearly all of the changes described. Principal differences between early and late sections were (a) the changes from small, widely scattered foci to larger, more frequently occurring foci, many of which had become confluent and; (b) in the latter sections, greater frequency of fragmentation of fibers and of myotubes. Sections of soleus muscle were examined microscopically and scored from 0 to 4 with increasing severity or frequency of each of the following criteria: (a) changes in staining reaction and nuclear “rowing”; (b) coagulation necrosis; (c) fracturing or splitting of fibers; (d) accumulations of polymorphonuclear neutrophils and mononuclear cells including macrophages in many affected areas. In some cases successful regeneration of muscle fibers occurred. It should be noted that these events seemed to occur in rapid succession so that, even in sections made from rabbits killed as early as the 16th day, small foci may be seen which exhibit nearly all of the changes described. Principal differences between early and late sections were (a) the changes from small, widely scattered foci to larger, more frequently occurring foci, many of which had become confluent and; (b) in the latter sections, greater frequency of fragmentation of fibers and of myotubes. Sections of soleus muscle were examined microscopically and scored from 0 to 4 with increasing severity or frequency of each of the following criteria: (a) changes in staining reaction and nuclear “rowing”; (b) coagulation necrosis; (c) fracturing or splitting of fibers; (d) accumulations of polymorphonuclear neutrophils and mononuclear cells; (e) accumulations of muscle fiber nuclei; (f) confluency of lesions and; (g) myotube formation. The final score for each animal is the mean of the scores for the several individual characteristics. Histopathology scores are

### Table IV

**Summary of statistical data on relationship of increased lysosomal enzymes and increased excretion of creatine, allantoin, and amino acids**

These results are from correlations of total activity of each enzyme from the leg muscle of rabbits with their urinary products. Thirty-one rabbits on a vitamin E-deficient diet were killed during the progression of the disease. Analyses are indicated in Experimental Procedure.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Creatine-creatinine</th>
<th>Allantoin-creatinine</th>
<th>Amino acids-creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease</td>
<td>0.80</td>
<td>0.01</td>
<td>0.66</td>
</tr>
<tr>
<td>Cathepsin</td>
<td>0.70</td>
<td>0.65</td>
<td>0.62</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>0.80</td>
<td>0.60</td>
<td>0.73</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>0.73</td>
<td>0.79</td>
<td>0.64</td>
</tr>
<tr>
<td>Aryl sulfatase</td>
<td>0.81</td>
<td>0.66</td>
<td>0.72</td>
</tr>
</tbody>
</table>

*For all correlation coefficients, p < 0.001 except for cathepsin versus amino acids, in which p < 0.01, and for β-glucuronidase versus amino acids in which p = 0.001.*
correlated in Fig. 1 with increased lysosomal enzymes and creatine and amino acids. Histopathological evidence also reached a maximum in 24 days. Histopathological evidence of muscular dystrophy correlates well with increased lysosomal enzymes and increased creatine, amino acids, and allantoin.

**DISCUSSION**

Demonstration of the particulate nature of lysosomal enzymes in vitamin E-deficient rabbit leg muscle and their detergent-induced activation (Tables I and III) provides evidence for similarity in subcellular localization with other more extensively studied normal tissues. However, whereas lysosomal enzyme activity concentrates together with mitochondria in subcellular fractions of rat liver (17), it is apparent that in the leg muscle of vitamin E-deficient rabbits there is considerable activity in the small particle fraction. The enzyme distribution data of Table III provide additional evidence for heterogeneity of distribution of lysosomal enzymes. It is of interest that similar distribution patterns for lysosomal enzymes have been found in pectoral muscle of genetically dystrophic chickens (30). In addition, increased cathepsin activity in the vitamin E-deficient rabbit (31), rat, and hamster (32) has been reported. Control experiments in our laboratory with rabbits deprived of food verified that the activity of these hydrolyses in skeletal muscle is not affected by variations in food intake.

Although conclusive evidence for the functions of lysosomes and their enzymes in metabolism is not available, it is generally agreed that they are of fundamental importance in catabolism, pathological necrosis and autolysis, and phagocytosis (17, 28, 33, 34). It is therefore striking that in nutritional muscular dystrophy, the rabbit, which is characterized both histologically and biochemically by accelerated catabolism, large increases of lysosomal enzymes are observed. It is obvious that increased amounts of the lysosomal complement of enzymes acting upon their respective tissue substrates could contribute to the muscle degeneration and necrosis (8, 9), increased turnover rates (10), catabolism (11,13), autolytic activity (12), and increased excretion of degraded tissue components (14–16), which are characteristic of vitamin E deficiency in experimental animals. In addition to nutritional muscular dystrophy in the rabbit there is involvement of the acid hydrolases in liver necrosis in the rat (35). Electron microscope studies by Piccardo and Schwarz (36) also indicate lysosome involvement in necrotic liver degeneration. The latter authors present evidence for increases in the number of electron-dense microbodies in necrotic livers; microbodies are believed to be identical with lysosomes (37, 38).

That rupture of lysosomes in situ does initiate tissue necrosis or autolysis has been demonstrated (33, 34). In addition to these cases of increased lysosomal enzymes in vitamin E deficiency, a similar involvement has been found in two cases of muscle degeneration in vivo due to non-nutritional causes. Both the genetically dystrophic mouse and chicken exhibit increased activity of the acid hydrolases at times when muscle catabolism is elevated (30). Increased catabolism of skeletal muscle protein in rats during fever correlates with detergent-activatable cathepsin (39). These five instances demonstrate the importance of further consideration of lysosomes in metabolism.

A question of great importance is at what stage in the vitamin E deficiency syndrome the increases in lysosomal enzymes occur. The experimental evidence summarized in Fig. 1 and Table IV was obtained in attempts to provide some answers. Creatinuria, which is considered one of the earliest detectable signs of nutritional muscular dystrophy and which precedes gross physical and histopathological signs of dystrophy, was chosen as a primary chemical index for muscle damage. The highly significant statistical correlation of increased lysosomal enzymes with increases in creatine, amino acids, and allantoin shows that lysosomal enzymes increase with the biochemical indices of muscular dystrophy. The early course of enzyme change shows in Fig. 1 that increases in cathepsin, ribonuclease, β-galactosidase, and β-glucuronidase precede or increase concurrently with creatinuria and that aryl sulfatase shows large increases shortly after creatinuria. It is therefore probable that the increase of this group of enzymes, with the capacity for muscle catabolism, is of importance in development of muscle damage.

It is of interest that there is a precedent for lysosomal involvement preceding histologically recognizable alteration. Increased free and total activity of several of the acid hydrolases was found before signs of liver necrosis appeared in rats (35). In a histological study of anoxic and anoxic-ischemic encephalopathy in rats, lysosomal swelling and opening were found to precede brain damage (33). A reasonable hypothesis, in accord with the experimental data, can be formulated to explain involvement and increases of acid hydrolases found in dystrophic leg muscle of the rabbit. The primary function of vitamin E is to inhibit unsaturated lipid peroxidation in the tissues of animals, lipoprotein membranes containing the highly unsaturated fatty acids being particularly vulnerable. In view of chemical evidence for lipid peroxidation in vivo in tissues of the vitamin E-deficient rabbit (4), including leg muscle (40), the first consequence of vitamin E deficiency may be free radical damage to lipoprotein membranes of the cell and its subcellular organelles. Invasion of the injured tissue by macrophages and phagocytic leukocytes is known to occur following many causes of cell injury (41, 42). Macrophages and leukocytes are rich sources of acid hydrolases (43–45); therefore, such a change in cell population could account for increased total activity of these enzymes. Further, we have analyzed macrophages and found them to be particularly rich sources of lysosomal enzymes (16), so that once they invade the muscle their presence may be sufficient to account for the increased total activity of lysosomal enzymes found here. Further, phagocytic cells entering regions of lipid peroxidation could undergo rupture due to peroxidation damage. This process would contribute additional free lysosomal enzymes over and above those released during active phagocytosis. Over-all effects of such events could appear histologically as necrosis and degeneration, and biochemically as increased catabolism and excretion of tissue constituents.

These results, showing involvement of lysosomal enzymes in muscular dystrophy, indicate how the chemical function of vitamin E can be related step by step to the gross pathology which occurs in the deficiency state.

**SUMMARY**

Detergent-induced activation of particle-bound β-glucuronidase, β-galactosidase, cathepsin, aryl sulfatase, acid phosphatase, and acid ribonuclease from rabbit leg muscle suggests that they are bound in lysosomes. In leg muscle from vitamin E-deficient rabbits the free and total activities of four lysosomal indicator enzymes were increased. Increases of total activity...
were: ribonuclease, 11-fold; cathepsin, 15-fold; β-galactosidase, 61-fold; aryl sulfatase, =. The increased activities correlate with the increased urinary excretion of creatine, allantoin, and amino acids. Histopathological signs of muscle degeneration appeared shortly after these acid hydrolases began to increase. The results suggest that these lysosomal acid hydrolases cause the muscle degeneration in vitamin E-deficient dystrophic rabbits.

REFERENCES

1. DAM, H., Esperientia, 1, (suppl.), 193 (1955).
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