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Effects of Vitamin E-Deficiency On Guinea Pig Lysosomes [1] (38157)

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(Introduced by S. G. Bradley)

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Vitamin E is thought to inhibit the peroxidation of unsaturated lipids in animal tissues (1). It has been suggested that vitamin E deficiency results in damage to intracellular membranes and subcellular organelles due to lipid peroxidation (2), and that this in turn may result in macrophage and leukocyte invasion of the injured tissue. Zalkin *et al.* (3) found increases in the total activity of lysosomal enzymes in leg muscle of vitamin E-deficient rabbits and suggested that these increases were due to the presence of macrophages, which have a high content of lysosomal hydrolases. The increases in these hydrolases preceded or occurred concomitantly with creatinuria, one of the earliest symptoms of muscular dystrophy.

A number of recent studies have demonstrated the presence of lysosomes indigenous to muscle cells (4–7), and these organelles could conceivably play an etiological role in the development of nutritional dystrophy prior to macrophage invasion. Lipid peroxidation in the cell, or of lysosomal membranes, could result in a release of the hydrolases into the cytoplasm with consequent cellular damage. To test this hypothesis in the present study, soluble and particle-bound cathepsin activities were determined in guinea pig gastrocnemius muscle and liver after 15 or 21 days on a vitamin E-deficient diet. These time periods (15 and 21 days) were chosen because it has been established by others (8, 9), using the same dietary protocol and species, that the earliest biochemical changes detectable in guinea pig tissues occur at 15 days and by 21 days the typical pathology of dystrophy is evident. Early changes (15 days) include increased myoglobin concentrations and a random loss of cross-striations in gastrocnemius muscle. By 21 days of diet, histopathological lesions and creatinuria are evident. If damage to lysosomes plays a causal role in the genesis of dystrophy, shifts in the distribution of lysosomal enzymes (from the particle-bound to the soluble form) might be expected to occur in the early stages of the disease. These shifts might reflect a release of the enzymes from lysosomes *in vivo* or a decreased stability of lysosomal membranes leading to leakage of enzymes during homogenization.

Materials and Methods. Guinea pigs and diet. Male English strain guinea pigs were purchased at 325 g (approx 35 days of age) and fed a normal diet of "Purina Guinea Pig" pellets for approximately 10 days. They were then maintained on one of the three following dietary regimes: normal "Purina Guinea Pig" pellets (N); synthetic vitamin E-deficient diet (10) (E^-); or the synthetic diet supplemented with *dl*-α-tocopherol (E^+). The *dl*-α-tocopherol, suspended in olive oil (15 mg/0.5 ml), was administered orally every third day. Food was withheld for 4 hr prior to supplementation and replaced 4 hr after supplementation. On days of tocopherol supplementation, animals on normal and vitamin E-deficient regimes received 0.5 ml of olive oil without tocopherol. All animals received 0.5 ml of cod liver oil on days tocopherol supplement was not given. Animals received approximately 125 mg of sodium ascorbate per week in the drinking water. Fresh synthetic diet was prepared twice a week.

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Preparation of tissue fractions. Animals were anesthetized with ether and killed by opening the chest cavity. Liver slices and both gastrocnemius muscles were quickly extirpated, washed with cold sucrose and chilled in the solution for 5 min. Liver was quickly weighed, cut into small pieces and placed in a conical glass tissue grinder with cold sucrose solution in the ratio of 1 g of liver to 9 ml of solution. The gastrocnemius muscles were trimmed of as much fat and fascia as possible and treated similarly. Tissues were kept in an ice bath or cold room at 4° at all times. Since mechanical disruption, such as grinding, can cause the liberation of lysosomal enzymes, care was taken to repeat the grinding procedures as reproducibly as possible. The technique is published in detail elsewhere (11). The suspension medium for the tissues was always 0.25 *M* sucrose plus 1 m*M* ethylenediamine-tetraacetate (EDTA), adjusted to pH 7 with NaOH.

The procedure used to fractionate homogenates was based on the work of de Duve *et al.* (12). Liver homogenate was divided into a nuclear fraction and a cytoplasmic extract by centrifuging for 7 min at 1000*g* at 4°. The resulting precipitate (nuclear fraction) was brought back to the original volume by the addition of sucrose solution. Triton X-100, at a final concentration of 0.2% (v/v), was homogenized into the nuclear fraction and into a portion of the cytoplasmic extract for measurements of total cathepsin activity in these fractions. The sum of the cathepsin activities found in the nuclear fraction and the cytoplasmic extract is the total activity. Portions of the cytoplasmic extract were further fractionated at 70,000*g* for 45 min. The activity in this resulting supernatant fraction is operationally defined as the soluble activity.

Homogenized muscle was passed through several layers of gauze and the resulting suspension was the muscle homogenate. A portion of the homogenate was used to measure the total activity, and the remainder was separated at 70,000*g* for 45 min to yield a particulate fraction and supernatant fraction. The particles were taken up in sucrose solution to the same volume from which they were centrifuged. Triton X-100 was homogenized into the whole homogenate and particulate suspensions in order to measure total cathepsin activity present in these fractions. The addition of Triton X-100 to muscle or liver supernatant fractions did not affect the enzyme activity.

Cathepsin assay. The procedure for determination of cathepsin activity of the tissue fractions was based on the Anson method (13), which measures the release of tyrosine residues from hemoglobin at pH 3.8. Activities are expressed as micrograms of tyrosine released per milligram of protein (or per milliliter of sample) in 10 min for liver or 30 min for muscle. The enzyme satisfied the linearity requirements of valid assay conditions.

Protein determinations. The protein concentrations of all tissue samples were determined by the method of Lowry *et al.* (14), using bovine serum albumin as the standard.

Statistical calculations. The data comparing the results of the different dietary regimes, E^-, E^+, and N, on cathepsin activity in liver and muscle tissue fractions at 15 and 21 days were analyzed by Student's small sample *t* test.

Results. Comparisons of cathepsin activities in muscle tissue, as influenced by diet, are presented in Table I. The specific activities of cathepsins in whole homogenates, particles or supernatant fractions were the same for the three dietary groups compared at 15 or 21 days. The percentage of cathepsin activity which was soluble was significantly greater in muscle from E^- animals than N animals at 21 days. However, there was no significant difference in the percent solubilized between the deficient (E^-) and the supplemented (E^+) animals.

The cathepsin activity of the particulate fraction plus the activity of the supernatant fraction was compared to the activity found in the homogenate from which these fractions were obtained (Table II). The sum of the activities of the parts was greater than the activity in homogenates of muscles from animals maintained on vitamin E-deficient diets for 21 days.

Table III shows the cathepsin activities in livers of animals given the different dietary regimens. The specific activities of the enzyme in cytoplasmic extracts, nuclear frac-

TABLE I. Cathepsin Activities of Muscle Fractions.[a]

Dietary group	Days on diet	Specific activity			Soluble activity (% of total)
		Homogenate	Supernatant	Particles	
N	15	4.7 ± 0.5	3.7 ± 0.4	4.7 ± 0.4	16.0 ± 1.3
E⁻	15	5.4 ± 0.4	5.8 ± 0.9	5.5 ± 0.7	19.0 ± 2.5
E⁺	15	6.7 ± 1.3	8.1 ± 2.8	5.9 ± 0.6	21.1 ± 4.6
N	21	4.5 ± 0.3	4.8 ± 0.8	4.9 ± 0.1	20.8 ± 1.7
E⁻	21	5.3 ± 0.3	8.4 ± 1.7	5.7 ± 0.4	30.5 ± 3.3[b]
E⁺	21	4.5 ± 0.1	5.8 ± 0.7	4.8 ± 1.0	25.3 ± 1.7

[a] Values represent means ± standard errors of the means in vitamin E-deficient (E⁻), vitamin E-supplemented (E⁺), and normal (N) animals after 15 and 21 days of the dietary regimens. The fractions were separated as described in Methods. There were five animals in each group. Cathepsin activity for all fractions was measured in the presence of Triton X-100 and is expressed as μg tyrosine released/30 min/mg protein. The percent of activity that was soluble was calculated by dividing cathepsin activity/ml of supernatant by activity/ml of homogenate and multiplying by 100.

[b] Different from 21-day normal ($P < 0.05$).

tions, or supernatant fractions were the same for the different dietary groups compared at 15 or 21 days. The percent of cytoplasmic extract activity that was soluble was significantly greater in livers of animals maintained on deficient (E⁻) diets for 21 days than in the livers of supplemented controls (E⁺).

Discussion. After 15 days of vitamin E-deficient diet, there was no significant shift of cathepsin activity from the particle-bound to soluble form. Because it has been previously established that mild degenerative changes occur in muscle tissue by 15 days along with increases in the myoglobin concentration (8) and oxygen consumption (9), release of lysosomal cathepsins into the cytoplasm is probably not a primary event in the development of nuitritional muscular dystrophy in guinea pigs.

The release of cathepsins into the supernatant fraction of preparations from liver or muscle after 21 days on the vitamin E-de-

TABLE II. Total Catheptic Activities of Muscle Tissue. Activity in Homogenates vs Sum of the Soluble + Particulate Fractions.[a]

Dietary group	Days on diet	*A* Homogenate activity	*B* Sum of supernatant plus particle activity	Difference between *B* and *A*
N	15	86.0 ± 8.7	86.4 ± 5.2	+ 0.4 ± 4.0
E⁻	15	95.3 ± 12.5	95.7 ± 15.7	+ 0.4 ± 7.5
E⁺	15	100.0 ± 13.7	95.0 ± 12.1	− 5.0 ± 5.4
N	21	75.1 ± 3.8	73.1 ± 4.2	− 2.0 ± 4.7
E⁻	21	78.6 ± 4.7	92.8 ± 4.7	+ 14.2 ± 3.5[b]
E⁺	21	72.3 ± 4.2	76.0 ± 4.5	+ 3.7 ± 3.2

[a] Values represent means ± standard errors of the means. There were five or six animals in each group. The cathepsin activity in each fraction is expressed as μg tyrosine released /min /ml of the fraction. Homogenates and fractions, prepared as described in Methods, were treated with Triton X-100.

[b] Significant difference between activity in homogenates and that found in the sum of supernatant plus particulate fractions ($P < 0.05$).

TABLE III. Cathepsin Activities of Liver Fractions.[a]

Dietary group	Days on diet	Specific activity				Soluble activity (% of cytoplasmic extract activity)
		Cytoplasmic extract	Nuclear fraction	Total activity	Supernatant	
N	15	11.5 ± 2.8	23.3 ± 1.9	15.4 ± 1.4	4.2 ± 1.3	14.3 ± 4.2
E^-	15	14.2 ± 2.2	22.6 ± 1.0	17.0 ± 1.5	7.5 ± 1.4	24.7 ± 5.5
E^+	15	12.1 ± 1.0	20.7 ± 1.2	15.3 ± 0.8	7.3 ± 1.2	23.3 ± 4.0
N	21	12.9 ± 0.9	19.9 ± 1.1	15.6 ± 0.7	3.6 ± 0.8	16.5 ± 2.4
E^-	21	14.6 ± 1.6	17.8 ± 2.5	15.8 ± 1.8	6.3 ± 1.6	21.5 ± 2.6[b]
E^+	21	13.6 ± 1.6	17.4 ± 1.3	16.8 ± 2.0	3.1 ± 0.7	13.4 ± 1.7

[a] Values represent means ± standard errors of means in vitamin E-deficient (E^-), vitamin E-supplemented (E^+), and normal (N) animals, after 15 and 21 days of the dietary regimens. The fractions were separated as described in the text. There were five animals each group. Cathepsin activity for all fractions was measured in the presence of Trition X-100 and is expressed as μg tyrosine released/10 min/mg protein. The total specific activity of liver homogenates was determined from the sum of the activities in the cytoplasmic extract and nuclear fraction divided by the total protein in these fractions. The percent of activity that was soluble was calculated on the basis of cathepsin activities /ml of supernatant and cytoplasmic extract fractions.

[b] Different from 21-day E^+ ($P < 0.05$).

ficient diet was the same as, or modestly increased, compared with preparations from animals given control diets. Histopathological lesions and creatinuria are evident by this time (9). The increases that did occur in soluble activity may reflect increased mechanical fragility of larger secondary lysosomes (15). Several physiological insults, such as starvation for short periods (5), denervation (16), and vitamin E-deficiency in weanling rabbits (17) have been shown to induce ultrastructural alterations resulting in the formation of large secondary lysosomes.

In contrast to studies with vitamin E-deficient rabibts (3), guinea pig tissues did not show an increase in the total activity of cathepsins at the time creatinuria is observed (21 days). However, this species difference could be due to the presence of an inhibitor of proteolytic activity in the muscle tissue of vitamin E-deficient guinea pigs. Muscle tissue of 21-day E^- guinea pigs differed from other groups in that the sum of the activity in the particulate and supernatant fraction was greater than the total activity of the homogenates. The presence of an inhibitor in one fraction could explain this effect and mask an increased cathepsin activity in homogenates. Finkenstaedt (18) found that when mitochondrial and supernatant fractions of normal rat liver were assayed together, a marked reduction in the total cathepsin activity was observed, and he suggested the presence of an inhibitor. We found no evidence of this effect in normal guinea pig liver or muscle, but only in muscles of animals on vitamin E-deficient diets for 21 days.

Summary. The specific activities of cathepsins in whole homogenates, cell particles, or soluble fractions of muscle or liver were the same in guinea pigs given vitamin E-deficient diets as those on control diets. The proportion of total cathepsin activity in supernatant fractions (70,000*g* for 45 min) was not affected in animals given the deficient diet for 15 days. The proportion of total cathepsin activity in the supernatant fraction of liver was increased in animals given the deficient diet, compared with the supplemented diet, for 21 days. The sum of the activities found in the soluble plus particulate fractions of muscle from animals maintained on vitamin E-deficient diets for 21 days was greater than activity found in whole homogenates. An inhibitor of cathepsin activity may be present in muscle from the vitamin E-deficient animals.

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