Lipid Peroxidation in Nutritional Muscular Dystrophy.* (29577)

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The function of vitamin E in animal tissues, and more specifically as related to nutritional muscular dystrophy, remains to be demonstrated. Evidence from many laboratories has suggested that nutritional muscular dystrophy, induced by feeding vit. E-deficient diets, is the result of loss of antioxidant function, attributable to decreased tissue concentrations of tocopherol.

Vitamin E-deficient muscle is composed of muscle fibers showing varying degrees of sublethal injury, depending upon the stage of depletion in the animal. Much of the work reported in vit. E deficiency has been done after prolonged states of deficiency, making it difficult to determine the primary biochemical lesion. The major interest in our laboratory has been the study of nutritional muscular dystrophy in its pre-clinical stages, *i.e.*, before histological evidence or creatinuria. Schottelius and his co-workers using the same dietary protocol and species have demonstrated an increased energy expenditure for muscle shortening(1), and metabolic evidence for dystrophy such as significantly increased myoglobin concentration(2,3) and oxygen consumption(4) in guinea pig tissues after 15 days on a vit. E-deficient diet. This evidence precedes the appearance of significant histopathological lesions and elevated urinary creatine. However, after 21 days on a diet, the typical pathology of dystrophy is evident, with a concomitant creatinuria; and at 30 days of diet there are gross clinical symptoms.

Several investigators have demonstrated a significant inhibition of lipid peroxidation by tocopherol, and postulate that the vitamin exerts its effect by neutralizing free radicals which arise in the initial stages of an antioxidative chain reaction(5,6,7). In vit. E deficiency, gross symptoms are supposedly a result of peroxidation damage to cellular structures. Oxidation of polyunsaturated fatty acids yield unstable peroxides and hydroperoxides which result in products which include malonaldehyde, and other compounds which yield malonaldehyde on acid hydrolysis(8). Malonaldehyde reacts with 2-thiobarbituric acid (TBA) to form a pink chromogen (absorption maximum at 535 mμ) which many investigators agree may be employed as an index of lipid peroxidation(9,10, 11).

Due to conflicting data in the literature concerning the function of vit. E in the genesis of experimental dystrophy, it was felt necessary to investigate lipid peroxidation in several tissues of our animals at the initial stage of dystrophy.

Materials and methods. Thirty-day-old English strain guinea pigs were randomly divided and assigned to one of 4 dietary regimens: normal ("Purina Guinea Pig Chow" pellets) (N); normal plus cod liver oil (NCLO); synthetic E-deficient diet† (1) plus cod liver oil (E—); and synthetic diet supplemented with dl-α-tocopherol and cod liver oil (E+). The dl-α-tocopherol was suspended in olive oil (15 mg/0.5 cc) and 0.5 cc administered orally every third day. Food was withheld 4 hours prior to supplementation and replaced 4 hours after supplementation. On days of tocopherol supplementation, animals on (NCLO) and (E—) regimens re-

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† The synthetic vitamin E-deficient diet consisted of the following: brewers yeast extracted with petroleum ether (Nutritional Biochemical Corp.), 5.6%; vitamin free casein (Nutritional Biochemical Corp.), 16.8%; corn starch, 44.5%; sucrose, 12.6%; H.M.W. salt mixture, 2.2%; alpha cellulose, 16.8%; vitamin E-free vitamin fortification mixture (Nutrional Biochemical Corp.), 0.3%; 1-arginine-HCl, 0.5%; stripped lard (Distillation Products Industries), 0.2%; agar (extracted with petroleum ether) as gel (59 g/1400 cc water), 0.5%.

ceived 0.5 cc of olive oil without tocopherol. All animals except (N) received 0.5 ml of cod liver oil orally on days tocopherol supplement was not given. All animals received 25 mg of sodium ascorbate thrice weekly. Fresh diet was prepared twice a week.

Animals were sacrificed after feeding durations of 15 or 21 days by decapitation, following light ether anesthesia. Gastrocnemius, masseter, heart and liver tissues were rapidly extirpated, weighed on a Mettler balance, and immediately placed on dry ice. The frozen tissues were shaved with a scalpel to facilitate rapid preparation of a 5% homogenate in 0.1 M phosphate buffer (pH 7.4, 4°C) by a motor-driven, glass conical tissue grinder. A 3 ml aliquot of homogenate was immediately pipetted into 4.5 ml of 10% trichloroacetic acid for control, and 2 other 3 ml aliquots were prepared for incubation. Into one of the incubation samples was added 0.2 ml of a dl-α-tocopherol acetate suspension (1.0 mg dl-α-tocopherol acetate/ml phosphate buffer, suspended by sonification). The 3 preparations were then subjected to sonification (20 kc/sec) by a Branson Model S-75 Sonifier (1.7 amperes for 15 seconds). All procedures to this point were carried out in a cold room at 4°C. Samples were then incubated in closed test tubes at 37°C in a water-bath shaker (120 cycles/min). Lipid peroxidation was determined by the 2-thiobarbituric acid test(12), using the procedure of Bieri and Anderson(9). All samples were run in duplicate.

In another group of experiments tissue α-tocopherol and ubiquinone concentrations were assayed simultaneously by strict adherence to the method of Edwin *et al*(13) involving Soxhlet extraction, floridin earth column chromatography and 2-dimensional paper chromatography. The organs of 6 to 8 animals were pooled to make sufficient mass for each of 3 determinations, run in triplicate.

Students' small sample "t" test was used for the statistical analysis with a probability of ≤ 0.05 being accepted as revealing a significant difference.

Results. Growth data. Weight gains of the animals on special diet are seen in Fig. 1.

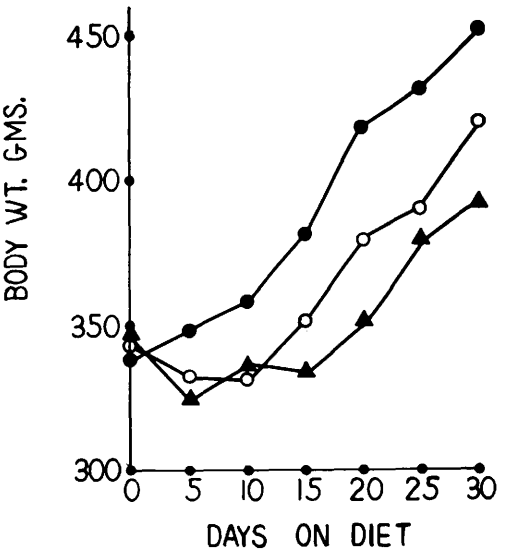

FIG. 1. Mean body weights of guinea pigs on special diets. ●——●, Normal + cod liver oil (NCLO); ○——○, vitamin E-deficient supplemented with dl-α-tocopherol and CLO (E+); ▲——▲, vitamin E-deficient + CLO (E−). Each point represents the mean of 7 to 11 animals. Normal animals (without cod liver oil) had the same growth curve as NCLO animals.

Loss of weight during the first 5 to 10 days in the E+ animals may be attributed to decreased food consumption and adjustment to the diet. After 10 days, the E+ animals have the same growth rate as the NCLO animals, but do not attain the same absolute weight. The E− animals do not increase their growth rate until after 15 days, and then have the same rate of growth as the other groups until 25 days. After approximately 30 days on diet, the E− animals start losing weight rapidly and usually die by 45 days on diet (not shown on Fig. 1).

Tocopherol and ubiquinone determination. Table I gives the mean α-tocopherol tissue concentrations for the 2 feeding durations. The animal tissues from each dietary regimen tested showed a constancy of α-tocopherol for the 2 feeding durations, with the exception of E+ heart, and these concentrations were significantly different between the heart, liver and muscle.

Vitamin E-supplementation significantly increased the α-tocopherol concentration above NCLO skeletal muscle at both 15 and

TABLE I. Mean and Standard Error of α-Tocopherol and Ubiquinone Concentrations (μg per g Wet Weight) for Tissues of the Different Dietary Regimens for Feeding Durations of 15 and 21 Days. Each value represents mean of 3 determinations, and each determination consisted of pooling of tissues from 6 to 8 animals.

Tissue	Diet	α-Tocopherol (μg/g) 15 day	α-Tocopherol (μg/g) 21 day	Ubiquinone (μg/g) 15 day	Ubiquinone (μg/g) 21 day
Heart	NCLO	18.2 ± .8	18.7 ± .8	13.4 ± .5	13.7 ± .9
	E+	13.5 ± .1	8.1 ± .9	10.9 ± .5	9.3 ± .2
	E—	6.2 ± .9	5.1 ±1.0	4.7 ±1.1	7.9 ± .4
Skeletal muscle	NCLO	1.5 ± .02	1.5 ± .04	.9 ± .1	2.1 ± .3
	E+	3.7 ± .4	2.7 ± .2	9.6 ± .9	7.8 ± .5
	E—	.5 ± .1	.8 ± .1	.8 ± .4	1.7 ± .1
Liver	NCLO	2.1 ± .01	1.9 ± .1	2.5 ± .3	2.6 ± .1
	E+	2.8 ± .2	3.3 ± .2	1.0 ± .2	1.3 ± .2
	E—	1.2 ± .02	1.0 ± .01	ND	

NCLO = Normal + cod liver oil.
E+ = Vitamin E-deficient diet supplemented with dl-α-tocopherol and cod liver oil.
E— = Vitamin E-deficient diet + cod liver oil.
ND = Non-detectable.

21 day feeding durations. Supplementation tended to increase liver tissue concentrations over NCLO, but the increase was not significant at 15 days; and vitamin E-supplemented heart tissue had significantly less α-tocopherol concentration than NCLO heart tissue. In all cases α-tocopherol concentrations of E+ tissues were significantly greater than the α-tocopherol concentrations of E— tissues.

Ubiquinone concentrations show a pattern quite similar to that of α-tocopherol concentrations (Table I). The concentrations are statistically the same within each dietary regimen for the 2 feeding durations. Tissues from E+ animals have significantly greater ubiquinone concentrations than tissues from E— animals. The E+ skeletal muscle is significantly greater than both NCLO and E— skeletal muscle.

Lipid peroxidation. Prior to incubation there were no significant differences in production of TBA chromogen between the same tissues of the 15 or 21 day feeding durations. After incubation at 37°C for 60 minutes, all tissues from all the dietary regimens at both feeding durations showed significant increases in production of TBA chromogen (Table II). Inclusion of dl-α-tocopherol in the E+ diet was found significantly to inhibit lipid peroxidation in the 15 and 21 day tissues when compared to either the E— or NCLO tissues; and, the E+ tissues were found to be not significantly different than N tissues.

Addition of dl-α-tocopherol acetate to the incubating tissue homogenates caused significant inhibition of lipid peroxidation only in E— tissues when compared to the homogenates of tissues without the added dl-α-tocopherol acetate. When comparing the tissues within the groups incubated with dl-α-tocopherol acetate it was found that E— tissues still produced greater TBA chromogen than E+ or N tissues, with the exception of gastrocnemius and masseter muscles at 15 days of diet.

Discussion. A point of emphasis in this communication is the investigation of animals in the early stages of nutritional muscular dystrophy, induced by feeding a diet low in vit. E content. Secondly, we have used a young herbivore in which the muscle fibers are still in the process of biochemical and structural maturation(14), and are more sensitive to the presence of highly unsaturated fatty acids such as are provided by supplementation of cod liver oil to the vitamin E-deficient diet. Older animals acquire greater tissue concentrations of vit. E through dietary sources(13) and relatively long periods of time are required to induce the manifestations of vit. E deficiency, plus the added problem of the adequacy of the diet as regards balance of essential nutrients.

Growth of the guinea pigs on the synthetic diet (Fig. 1) was not optimal even when the diet was supplemented with reason-

TABLE II. Means and Standard Errors of Differences in O.D. × 10^3 Between Control and Incubated Tissues, from the Different Dietary Regimens for Feeding Durations of 15 and 21 Days. Incubated + E is incubation with addition of dl-α-tocopherol acetate. Values for N diet are used as the same for the 15 and 21 day feeding durations.

Tissue	Diet	Incubated		Incubated + E	
		15 days	21 days	15 days	21 days
Heart	N	(5) 10.1 ± 4.0		8.1 ± 4.0	
	NCLO (5)	75.1 ± 11.5 (6)	25.2 ± 7.5	78.3 ± 21.7	21.1 ± 8.3
	E+ (5)	27.1 ± 11.6 (7)	13.2 ± 3.6	35.0 ± 15.2	12.0 ± 2.6
	E— (7)	65.4 ± 13.6 (6)	82.0 ± 9.4	25.4 ± 10.4	63.0 ± 8.3
Liver	N	(5) 11.2 ± 1.4		12.3 ± 2.2	
	NCLO	30.0 ± 4.6	25.2 ± 6.5	22.2 ± 13.8	16.4 ± 3.7
	E+	19.0 ± 8.4	19.3 ± 8.2	14.1 ± 10.0	15.3 ± 4.6
	E—	170.0 ± 4.9	138.0 ±33.0	123.3 ± 37.2	87.0 ±33.4
Gastrocnemius	N	(5) 39.3 ± 11.5		29.0 ± 11.2	
	NCLO	56.1 ± 14.2	71.3 ± 9.4	48.0 ± 16.3	61.0 ±13.4
	E+	42.0 ± 11.0	50.0 ± 7.3	14.0 ± 8.2	48.4 ± 6.8
	E—	59.3 ± 13.0	94.0 ± 8.2	31.0 ± 8.9	69.4 ±10.1
Masseter	N	(5) 27.1 ± 6.1		19.1 ± 5.5	
	NCLO	37.3 ± 12.3	31.0 ± 6.2	25.1 ± 13.1	24.3 ± 6.7
	E+	12.3 ± 5.2	19.0 ± 2.4	6.0 ± 9.2	17.4 ± 2.6
	E—	28.1 ± 6.7	49.0 ± 8.4	13.1 ± 4.2	34.0 ± 5.0

N = Normal.
NCLO = Normal + cod liver oil.
E+ = Vitamin E-deficient diet supplemented with dl-α-tocopherol and cod liver oil.
E— = Vitamin E-deficient diet + cod liver oil.
Numbers in parentheses indicate No. of animals, which is the same for all tissues of the same group of animals.

ably large amounts of vit. E. However, after the initial 5 days the E+ animals had the same growth rate as normal animals for a period longer than the duration of the experiment. We have also used this E+ diet in feeding rats, born from mothers maintained on the E+ diet, from weaning to 75 days of age and found the same growth rate as normal litters fed Purina lab chow (unpublished data).

Tissue concentrations of α-tocopherol and ubiquinone reported here qualitatively parallel those of Edwin *et al*(13) for the rat, and Green *et al*(15) for the rabbit; quantitatively, guinea pigs have a lower basal level than rats or rabbits and show a pronounced liability to vit. E deficiency. Fluctuating concentrations of α-tocopherol (*i.e.* heart) suggest there are local accumulations as deficiency of the vitamin progresses. Edwin *et al*(13) have pointed out that local tissue vit. E deficiency may occur to the point of causing death, although no absolute state of deficiency exists in the organism as a whole.

Low basal levels of α-tocopherol for guinea pigs, as compared to rat and rabbit, may be due to species differences, or the fact that the tissues were from young animals who had not acquired appreciable stores. However, investigators have shown that tissue vit. E levels were depressed when rats were fed diets containing cod liver oil and other unsaturated fatty acids(16), or diets containing high levels of vit. A(17,18). The guinea pigs in this study received 0.5 ml of cod liver oil 4 times a week, which would supply at least 425 U.S.P. units of vit. A. Vitamin A requirements of the guinea pig are not known, but the quantity given in these experiments was 10 × the requirement of an adult man compared on a weight basis.

Ubiquinone levels in this investigation seem to be some function of the α-tocopherol levels in the various tissues, which is in agreement with Green and his colleagues(13, 19). Schwarz(20) has shown that ubiquinones in the oxidized (quinone) form prevent respiratory decline in the same magnitude as α-tocopherolquinone when supple-

mented to whole homogenates. Martius(21) has recently demonstrated that α-tocopherol and tocopherol analogs are converted in tissues into a trimethyl-p-benzoquinone with a C_{50} side chain which could be called "deoxyubiquinone," and states "the assumption that the similarity in structure may be paralleled by a similarity in function in the cell appears to me at least not entirely illogical."

The different *in vitro* increases in TBA chromogen during the 60 minute incubation period must be attributed to the inherent capabilities of the tissues. Table II shows that the increase in lipid peroxidation during incubation was greatest in NCLO and E— tissues, whereas tissues from E+ animals were in the same range as normal tissues by 21 days of diet. High peroxidation in NCLO heart, liver and gastrocnemius tissues at 15 days must be explained by the inclusion of cod liver oil in the diet. Horwitt and his co-workers have shown that the peroxidizability of unsaturated fatty acids in specific tissues is a function of the tocopherol-unsaturated fatty acid concentrations, and that high concentrations of polyenes in cod liver oil were most effective in producing the creatinuria associated with dystrophy(16,22,23). The NCLO animals in this study showed no evidence for dystrophy, and it was interesting that heart tissues appeared to accommodate cod liver oil after 21 days of diet. Low peroxidizability of tissues from E+ animals (which were also given cod liver oil in the same amounts as NCLO and E— animals) supports Horwitt's thesis, in that supplementation with dl-α-tocopherol thrice weekly brought the E+ lipid peroxidation values within range (or lower in the case of masseter tissue) of normal tissue.

Addition of dl-α-tocopherol acetate suspended in phosphate buffer by sonification, and further sonification after addition to the homogenate seemed to make the dl-α-tocopherol acetate available to the tissues, as evidenced by the significant decrease in lipid peroxidation in the E— tissues. We are presently not able to state why the dl-α-tocopherol acetate was not effective in inhibiting lipid peroxidation in tissues from the other dietary regimens. Addition of 0.2 mg of dl-α-tocopherol acetate to an aliquot of homogenate was equivalent to 1.3 mg/g wet weight of tissue, which greatly exceeded any of the concentrations of α-tocopherol found in the tissue (Table I). The significance of this inhibition as pertains an *in vivo* situation could be questioned due to the relatively large amounts added to the homogenates. Bunyan *et al* (24) found that dietary concentrations of vit. E sufficient to completely prevent liver necrosis in the rat (10 p.p.m.) inhibited lipid peroxidation by only 10%.

Previous studies have shown a significant increase in oxygen consumption of E— soleus muscle at 15 days of diet(4). The small mass of soleus muscle was prohibitive for studying individual muscles in this investigation, but gastrocnemius muscle was found not to have a significant increase in lipid peroxidation until 21 days of diet when compared to NCLO and E+ gastrocnemius muscles. Bender *et al*(2), using guinea pigs and the same dietary regimens, found a significant increase in myoglobin concentration in E— gastrocnemius muscle at 15 days of diet.

Summary. α-Tocopherol and ubiquinone concentrations, and lipid peroxidation were investigated in gastrocnemius, masseter, heart and liver tissues of the guinea pig during the initial stages of nutritional muscular dystrophy. α-Tocopherol and ubiquinone concentrations were significantly less in tissues from animals fed E— diet than from animals fed E+ diet, at 15 and 21 days on diet. Lipid peroxidation was significantly greater in E— than E+ heart and liver tissues at 15 days, but E— gastrocnemius and masseter muscles did not show significant increases in lipid peroxidation until 21 days of diet. Supplementation of animals on E— diet with dl-α-tocopherol reduced lipid peroxidation in all tissues comparable to the lipid peroxidation from tissues of normal animals. Addition of dl-α-tocopherol acetate to incubating homogenates from tissues of all dietary regimens reduced lipid peroxidation only in E— tissues. The well established presence of nutritional muscular dystrophy in gastrocnemius

muscle at 15 days of this vitamin E-deficient diet, by several investigators, continues to raise the question as to the primary function of tocopherol in the origin of nutritional muscular dystrophy.

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Clearance of Plasma Enzymes in Normal and LDH Agent Infected Mice.* (29578)

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The presence of a viral-like agent in many transplanted mouse tumors which causes elevation of plasma lactic dehydrogenase (LDH)† is now well established(1-5). The agent can be transmitted through many generations in the absence of a tumor by injection of infectious plasma alone. This is followed by a 5- to 10-fold increase in the plasma LDH beginning in 30 hours and persisting through the lifetime of the animal. The mechanism of elevation of LDH enzyme is not known. Explanations which have been proposed but not yet substantiated include:

1) Destruction of some target organ or tissue releasing LDH to plasma; 2) Increased cellular permeability to enzyme; 3) Decreased clearance of enzyme from plasma; 4) Overproduction of enzyme.

1) and 2) above presumably would entail elevation of additional enzymes in plasma. However, we have pointed out previously(7) that without knowledge of the clearance rate of different enzymes, the degree of elevation could not be predicted. This paper describes experiments in which the clearance of LDH

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† Abbreviations: LDH—lactic dehydrogenase; MDH—malic dehydrogenase; PHI—phosphohexose isomerase; GOT—glutamic-oxalacetate-transaminase; GPT—glutamic-pyruvic-transaminase; and RBC, WBC—red and white blood cells, respectively.