Comparative Effects of Alpha-Tocopherol, DPPD and Other Antioxidants on Muscular Dystrophy in Guinea Pig.* (23188)

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Considerable data are available indicating that various antioxidants unrelated chemically to the tocopherols can prevent various manifestations of vit. E-deficiency in experimental animals. Methylene blue, thionine and thiodiphenylamine, for example, were all active in promoting growth and preventing exudative diathesis in chicks fed a diet deficient in vit. E(1). Methylene blue and DPPD (N, N'-diphenyl-p-phenylenediamine) prevented encephalomalacia in chicks(1,2) and resorption gestation in rats(3-5) fed diets deficient in vit. E. Methylene blue was also active in preventing dystrophy in calves fed a diet containing cod-liver oil(6) while DPPD showed similar activity in preventing symptoms of stiff lamb disease and histological degeneration of the skeletal and cardiac muscles of lambs fed a diet deficient in vit. E(7). There is evidence to indicate, however, that the above antioxidants will not completely replace vit. E in the diet but that the apparent tocopherol-like potency of these materials is due to their sparing effect on small residual quantities of vit. E in the diet and/or the body tissues. This is indicated by findings that the above antioxidants were devoid of vit. E activity as judged by the prevention of fetal resorptions when the diet was rigorously freed of tocopherol and the depletion period was extended(8,9). In the present communication further data are presented on the comparative effects of alpha-tocopherol and various antioxidants on the incidence and time of onset of muscular dystrophy in guinea pigs fed a highly purified diet deficient in vit. E.

Procedure. The basal vit. E-free ration employed consisted of casein,‡ 30%; cornstarch, 20%; dextrose, 11%; sucrose, 10%; cellulose,§ 10%; salt mixture,‖ 6%; lard,¶ 5%; agar, 5%; potassium acetate, 2.5%; and magnesium oxide, 0.5%. To each kg of the above diet were added the following vitamins: thiamine hydrochloride, 16 mg; riboflavin, 16 mg; pyridoxine hydrochloride, 16 mg; calcium pantothenate, 40 mg; nicotinic acid, 200 mg; biotin, 1 mg; folic acid, 10 mg; vit. B_{12}, 100 μg; 2-methyl-naphthoquinone, 5 mg; para-aminobenzoic acid, 100 mg; inositol, 2 g; choline chloride, 2 g; vit. A, 5000 U.S.P. units and vit. D_2, 500 U.S.P. units. The vitamins were added in place of an equal amount of cornstarch. Each guinea pig also received daily (6 times/week) an oral supplement of 10 mg ascorbic acid in 0.1 ml water. In preparation of the diets the various ingredients (minus the agar and the lard) were thoroughly mixed. The agar was dissolved in hot water (approximately 400 cc water/kg of dried diet), and the agar-water mixture was then mixed with the rest of the diet after which the lard was added. The mixture was then strained through an electric meat grinder and dried in an oven at a temperature of 110° to 120°F. In addition to the basal vit. E-free ration, tests were also conducted with animals fed the basal ration plus the following antioxidants: (1) 0.025% DPPD (N, N' - diphenyl - p-phenylenediamine), (2) 0.025% Santoquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline), and (3) 0.025% DBH (2,5-di-tert-butylhydroquinone). The above supplements were incorporated in the diet in place of an equal amount of cornstarch. Sixty

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† Deceased April 17, 1956.

‡ Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

§ Solka-floc BW 200, Brown and Co., Berlin, N. H.

‖ Salt Mix Wesson Modified (Osborne-Mendel), General Biochemicals, Chagrin Falls, O.

¶ Stripped Lard, Distillation Products Industries, Rochester, N. Y. Tocopherol content of this material according to manufacturers is less than 5 μg/g.

female guinea pigs were selected, average body weight 224 g (range 204 to 257 g). Animals were divided into 5 comparable groups of 12 guinea pigs each. One group was fed the basal ration; a second group received basal ration plus an oral supplement of 5 mg α-tocopherol acetate animal (in 0.1 ml propylene glycol) 6 times weekly; the remaining 3 groups were fed the diets containing DPPD, Santoquin and DBH. Animals were placed in metal cages with raised screen bottoms (2 animals/cage) and were fed the above diets and water *ad libitum* for 1 year or until death, whichever occurred sooner. Diets were prepared biweekly and were stored under refrigeration when not in use. Animals were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet.

Results. For the first 12 weeks of feeding no significant differences in weight or gross appearance were observed between the various groups. All animals survived and were indistinguishable grossly from guinea pigs of a similar age which had been fed a natural food stock ration. The average body weight after 12 weeks of feeding on the various diets was as follows: basal ration, 506 g; basal ration plus alpha-tocopherol acetate, 537 g; basal ration plus DPPD, 534 g; basal ration plus Santoquin, 545 g; and basal ration plus DBH, 510 g. Animals of a comparable age on the natural food stock ration averaged 589 g. During the 13th week, 3 of the guinea pigs on the basal vit. E-free ration developed clinical symptoms of dystrophy (dragging of the hind limbs and delayed righting when the animals were placed on their back). At this time the 3 dystrophic animals and one non-dystrophic animal from this group and 4 guinea pigs from each of the other groups were autopsied and sections of the gastrocnemius muscle were prepared for histological study. The dystrophic animals exhibited the typical Zenker degeneration noted by Pappenheimer(10) and others as characteristic of the dystrophic voluntary muscles of vit. E-depleted animals. The musculature of the other animals appeared normal in all respects. In contrast to results obtained on the basal vit. E-free ration, animals fed diets containing DPPD, Santoquin or DBH did not develop clinical symptoms of dystrophy until after the 25th week of feeding, whereas no dystrophy occurred in any of the animals administered alpha-tocopherol acetate. Results are summarized in Table I.

TABLE I. Comparative Effects of Alpha-Tocopherol Acetate, DPPD and Other Antioxidants on Incidence and Time of Onset of Muscular Dystrophy in Guinea Pigs Fed a Vit. E-Deficient Ration (8 Animals/Group).

Supplements fed with basal vit. E-deficient ration	Incidence of dystrophy (%)	Avg time of onset of dystrophy* (days)
None	100	139
α-tocopherol acetate	0	
DPPD	62.5	235
Santoquin	87.5	182
DBH	100	231

* Data were based only on animals with dystrophy.

Findings indicate that DPPD, Santoquin and DBH delayed but did not prevent occurrence of dystrophy in guinea pigs fed a highly purified diet deficient in vit. E. In contrast to the results obtained with these antioxidants, alpha-tocopherol acetate was fully protective in this regard. Since the basal diet employed in these studies was free of vit. E and contained no cod-liver or other highly unsaturated oils, it would appear that the delay in onset of dystrophy on diets containing DPPD, Santoquin and DBH was due not to the sparing effect of these antioxidants on residual quantities of vit. E (which is readily destroyed in the presence of unsaturated fats, particularly fish oils) in the diet but rather to the sparing effect of these antioxidants on vit. E in the tissues. Findings suggest that as long as tissue stores of vit. E were adequate, dystrophy did not occur. Once tissue stores of this vitamin were depleted, however, the above antioxidants were ineffective in preventing dystrophy in contrast to the continued effectiveness of alpha-tocopherol acetate in this regard.

Summary. DPPD, Santoquin and DBH delayed but did not prevent the occurrence of muscular dystrophy in guinea pigs fed a highly purified diet deficient in vit. E. In contrast to these results, alpha-tocopherol ace-

tate was fully protective in this regard.

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Adaptation of ECHO Viruses in HeLa Cells; Their Use in Complement Fixation.* (23189)

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The adaptation of ECHO prototype viruses (1) or primary isolation of ECHO viruses in cultures of HeLa cells has not been reported previously. Twelve of the original 13 ECHO viruses have been propagated successfully in cultures of HeLa cells maintained in this laboratory. ECHO virus type 4 failed to grow in HeLa cells.

During preparation of ECHO hyperimmune sera in monkeys, difficulties were encountered in complement fixation (CF) tests with viral antigens derived from infected renal cells. Viral antigens derived from HeLa cells proved to be useful in delineating homotypic antibody, and in exposing serologic cross-relationships observed in neutralization tests. The present report describes the methods used in adaptation of ECHO viruses to HeLa cells, and the application of viral antigens derived from HeLa cells in the CF test.

Materials and methods. Viruses. The prototype ECHO viruses were furnished by J. L. Melnick, Wm. McD. Hammon and A. B. Sabin. We passaged the viruses once or twice in monkey renal epithelial cells(2) when a large pool of stock virus was prepared. The passage history and titers of viruses propagated in cultures of renal and HeLa cells appear in Table I. *Tissue cultures.* The line of HeLa cells was obtained from J. T. Syverton in 1952(3). Since 1955 the cell line has been propagated in yeast extract medium(4) modified as follows: 1% yeastolate (Difco), 8.0%; 20% glucose, 1%; Hank's BSS, 68.0%; 2.8% $NaHCO_3$, 3%; and human serum, 20.0%. Following satisfactory growth in tubes or in bottles the cells were washed 3 times with phosphate buffered saline (PBS). The maintenance solution used in this laboratory(5) consists of mixture 199, 63.5%; 5% $NaHCO_3$, 3%; 20% glucose, 1%; tryptose phosphate broth, 25%; and calf serum, 7.5%. Following addition of maintenance solution cells were ready for inoculation with ECHO prototype viruses. *Virus titrations.* The stock virus pools were titrated in roller tube cultures of monkey kidney epithelial cells. ECHO viruses adapted to HeLa cells were titrated in renal epithelial cell cultures in the colorimetric test originally described by Salk, Youngner, and Ward(6) as modified by Melnick and Opton(7). *Antisera.* Sera were obtained from monkeys (*Macaca mulatta*) hyperimmunized with 13 prototype ECHO

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