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Chronic Toxicity of Methyl Linoleate Hydroperoxide for the Rabbit. (31459)

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Although the toxicity of oxidized fats for animals is well known(1-6), the explanation of their toxic effect is still unclear. There is evidence that the oral toxicity of air-oxidized oils is associated with their peroxide concentration; however, since peroxides apparently are destroyed in the intestine their toxicity presumably is exerted at this site(5,6). Whether fatty acid peroxides are formed in the tissues of animals in general, and of vitamin E deficient animals in particular, is a subject of current controversy. Clearly, if peroxides are formed *in vivo* they must be rapidly decomposed, as no appreciable accumulation occurs and their continued presence would have serious implications for the structural integrity of cell membranes and subcellular particles(7).

The muscular dystrophy which results from a deficiency of vit E may be attributable to peroxidation of lipids in the cellular and subcellular membranes which proceeds in the absence of this vitamin(8). If this explanation is correct, chronic parenteral administration of small amounts of preformed hydroperoxides might be expected to enhance the appearance of the disease by increasing the rate of vit E consumption in the tissues for decomposition of the peroxide or for destruction of additional free radicals whose formation may be catalyzed in its presence. This hypothesis was tested in the present study by determining the effects on the occurrence of vit E deficiency of administering small doses of purified methyl linoleate hydroperoxide to rabbits over protracted periods of time.

Experimental. New Zealand White weanling rabbits weighing 1100-1600 g were housed individually in metal cages and maintained on a vit E deficient diet for 10 days to deplete their tocopherol reserves. The composition of the diet was as follows(%): Labco casein 20.0, glucose (Cerelose) 20.4, starch 40.0, distilled lard 7.0, salts 4164(9) 4.0, cellulose 5.0, cod liver oil 3.0, choline chloride 0.1 and vit premix(9) 0.5. Methyl linoleate hydroperox-

ide (MLHP), prepared fresh according to a method described previously(10), was suspended in isotonic saline with the aid of 2% Tween 80. An emulsion of pure methyl linoleate was prepared in the same way.

In Experiment I, 2 groups of 6 vitamin E-depleted rabbits each were injected daily over a period of 10-14 days, one group with 1 ml of an emulsion containing 50 mg of MLHP and the other with the same quantity of emulsified methyl linoleate. The dose was administered intravenously through an ear vein. During the course of injections observations were made on the excretion of creatine and creatinine in the urine(11), body weight changes and development of muscular incoordination. At the end, the animals were examined for gross lesions of the internal organs and the livers were preserved in 10% formalin for histopathological examination.

Experiment II was designed to determine the influence of tocopherol or selenium administration on the chronic toxicity of MLHP. Thirty-two animals depleted of vit E reserves as described above were divided into 5 groups. Group 1 was maintained as an untreated control, Group 2 received daily intravenous injections of 50 mg of methyl linoleate in emulsion and the remaining animals were given a similar daily dose of MLHP. Group 5 received, in addition, a daily oral supplement of 100 mg of d-α-tocopheryl acetate prior to the injection, and Group 6 was given a supplement of 1 μg of selenium per g of diet in the form of sodium selenite. All the animals were killed after 10-14 days and histological examinations made made of their livers.

Experiment III was carried out to determine whether the incipient signs of vit E deficiency could be induced in rabbits depleted of their tocopherol stores by continuous intravenous infusion of MLHP. As peroxides are known to be rapidly metabolized *in vivo*, continuous administration of these compounds is probably necessary to maintain a circulating titer. After being maintained on a vit E deficient diet for 12-14 days, weanling animals were shaved in the neck region and fine polyethylene tubing was introduced 2-3 inches into the jugular vein through an 18-gauge heparinized hypodermic needle. The rabbits subsequently were kept in a restraining cage and given free access to food and water. Fresh emulsions of MLHP (peroxide value 4800-5400 meq per kg) were prepared in isotonic saline containing 100 mg% Tween 80 and 50 mg% animal lecithin. The hydroperoxide was dispersed in this medium at a concentration of 2 mg per ml by shaking gently and then bubbling nitrogen through the mass vigorously for 5 minutes. Emulsions thus prepared were stable for the duration of the infusion. As intravenous administration of fat emulsions is known to result in some physiological reactions which may be confused with those observed in vit E deficiency(12), a group of control rabbits was infused with a preparation containing methyl linoleate in place of MLHP. The emulsions were administered by means of a continuous infusion pump at a rate of 0.103 ml (206 μg MLHP) per minute for a period of 26-30 hours. The parameters chosen as criteria of incipient vit E deficiency were red blood cell hemolysis and creatinuria. Samples of blood and urine were taken at the beginning and end of the infusions (and, where possible, during the experiment) for analysis by published methods(11,13).

Results. Experiment I. The rabbits which received daily MLHP injections underwent a sharp increase in the ratio of creatine to creatinine in the urine during a 10-14 day period (Table I) and exhibited either a general loss of muscle tone or gross symptoms of incoordination which were evident in the righting reaction. In the methyl linoleate-treated group, no gross signs of dystrophy were detected and most of the creatine:creatinine ratios remained within the normal range. Two animals in the control group exhibited a mild creatinuria as might be expected of rabbits maintained on the deficient diet for 3 weeks. On the other hand, 5 of the 6 MLHP-injected rabbits experienced pronounced creatinuria as indicated by values in excess of 1.00. The range of ratios for the 12 animals at the start of the injection period was 0.03-0.33. Whereas the linoleate-treated group gained some weight during the experiment, the weights of most of the hydroperoxide-treated animals remained about constant or declined.

TABLE I. Effect of Intravenous Administration of Methyl Linoleate or MLHP on Urinary Creatine Excretion and Occurrence of Gross Symptoms of Muscular Dystrophy in Rabbits.

Rabbit No.	Methyl linoleate group* Body wt Initial	Final	Urinary creatine/creatinine Initial	Final	Righting reaction	MLHP group* Body wt Initial	Final	Urinary creatine/creatinine Initial	Final	Righting reaction
1	1100	—	.16	.04	—	1350	—	.10	.65	—
2	1570	1900	.10	.54	—	1550	1440	.03	2.92	+
3	1470	1510	.12	.73	—	1380	1080	.10	1.38	+
4	1095	1190	.33	.20	—	1090	1110	.17	1.73	—
5	1490	1570	.11	.12	—	1400	1380	.09	1.81	+
6	1320	1390	.16	.08	—	1470	1580	.10	1.08	—

* 50 mg per day injected intravenously for 10-14 days. Peroxide value of the MLHP preparation was 5000 meq/kg.

Gross examination of the livers of the MLHP-injected rabbits revealed the presence of white mottled areas on the surface of the lobes. Histopathological examination showed that these areas were marked by diffuse centrolobular fatty changes of varying degree with scattered foci of necrosis and calcification. Giant cell formation and bile duct proliferation also were observed in some livers. These lesions were not observed in any of the animals which received methyl linoleate.

Experiment II. Administration of large amounts of α-tocopherol orally prevented the occurrence of creatinuria and other gross signs of toxicity in rabbits injected with small daily doses of MLHP, but did not entirely prevent the liver lesions (Table II). Selenium had no influence on the incidence of hepatic lesions or on the increased creatine excretion. Interestingly, one case of hepatic lesions was observed in the methyl linoleate group, suggesting that peroxidation of this compound occurred in the vit E-depleted liver of this animal.

Experiment III. Table III shows that the continuous infusion of either methyl linoleate or MLHP for 26-30 hours was associated with an increase in erythrocyte hemolysis and creatine excretion. However, the hemolysis values for the MLHP-treated rabbits were consistently greater than those for the control group, and in 3 animals the creatine:creatinine ratios reached exceptionally high levels. The results suggest that the hydroperoxide induced a degeneration of red blood cell membranes and initiated additional destructive processes affecting other cells. These effects appear to be more extensive than can be attributed to the non-specific effect of infusing lipid emulsions.

Discussion. The rate of MLHP administration used in these studies (about 12 μM per 100 g body weight per day) was well below the LD_{50} intraperitoneal dose of 150-170 μM per 100 g reported for rats(6) and a comparable figure reported for mice(4). The toxicity of linoleate hydroperoxides is much greater by the intraperitoneal route than by

TABLE II. Influence of Vit E or Selenium on Toxic Effects of Intravenously Administered MLHP.

Treatment*	Oral supplement†	No. of rabbits In group	With liver lesions	With incoordination	Urinary creatine/creatinine Avg	Range
None	none	3	0	0	.15	.10– .20
Methyl linoleate	"	8	1	2	.14	.08– .30
MLHP	"	6	5	3	.69	.13–1.81
"	vit E	7	3	1	.12	.04– .20
"	Se	8	8	1	.64	.23–1.67

* 50 mg per day injected intravenously for 10-14 days. Peroxide value of the MLHP preparation was 5800 meq/kg.

† 100 mg d-α-tocopheryl acetate per day; 1 ppm Se as sodium selenite.

TABLE III. Effect of Continuous Infusion of Methyl Linoleate or MLHP on Erythrocyte Hemolysis and Urinary Creatine Excretion in Rabbits.

Rabbit No.	Methyl linoleate group			MLHP* group		
	Infusion time, hr	% RBC hemolysis	Urinary creatine/creatinine	Infusion time, hr	% RBC hemolysis	Urinary creatine/creatinine
1	0	0	.11	0	0	.13
	29	31	.34	30	49	.59
2	0	0	.01	0	0	.32
	30	9	.73	30	100	3.12
3	0	0	.31	0	0	.08
	30	32	.37	30	49	12.90
4	0	0	.004	0	0	.20
	26	21	.38	30	100	3.80

* Peroxide values of the MLHP preparations ranged from 4800 to 5400 meq/kg. Infusion rate was 206 μg/min.

the oral route(3,4,6), and at acute doses seems to be unaffected by tocopherol administration (6). The ameliorating effect of vit E on peroxide-induced creatinuria observed in the present study indicates that if the amounts of hydroperoxide administered are small the vitamin is capable of partially destroying them *in vivo,* probably by reduction to the corresponding hydroxy acid. Considering the large amounts of vit E employed in this experiment, however, and the incomplete protection obtained, it is not surprising that at much higher rates of hydroperoxide administration no protective effect was discernible(6).

The results of these experiments indicate that the chronic administration of small doses of hydroperoxide leads to a more rapid destruction of tocopherol in the tissues and an accelerated appearance of deficiency symptoms (creatinuria, erythrocyte hemolysis, muscular dystrophy). It has been reported by several workers that the concentration of vit E in the tissues of exhaustively depleted animals declined to undetectable levels. Under these conditions the formation of hydroperoxides *in vivo* may be presumed to increase, and there is considerable evidence that the damaging effect of these compounds on subcellular particles, sulfhydryl enzymes and other proteins(14) may account for the biochemical and histological changes observed in vit E deficiency. It has been reported(15) that cerebellar disorders characteristic of tocopherol deficiency can be induced in chicks by intravenous injection of 10 mg of methyl linoleate hydroperoxide.

The fatty degeneration of the liver found in this study does not occur in vit E deficient rabbits and has not been reported in other animals following short-term hydroperoxide administration. The lesion is distinct from the dietary necrotic liver degeneration described by Schwartz(16), as indicated by the histopathology and the failure of selenium to exert a protective effect. Intravenous administration of preformed hydroperoxides may lead to rapid localized liver damage before these compounds are reduced by tissue antioxidants. Holman and Greenberg(1) have observed that a hydroxy acid with a conjugated diene system (the likely reduction product of a linoleate hydroperoxide) is much less toxic for rats than the corresponding hydroperoxide.

Summary. Small quantities of purified methyl linoleate hydroperoxide (MLHP) were administered by daily intravenous injection (50 mg/day) or by continuous intravenous infusion (206 μg/min) to rabbits depleted of their vitamin E stores. After 10-14 days the injected animals exhibited fatty degeneration and necrosis of the liver, creatinuria and an increased incidence of muscular incoordination. The creatinuria was prevented by large oral doses of vitamin E (100 mg/day) but sodium selenite had no effect on the creatinuria or the incidence of liver lesions. Infusion of MLHP for 26-30 hr led to an increased fragility of the red blood cells and a marked creatinuria. These results indicate that chronic administration of small quantities of MLHP leads to a more rapid de-

struction of vitamin E in the tissues and an accelerated appearance of deficiency symptoms.

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Thyroxine Augmentation of Growth Hormone-Induced Endochondral Osteogenesis.* (31460)

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The actions of thyroxine in enhancing body growth, skeletal and somatic, are among the most sensitive of this hormone's effects(1), but remain incompletely studied and understood. Thyroxine participates both in (*A*) incremental growth, and in (*B*) differentiation.

A, 1. It has long been known that thyroxine augments the action of pituitary growth hormone, in the absence of the pituitary and/or the thyroid glands, as well as in intact animals (2). Whether this augmentation is true synergism is unresolved, but the demonstration by Geschwind and Li(3) that a dose of thyroxine so minute as to have no discernible effect alone could yet increase the sensitivity of the "tibia line" assay for growth hormone, appears strong evidence for synergism.

A, 2. Alone, thyroxine induces a discernible, but slight and unsustained, elongation of bones in hypophysectomized rats, a transient effect which is thus independent of pituitary growth hormone(4).

B. In differentiation, thyroxine can induce the appearance of new epiphyseal ossification centers and subsequently their fusion to the diaphysis through resorption of the epiphyseal cartilage plate. This action occurs in the absence of the pituitary(4) or the thyroid(5,6), or both(7). It is apparently effected through continued erosion of cartilage in the absence of an equivalent rate of chondrogenesis; "chondroclasts" have appeared at the marrow-cartilage junction during thyroxine administration(8).

In most circumstances, the principles underlying these actions are not known; in one circumstance (growth in thyroxine-treated

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