Some Effects of Methyl Linoleate Hydroperoxide on Oxidative Phosphorylation in Rat Liver Mitochondria.* (31186)

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The effects of methyl linoleate hydroperoxide (MLHP), a peroxidation product of methyl linoleate, on the denaturation of the lipoproteins (Nishida and Kummerow(9)) and on certain pathologic disorders in animals simulating vitamin E deficiency is well known. Both the denaturation phenomenon and the pathologic disorders are alleviated by α-tocopherol supplementation (Nishida and Kummerow(10)).

Evidence is reported here which indicates that MLHP has an inhibiting effect on the coupling of phosphorylation with oxidation in rat liver mitochondria.

Materials and methods. Mitochondria were prepared from the livers of normal adult Sprague-Dawley male rats on a stock laboratory diet by the Hogeboom-method (Hogeboom(4)), using 0.25 M sucrose.

The conventional Warburg procedure was used to measure oxygen uptake over a 30-minute period at 30° following a 6-minute preincubation (see footnote to Table I for composition of media).

Incubation time was terminated by addition of 5% trichloroacetic acid (TCA). The inorganic phosphate remaining in each vessel was determined by Lowry's method(6). The P/O ratio was calculated from the values observed.

The protein content of each tissue sample was determined by the use of phenol reagent (Lowry and Lopez(7)).

The extent of peroxidation in mitochondria was determined by the barbituric acid (TBA) reaction method of Corwin(1), with the exception that 0.5 ml of the mitochondrial preparation was used in place of whole liver homogenate.

To prepare MLHP, 50 g of methyl linoleate urea complex (Hormel Institute, Univ. of Minnesota) was extracted with 100 ml of hot 1 N HCl, and the methyl linoleate was washed 3-4 times, each time with 100 ml of water. The pure methyl linoleate was extracted with 30 ml of Skelly Solve F (SSF). After evaporation of the SSF under nitrogen (yield 13 g), 1 g of the oily methyl linoleate residue was incubated overnight at 0°C in 100 ml of 0.2 M phosphate buffer, pH 8, containing 20 mg lipoxidase (10,000 μ/mg, Sigma) with constant stirring. The MLHP formed was extracted with SSF and the solvent evaporated under nitrogen. "The peroxide value" (Olcott and Doler(11)) was determined on each sample from the UV absorption at 234 mμ (Holman(5)). Various preparations gave peroxide values ranging from 300-3800. This lipid peroxide was suspended in 0.3% Tween 80, and 0.01-0.02 ml of this suspension was used per reaction flask.

DL-α-tocopherol,‡ DPPD§ (N,N'-diphenyl-p-phenylene diamine) and Ethoxyquin¶ (Santoquin, 1-2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) were dissolved in ethanol, and 0.01-0.02 ml of the solution was added per reaction flask as indicated in Table III.

In the first series of experiments listed in Table III, the MLHP was added to the buffer medium followed by the α-tocopherol and finally by the mitochondria, immediately prior to incubation at 30°C. In the second series of experiments in Table II the MLHP and mitochondria were added to the buffer at 0°C, and after 30 minutes at this temperature α-tocopherol was added and incubation at 30°C was carried out.

Results and discussion. It can be seen from Table I that the addition of MLHP to mitochondria decreased the P/O ratio when succinate or β-hydroxybutyrate were used as substrates. This change in ratio was pri-

* This work was supported by USPHS Grant AM 08300.

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‡ From Hoffmann-LaRoche Inc., Nutley, N. J.

§ From B. F. Goodrich Chemical Co., Akron, Ohio.

¶ From Monsanto Chemical Co., St. Louis, Mo.

marily due to a decrease in phosphorylation, although the MLHP did cause some decrease in oxygen uptake. In Table II are shown the inhibitory effects of different levels of MLHP and the effect of DL-α-tocopherol on this inhibition. It can be seen from the results of Series I that addition of the α-tocopherol to MLHP prior to the addition of the mitochondria restored or prevented almost completely the inhibitory effect of the peroxide, except in the case where MLHP of high peroxide value (3800) was used. In control

TABLE I. Oxidative Phosphorylation by Rat Liver Mitochondria Treated with MLHP.*

Substrate	No. of rats	ΔO, μatoms/mg protein	%	ΔO, μmoles/mg protein	P/O
Succinate	18	1.74 (.98–2.58)	100	3.71 (2.03–5.53)	2.13 ± .29
+ ML, 3.5 μmoles	7	1.69 (1.26–2.54)	93–104	3.56 (2.42–5.46)	2.11 ± .32
+ MLHP, 3.1 μmoles†	19	1.40 (.67–2.56)	25–108	.86 (0 –1.24)	.61 ± .52
β-Hydroxybutyrate	8	1.23 (.79–1.46)	100	3.29 (2.25–4.65)	2.76 ± .31
+ ML, 3.5 μmoles	7	1.20 (1.00–1.46)	76–102	3.66 (2.62–4.72)	3.06 ± .46
+ MLHP, 3.1 μmoles†	13	.71 (.25– .95)	29– 77	1.04 (0 –1.21)	1.47 ± .92

* Ranges are shown in parentheses. † Peroxide value = 3,800.

P/O ratios are expressed by average value and its standard error.

Reaction mixtures contained the following in μmoles/3.0 ml: 65 Tris buffer (pH 7.4); 50 phosphate (pH 7.4); 50 substrate; 0.03 cytochrome c; 3 ATP; 0.2 DPN; 15 NaF; and 30 $MgCl_2$ in the main compartment. 25 glucose and hexokinase (Sigma Chemical Co.) and 25 KM units were put in the side arm.

ML or MLHP in 0.01 ml of 0.3% Tween 80 was added to the solution prior to addition of mitochondrial suspension (equivalent to 200 mg fresh liver in 0.5 ml 0.25 M sucrose) per incubation.

TABLE II. Addition of α-Tocopherol to the Media Containing MLHP.

Substrate	No. of rats	ΔO, μatoms/mg protein	P/O
Series 1. Addition at zero time			
Succinate	6	2.06 (1.69–2.74)	2.01 (1.92–2.22)
3,800 no α-tocopherol	19	1.40 (.67–2.56)	.61 (0 –1.59)
+ "MLHP" (3,800)* + α-tocopherol, 0.5 μmoles	3	2.23 (1.15–2.47)	.73 (.30–1.03)
+ "MLHP" (500)* + α-tocopherol, 0.5 μmoles	3	1.32 (.95–1.69)	1.84 (1.68–2.10)
+ "MLHP" (500)*	3	1.46 (.90–2.62)	.80 (.26–1.43)
β-Hydroxybutyrate	3	1.19 (.96–1.52)	2.66 (2.21–2.95)
+ "MLHP" (1,000)* + α-tocopherol, 2.5 μmoles	5	.67 (.35– .82)	2.24 (1.36–3.03)
+ "MLHP" (1,000)*	5	.76 (.28–1.24)	.69 (0 –2.32)
Glutamate	5	1.19 (.89–2.01)	2.90 (2.67–3.24)
+ "MLHP" (1,350)* + α-tocopherol, 2.5 μmoles	5	.84 (.63–1.19)	2.48 (2.47–2.52)
+ "MLHP" (1,350)*	5	.68 (.47– .97)	1.84 (1.28–1.90)
Series 2. Addition of α-tocopherol after mitochondria had been incubated with MLHP for 30 min at 0°C			
Succinate			
+ MLHP (1,350)* + α-tocopherol, 2.5 μmoles	3	1.38 (1.16–1.52)	1.56 (1.36–1.77)
+ MLHP (1,350)*	3	.95 (.74–1.10)	1.10 (.72–1.82)
β-Hydroxybutyrate			
+ MLHP (1,350)* + α-tocopherol, 2.5 μmoles	3	.61 (.47– .72)	2.20 (2.02–3.02)
+ MLHP (1,350)*	3	.63 (.46– .85)	1.32 (1.35–1.87)

* Peroxide value.

TABLE III. Addition of Antioxidants and Other Substances. The values are average of 2-3 determinations.

Substance	ΔO, atom/mg protein	P/O	TBA reaction (optical density) O.D. at 532 mμ, % of MLHP control
(Substrate: Succinate)			
None	1.73	1.86	31
MLHP (1,020)*	.94	.76	100
+ "DPPD" 0.4 μmoles	1.76	1.70	47
MLHP (500)* + DPPD, 0.4 μmoles	1.15	1.21	—
MLHP (1,020)* + Ethoxyquin, 1.8 μmoles	1.17	.76	31
+ MLHP (1,020)* + Ethoxyquin, 3.6 μmoles	1.27	.26	47
MLHP (1,350)* + α-tocopherol, 2.5 μmoles	(see Table II)		
DNP 1 × 10⁻³ M	1.41	.03	—
+ "MLHP" (1,020)*	1.25	.07	—
(Substrate: β-Hydroxybutyrate)			
None	1.43	2.79	32
MLHP (1,020)*	1.07	1.16	100
+ "DPPD" 0.4 μmoles	.52	1.57	48
MLHP (500)* + DPPD, 0.4 μmoles	.64	1.44	39
MLHP (1,020)* + Ethoxyquin, 1.8 μmoles	.60	.60	70
MLHP (1,020)* + Ethoxyquin, 3.6 μmoles	.48	.30	45
MLHP (1,350)* + α-tocopherol, 2.5 μmoles	(see Table II)		35

* Peroxide value.

experiments in which α-tocopherol alone was added to mitochondria, no effect on either oxygen uptake or P/O ratio was found.

Since these results may well have been due to the ability of α-tocopherol to destroy the peroxide prior to the addition of mitochondria, the second series of experiments was carried out in which the peroxide was permitted to react at 0°C with the mitochondria for 30 minutes prior to addition of the α-tocopherol. It can be seen from Table II that the α-tocopherol restored mitochondrial oxidative phosphorylation almost to normal, whereas without the subsequent addition of α-tocopherol markedly depressed P/O ratios were obtained.

The results shown in Table III indicate that the zero time addition of DPPD to the system decreased MLHP inhibition and oxidative phosphorylation somewhat in the succinate system and to a lesser extent in the β-hydroxy-butyrate system. In both cases, the effect of DPPD is much less than that observed in Table II with α-tocopherol. The fact that DPPD is essentially equal in activity to α-tocopherol (Draper and Johnson(3)) for reproduction in the vitamin E-deficient female rat (Zalkin and Tappel(12)), possibly indicates that its slower activity in the *in vitro* system was due to its insolubility in the medium and thus its failure to penetrate the mitochondrial membrane; as in the control experiments with α-tocopherol control experiments with DPPD alone or Ethoxyquin alone had no effect on oxidative phosphorylation.

Because of the possibility of the DPPD's not being able to penetrate the mitochondrial membrane, an experiment was performed whereby 500 γ of DPPD suspended in 0.3% Tween 80 was homogenized with the mitochondria. This large amount of DPPD had no effect on the normal P/O ratio. However, with the addition of MLHP the reaction was inhibited indicating again no protection of

the mitochondria by DPPD.

A similar experiment with Ethoxyquin showed it to also be inactive. In each case, when an antioxidant was added into the media, development of the TBA-reactant color was significantly decreased to the level of color development found in normal mitochondria (Table III).

When the TBA reaction was carried out in the absence of mitochondria, only a slight yellow color was found due to MLHP, a color which was much less pronounced than the red color produced by the combination of MLHP and mitochondria.

These findings indicate that lipid peroxidation is occurring due to the action of MLHP on the mitochondria. However, there does appear to be a direct relationship between the TBA reaction and the P/O ratio, and the present data appear to indicate a rather specific activity of α-tocopherol, in restoring the ability of oxidized mitochondria to carry out oxidative phosphorylation.

Two possibilities exist, (1) that the added peroxide is oxidizing the phospholipid which is required in the oxidative phosphorylation reaction (McCay and Caputto(8)), or (2) that the added peroxide is destroying some protein component of the oxidative phosphorylation pathway, in particular some component involved in the coupling of the phosphorylation with oxidation. To test the latter hypothesis, samples of pure MLHP (peroxide No. 5840) were sent to Dr. Racker for assay as to their effect on his protein coupling factors. His experiments indicate that coupling factor F2 is inhibited by this peroxide.

The decrease of P/O ratios sometimes observed in liver mitochondria from vitamin E-deficient animals (Zalkin and Tappel(12) and McCay and Caputto(8)) and the alleviating effects of α-tocopherol and of certain other synthetic antioxidants, such as DPPD and Ethoxyquin on vitamin E deficiency *in vivo* (Draper and Johnson(3)) and *in vitro* respiratory decline of vitamin E deficiency (Corwin and Schwarz(2)), at least suggest that this peroxide inhibition may be the type of reaction which occurs in vitamin E deficiency.

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Received March 2, 1966. P.S.E.B.M., 1966, v122.

Effect of Heparin and Sulfated Polysaccharides on *in vitro* Hepatic Phagocytosis.* (31187)

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Previous investigations of the influence of heparin on the phagocytic function of the reticuloendothelial system (RES) are contradictory. Knisely *et al*(1) reported that heparin transiently retarded the phagocytic engulfment of India ink, kaolin, and graphite particles by the sinusoidal macrophages of frog liver. Similarly, heparin hindered hepatic

* This investigation was supported in part by U.S. Atomic Energy Commission.