Autoxidation of Methyl Linoleate: Effect of Sex Hormones and of Nicotinic Acid and Related Compounds.* (29053)

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In our studies of the oxidation of cholesterol-26-C^{14} to $C^{14}O_2$ by rat liver mitochondrial preparations we have observed that nicotinic acid alone among its homologs, analogs and derivatives will enhance the oxidation of cholesterol when added directly to the incubation mixture(1-3). We have also observed that mitochondrial preparations from livers of female rats or of chemically or surgically castrated male rats oxidize cholesterol to a greater extent than do similar preparations from livers of normal male rats(4). Neither male nor female hormones have any effect when added to the incubation mixture(5). In view of the report of Schuler and Meier(6) that estrogens inhibit the cortisone catalyzed oxidation of linoleic acid *in vitro* and of our preliminary findings(7) which indicate that epoxides of unsaturated fatty acids inhibit cholesterol oxidation by mitochondrial preparations, we have investigated the influence of various nicotinic acid homologs and of certain sex hormones on the ascorbic acid catalyzed oxidation of methyl linoleate, as measured by the thiobarbituric acid (TBA) reaction(8). The TBA reaction has been shown to be as effective as other standard methods (peroxide, absorption, aldehyde test) in following the oxidation of methyl oleate or linoleate(9).

Materials and methods. The system used was similar to that described by Wilbur *et al* (8). Methyl linoleate was dissolved in 95% ethyl alcohol to give a final concentration of 0.9 mg/ml. Addition of 0.2 ml of the stock solution of methyl linoleate per 2 ml of buffer solution containing 0.18 mg/ml of ascorbic acid yielded a solution of methyl linoleate whose concentration was 3×10^{-4} M. The solution was incubated with shaking in a water bath kept at 37°C and 2.0 ml aliquots were removed for assay at 0, 2, 4 and 6 hours. For color development, the 2.0 ml aliquot was treated with 1.0 ml of 20% trichloroacetic acid and 2 ml of 0.67% TBA solution. The mixture was held at boiling water bath temperature for 10 minutes, cooled, centrifuged and the absorbence of the resulting red solution was determined at 530 mμ. We carried out studies using phosphate buffer, pH 6.8 and, since our cholesterol oxidation studies were carried out in Tris buffer at pH 8.5, we also carried out a series of experiments in that buffer. Our mitochondrial studies were carried out in solutions containing 10% sucrose but use of sucrose in place of ascorbic acid gave excessive TBA color, as Wilbur *et al*(8) and Franke(10) had reported earlier.

In experiments with the various nicotinic acid analogs the test compounds were dissolved in the buffer to give a final concentration of 6.3×10^{-3} M. This corresponds to 0.77 mg of nicotinic acid/ml or 10 mg/13 ml, the concentration at which the compounds are used in the cholesterol oxidation experiments. The sex hormones were emulsified in the appropriate buffer with the use of Tween 20. The final concentration of hormone was 1.7×10^{-4} M and of emulsifying agent 1%.

Except as noted below, all compounds were purchased from commercial sources.

Results and discussion. The ascorbic acid catalyzed oxidation of methyl linoleate proceeds to the same extent whether carried out in phosphate buffer at pH 6.8 or in Tris buffer at pH 8.5, as can be seen from Table I.

The nicotinic acid-like compounds used in this study were nicotinic acid, nicotinamide, ω(3-pyridyl) acetic, propionic and butyric acids, picolinic acid and 3-pyridine sulfonic acid. In incubations carried out in phosphate buffer (pH 6.8) only picolinic and 3-pyridine sulfonic acids enhanced oxidation of methyl linoleate after 2 hours. At 4 hours enhance-

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TABLE I. Effect of Buffer on Ascorbic Acid Catalyzed Oxidation of Methyl Linoleate. (Thiobarbituric acid color at 530 mμ)

	Color yield × 100			
	0 hr	2 hr	4 hr	6 hr
Phosphate buffer, pH 6.8	18	20	18	20
Buffer + methyl linoleate (ML)*	32	31	45	50
Buffer + ascorbic acid (AA)*	32	80	75	65
Buffer + ML + AA	58	100	112	110
Tris buffer, pH 8.5	18	18	20	26
Buffer + ML	30	45	58	42
" + AA	28	40	40	38
" + ML + AA	45	110	115	110

* .18 mg/ml.

ment of oxidation was also shown by nicotinic and ω(3 pyridyl)-acetic acid and by six hours only the pyridyl propionic and butyric acids had no effect. When the incubations were carried out in Tris buffer (pH 8.5) picolinic acid inhibited oxidation of methyl linoleate but none of the other test compounds had any effect. The data of these experiments are presented in Table II. From these data, an influence upon oxidation of the fatty acids normally present in mitochondria cannot be invoked to explain the observed effects of the compounds tested upon the oxidation of cholesterol by rat liver mitochondrial preparations.

TABLE II. Influence of Nicotinic Acid and Related Compounds on Ascorbic Acid (AA) Catalyzed Oxidation of Methyl Linoleate (ML). (Thiobarbituric acid color at 530 mμ)

System	Color yield × 100			
	0 hr	2 hr	4 hr	6 hr
Phosphate buffer, pH 6.8				
Control (ML + AA)*	78	92	105	110
Nicotinic acid†	80	110	150	150
Nicotinamide	92	110	100	130
3 pyridylacetic acid	68	95	120	145
ω (3 pyridyl) propionic acid	80	108	120	108
ω (3 pyridyl) butyric acid	62	95	110	100
Picolinic acid	75	140	138	158
Pyridine 3-sulfonic acid	75	150	162	155
Tris buffer, pH 8.5				
Control (ML + AA)	65	110	105	98
Nicotinic acid	60	110	108	100
Nicotinamide	70	98	100	100
3 pyridylacetic acid	60	105	100	100
ω (3 pyridyl) propionic acid	70	100	98	90
ω (3 pyridyl) butyric acid	70	112	108	100
Picolinic acid	85	65	68	58
Pyridine 3-sulfonic acid	82	120	105	98

* 0.18 mg/ml each of methyl linoleate and ascorbic acid.
† 6×10^{-3} M.

Schuler and Meier(6) found that 4 different estrogenic compounds (estradiol, estrone, equilenin and diethylstilbesterol) inhibited the copper or cortisone catalyzed oxidation of linoleic acid(11). In their studies, carried out in phosphate buffer at pH 7.0 oxidation was determined by measuring oxygen uptake manometrically. In our experiments we used 3 androgens (testosterone, androsterone and epiandrosterone) and 4 estrogens (estradiol, estriol, estrone and hexesterol). In the experiments carried out in either phosphate buffer (pH 6.8) or Tris buffer (pH 8.5) none of the sex hormones had any effect. Estradiol may have had a slight inhibiting effect on methyl linoleate oxidation after 6 hours in Tris buffer. These results again suggest that the estrogen effect on cholesterol oxidation by rat liver mitochondrial preparations are due to biochemical sequelae of estrogen treatment and not to their chemical effects on the fatty acid constituents of the mitochondria. (Table III).

TABLE III. Influence of Sex Hormones on Ascorbic Acid (AA) Catalyzed Oxidation of Methyl Linoleate (ML). (Thiobarbituric acid color at 530 mμ)

System	Color yield × 100			
	0 hr	2 hr	4 hr	6 hr
Phosphate buffer, pH 6.8				
Control (ML + AA)*	55	50	95	58
Testosterone†	42	62	95	68
Androsterone	45	58	98	75
Epiandrosterone	35	48	89	75
Estradiol	40	42	95	80
Estriol	45	45	89	75
Estrone	50	48	98	78
Hexesterol	40	40	75	65
Tris buffer, pH 8.5				
Control (ML + AA)	40	50	58	50
Testosterone	30	40	50	45
Androsterone	20	35	48	48
Epiandrosterone	28	42	58	50
Estradiol	30	30	40	38
Estriol	35	38	45	50
Estrone	30	38	53	58
Hexesterol	22	40	60	40

* 0.18 mg/ml each of methyl linoleate and ascorbic acid.
† 1.7×10^{-4} M.

Summary. The influence of a number of nicotinic acid analogs and homologs (nicotinic acid, nicotinamide, picolinic acid, pyridine 3-sulfonic acid and ω(3-pyridyl) acetic, propionic and butyric acids) and sex hormones (testosterone, androsterone, epiandrosterone, estradiol, estriol, estrone, and hexesterol) upon the ascorbic acid catalyzed oxidation of methyl linoleate has been studied. Experiments were carried out in phosphate (pH 6.8) and Tris (pH 8.5) buffers. The extent of oxidation was measured by the thiobarbituric acid color reaction. None of the sex hormones had any effect on the oxidation of methyl linoleate in either buffer. In phosphate buffer only ω(3-pyridyl) propionic and butyric acids failed to enhance oxidation of methyl linoleate after 6 hours. In incubations carried out in Tris buffer picolinic acid inhibited oxidation of methyl linoleate. None of the other test compounds had any effect. In the absence of test compounds the type of buffer did not affect the rate of methyl linoleate oxidation.

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