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Effects of Tocopherol Depletion on the Regional Differences in Adrenal Microsomal Lipid Peroxidation and Steroid Metabolism*

D. A. STAATS, D. P. LOHR, AND H. D. COLBY

Department of Biomedical Sciences, University of Illinois College of Medicine, Rockford, Illinois 61107

ABSTRACT. Studies were done to assess the contribution of α-tocopherol to the regional differences in microsomal lipid peroxidation (LP) and steroid metabolism in the guinea pig adrenal cortex. In normal guinea pigs, ferrous ion (Fe^{2+})- and ascorbic acid-induced LP are far greater in microsomal preparations from the inner adrenal zone (zona reticularis) than in those from the outer zones (zona fasciculata plus zona glomerulosa). The amounts of unsaturated fatty acids, substrates for LP, are similar in the two zones, but α-tocopherol concentrations are 4–5 times greater in outer than inner zone microsomes. Tocopherol depletion by dietary deprivation had little effect on LP *in vitro* in inner zone microsomes, but substantially increased LP in outer zone preparations. As a result, tocopherol deficiency eliminated the zonal differences in microsomal LP. Unsaturated FFA concentrations were lower in tocopherol-deficient microsomal preparations than in those from tocopherol-sufficient animals, suggesting peroxidative losses *in vivo*. Tocopherol deficiency decreased steroid C-17,20 lyase activity in outer zone microsomes, but had no effect on activity in inner zone preparations, eliminating the normal zonal difference in activity (outer > inner). The results indicate that α-tocopherol is a major determinant of adrenal LP and is responsible for the regional differences in microsomal LP in guinea pig adrenal cortex; the effects of ascorbic acid on LP in each zone are also affected by α-tocopherol. α-Tocopherol may influence the functional zonation of the adrenal cortex by selectively protecting outer zone steroidogenic enzymes from oxidative degradation. (*Endocrinology* **123:** 975–980, 1988)

LIPID peroxidation (LP), the oxidative degradation of membrane unsaturated fatty acids, has been implicated in a variety of pathological as well as physiological processes (1–3). One of the organs in which particularly high levels of LP have been demonstrated is the adrenal cortex (4–7). It has been proposed that LP may contribute to the functional zonation of the adrenal cortex by effecting the destruction of certain membrane-bound steroidogenic enzymes in regions of high peroxidative activity (7). The resulting regional differences in enzyme activities could contribute to the unique secretory profile of each adrenal zone.

In support of the above hypothesis, we recently reported that LP was far greater in subcellular fractions from the guinea pig zona reticularis than in those from a combined zona fasciculata-zona glomerulosa preparation (8, 9). The region of high peroxidative activity is associated with relatively low activities of several steroidogenic enzymes required for corticosteroid synthesis (10, 11). We also found that the regional differences in peroxidative activity were inversely related to the microsomal and mitochondrial content of the antioxidant α-tocopherol and proposed a cause and effect relationship. To test the latter hypothesis, we have now evaluated the effects of α-tocopherol depletion on microsomal LP and steroid metabolism in the adrenal cortex. The results indicate that α-tocopherol is an important determinant of adrenal LP and may be responsible for some of the regional differences in steroid metabolism.

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Address all correspondence and requests for reprints to: Dr. H. D. Colby, Department of Biomedical Sciences, University of Illinois College of Medicine, 1601 Parkview Avenue, Rockford, Illinois 61107-1897.

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Materials and Methods

Male English short hair guinea pigs, weighing approximately 800–1000 g, were obtained from Camm Research Institute (Wayne, NJ). Animals were maintained under standardized conditions of light (0600–1800 h) and temperature (22 C) and received water *ad libitum*. All guinea pigs were fed a synthetic chow obtained from Teklad (Madison, WI). Control animals received a nutritionally complete modified Reid-Briggs guinea pig chow (catalog no. TD86284). The experimental group received chow which lacked tocopherol (catalog no. TD86283) but was otherwise identical to the control diet. Animals received the synthetic diets for 9–10 weeks, the time required to deplete adrenal glands of α-tocopherol, as determined by HPLC analysis.

Animals were killed by decapitation between 0800–0900 h, and adrenals were quickly removed and placed in cold 1.15%

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KCl-0.05 M Tris-HCl (pH 7.4) on ice. Tissues were trimmed free of fat and connective tissue and weighed. Adrenal glands were bisected longitudinally, and the dark brown inner zone, consisting of zona reticularis, was dissected away from the tan outer zone, consisting of the zona glomerulosa and zona fasciculata, as previously described by Martin and Black (12). Tissues were then homogenized in KCl-Tris buffer, and washed microsomal fractions were obtained by differential centrifugation, as previously described (13). Microsomal protein concentrations were determined by the method of Lowry *et al.* (14). Steroid 17α-hydroxylase, 21-hydroxylase, and C-17,20 lyase activities were evaluated by HPLC analyses, as previously described (11).

For LP analyses, microsomal fractions (0.2 mg protein/ml) suspended in 1.15% KCl-0.05 M Tris-HCl (pH 7.4) were incubated in 25-ml Erlenmeyer flasks at 37 C for 30 min under air. The total incubation volume in each flask was 2.5 ml. Where indicated, $FeSO_4$ or L-ascorbic acid (Sigma Chemical Co., St. Louis, MO) was added to the reaction flasks before the start of incubation. The formation of malonaldehyde (MDA), as measured by the thiobarbituric acid test, was used as an index of LP. MDA was measured by the method of Ottolenghi (15), as modified by Hunter *et al.* (16).

The α-tocophereol content of microsomal preparations was determined by the HPLC method of Driskell *et al.* (17). Lipids were saponified by the addition of 10 N KOH and incubation at 70 C for 30 min. The samples were then extracted with hexane, dried under nitrogen, and reconstituted in 200 μl HPLC grade methanol. Retinyl acetate was used as an internal standard. Aliquots (50 or 100 μl) of methanol were injected into a Waters HPLC system (Milford, MA) equipped with a radial compression system containing a 10-μm C_{18} cartridge. The cartridge was eluted with a linear gradient of 90% methanol-10% water to 100% methanol over 15 min. The flow rate was 2.0 ml/min, and the eluent was monitored by absorbance at 290 mn.

The FFA composition of adrenal inner and outer zone microsomal preparations was determined by the method of Allen (18). Lipids were extracted from the microsomes with hexane under acidic conditions (1 M phosphoric acid), and then from the hexane with 0.5 M potassium hydroxide. Methyl group esterification of the fatty acids was done by incubation with methyl iodide at 65 C. The fatty acid methyl esters were then extracted with ethylene chloride (25 μl) under acidic conditions (0.1 M phosphoric acid). Aliquots (1 μl) were injected into a Shimadzu CG-4CM series gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD) equipped with a flame ionization detector set at 280 C and a Hewlett-Packard model 3390A recording integrator (Palo Alto, CA). A 1.7-m × 5-mm od glass column (id, 3 mm) packed with 10% Silar at 10 C on 100/120-mesh Gas-Chrom Q II (Alltech/Applied Sciences, Deerfield, IL) was eluted with nitrogen (flow rate, 30 ml/min) and a temperature program of 2 C/min from 130–220 C. Fatty acid methyl esters were identified on the basis of their retention times compared to authentic standards (Sigma Chemical Co.). Nervonic acid (C24:1) was used as an internal standard. All data were analyzed by Student's *t* test or Newman-Keuls test (multiple comparisons), as appropriate; $P < 0.05$ was considered significant.

Results

Adrenal microsomes from guinea pigs fed the tocopherol-deficient diet were almost totally depleted of α-tocopherol (Table 1). In control animals outer zone microsomes contained 4–5 times more α-tocopherol than inner zone preparations, as reported previously (8). Tocopherol deficiency did not affect cystolic ascorbic acid content (Table 1). Ascorbic acid levels in the inner and outer zones from control and tocopherol-deficient animals were similar.

Addition of ferrous ion (Fe^{2+}) to adrenal inner or outer zone microsomal preparations from control or tocopherol-deficient guinea pigs stimulated LP in a concentration-dependent manner (Fig. 1). In microsomes from control animals LP was significantly greater ($P < 0.05$) in the inner than in the outer zone at Fe^{2+} concentrations of 1×10^{-4} and 1×10^{-3} M. However, in tocopherol-depleted tissues MDA production was similar in the two zones. The latter was the result of tocopherol deficiency increasing LP in the outer zone but having little effect on LP in inner zone adrenal microsomes.

The time courses for Fe^{2+}-induced LP in adrenal inner and outer zone microsomal preparations from control and tocopherol-deficient guinea pigs are shown in Fig. 2. Tocopherol deficiency had little effect on the rate of MDA production by inner zone microsomes. In outer zone preparations, in contrast, tocopherol deficiency substantially increased the rate of LP. In outer zone microsomes from control animals, there was an initial lag period of 10–20 min, followed by a sharp increase in the rate of MDA production. The lag period was absent in outer zone microsomal incubates from tocopherol-deficient animals. As a result, MDA levels were significantly ($P < 0.05$) greater in the tocopherol-depleted than in control adrenal outer zone microsomal preparations at all incubation times.

The effects of tocopherol deficiency on the prooxidant (LP-inducing) actions of ascorbic acid in adrenal inner and outer zone microsomal preparations are shown in Fig. 3. In control microsomal preparations from both

TABLE 1. Cytosolic ascorbic acid and microsomal α-tocopherol concentrations in adrenal cortices from control and tocopherol (E)-deficient guinea pigs

Tissue	Ascorbic acid (mg/g tissue)	α-Tocopherol (μg/g tissue)
Inner zone		
Control	1.06 ± 0.06	30.8 ± 4.1
E-deficient	1.05 ± 0.05	ND
Outer zone		
Control	0.98 ± 0.07	136.0 ± 32.6[a]
E-deficient	1.02 ± 0.04	3.3 ± 2.7

Values are expressed as the mean ± SE of six experiments. ND, Not detectable (<1.0 μg).

[a] $P < 0.05$ *vs.* corresponding inner zone value.

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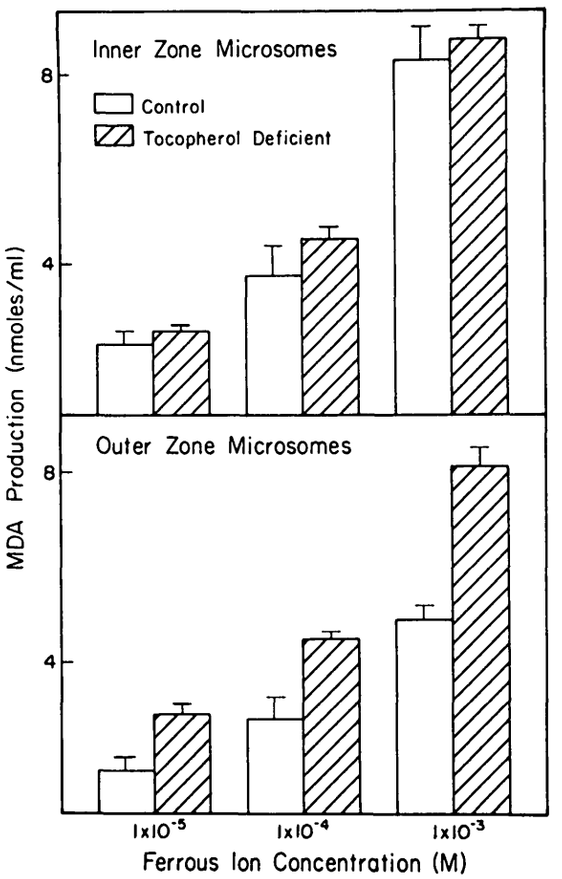

FIG. 1. Concentration-dependent effects of ferrous ion (Fe^{2+}) on MDA production by adrenal inner and outer zone microsomal preparations from control and tocopherol-deficient guinea pigs. Microsomes (0.1 mg protein/ml) were incubated for 30 min at 37 C with the concentrations of Fe^{2+} indicated, and MDA production was determined as described in *Materials and Methods*. Values are the means ± SE of four to six experiments.

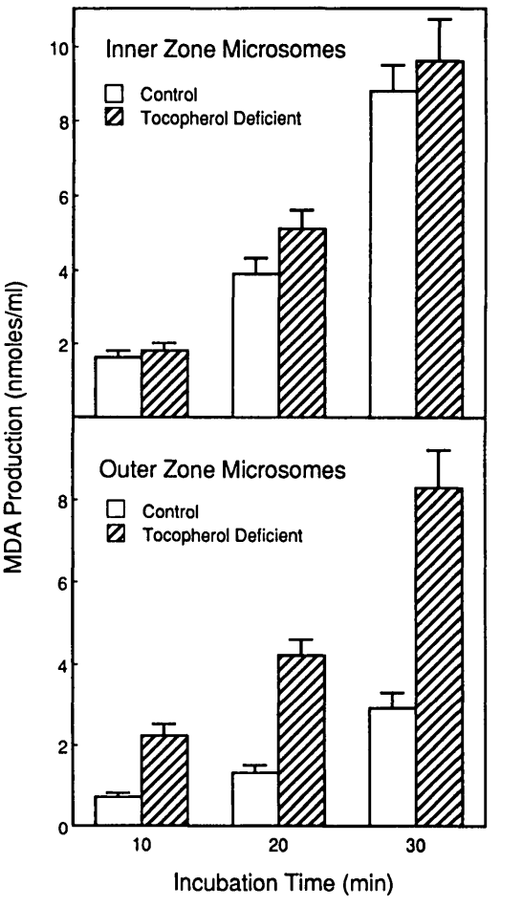

FIG. 2. Time courses for Fe^{2+}-induced MDA production by adrenal inner and outer zone microsomes from control and tocopherol-deficient guinea pigs. Microsomal preparations (0.1 mg protein/ml) were incubated with 1.0 mM ferrous sulfate at 37 C for the times indicated, and MDA production was determined as described in *Materials and Methods*. Values are the means ± SE of four to six experiments.

zones, LP increased with increasing ascorbic acid concentrations up to approximately 0.1 mM. MDA formation progressively declined with higher ascorbic acid concentrations until, at physiological ascorbic acid levels (~1–5 mM), very little LP occurred. At most concentrations of ascorbic acid, LP was greater with inner than outer zone microsomes ($P < 0.05$ at 1×10^{-4}, 2×10^{-4}, and 5×10^{-4} M ascorbic acid). Tocopherol deficiency increased ascorbic acid-induced MDA production to a greater extent in outer than in inner zone preparations (Fig. 3), virtually eliminating the differences between the two zones. Nonetheless, the dose-dependent nature of ascorbic acid-induced LP was similar to that in control microsomes; maximal stimulation was provided by approximately 0.1 mM ascorbic acid. However, in microsomes from tocopherol-deficient animals, even physiological concentrations of ascorbic acid stimulated LP, particularly in the outer zone.

To determine if changes in substrate availability contributed to the effects of tocopherol deficiency on adrenal LP, fatty acid concentrations in outer and inner zone microsomes were evaluated (Table 2). The fatty acid compositions of inner and outer zone microsomal preparations from control animals were very similar. The quantiatively major unsaturated fatty acids found in both zones were oleic acid (18:1), arachidonic acid (20:4), and linoleic acid (18:2). Tocopherol deficiency caused significant declines in both inner and outer zone linoleic acid and arachidonic acid concentrations (Table 2).

As previously reported (11), in microsomes from control animals 21-hydroxylase activity was greater in the inner than the outer zone, but 17α-hydroxylase and C-17,20 lyase activities were greater in the outer zone (Table 3). Tocopherol deficiency had no effect on 17α-hydroxylase or 21-hydroxylase activity in either zone or on C-17,20 lyase activity in inner zone microsomes. However, outer zone C-17,20 lyase activity was decreased by tocopherol deficiency, eliminating the zonal differences

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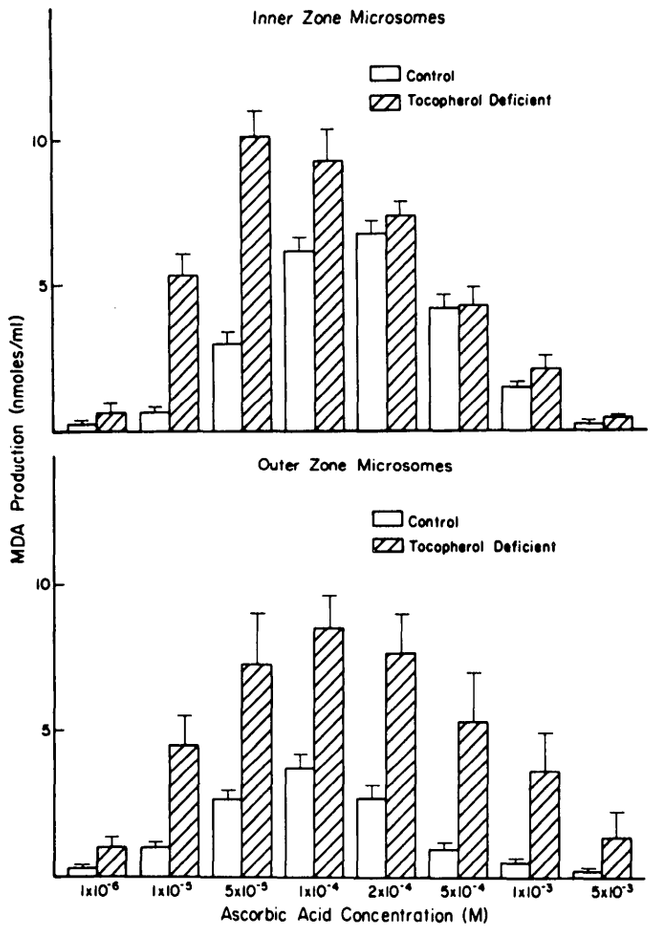

FIG. 3. Concentration-dependent effects of ascorbic acid on MDA production by adrenal inner and outer zone microsomes from control and tocopherol-deficient guinea pigs. Microsomal preparations (0.1 mg protein/ml) were incubated for 30 min at 37 C with the concentrations of ascorbic acid indicated, and MDA production was determined as described in *Materials and Methods*. Values are the means ± SE of four to six experiments.

in enzyme activity. Although the 17α-hydroxylase and C-17,20 lyase reactions appear to be catalyzed by a single cytochrome P-450 isozyme, the two activities can be independently modulated by various factors (see Refs. 19 and 20 for reviews).

Discussion

We previously reported that microsomal and mitochondrial preparations from the zona reticularis (inner zone) of the guinea pig adrenal cortex were far more susceptible to LP than were those from the outer zones of the gland (8, 9). The inverse relationship between LP and membrane content of α-tocopherol in the two zones suggested that differences in antioxidant levels might be responsible for the regional differences in LP. The results presented in this report indicate that these zonal differences in microsomal LP are eliminated by depletion of adrenal α-tocopherol. The fatty acid profiles of inner and outer zone microsomes are virtually identical, suggesting that substrate availability for LP is similar in the two zones. Thus, the regional differences in microsomal LP seem fully attributable to the differences in α-tocopherol content.

The adrenal cortex contains higher concentrations of ascorbic acid and α-tocopherol than any other organ, but their physiological role(s) in the adrenal, if any, has not been resolved (see Ref. 7 for review). It has been proposed that ascorbic acid and α-tocopherol interact as part of an electron transport system to terminate free radical chains (21). However, ascorbic acid can exert either prooxidant or antioxidant effects, depending on various factors, including the concentration employed (7). Our results and prior observations by Kitabchi and co-workers (4, 22–24) indicate that subphysiological concentrations of ascorbic acid exert potent prooxidant effects on adrenal membrane preparations, initiating LP. However, concentrations in the physiological range (~1–10 mM) had little or no effect on LP in microsomes from control guinea pigs. In tocopherol-deficient microsomes, in contrast, even the higher concentrations of ascorbic acid initiated substantial amounts of LP. Nathans and Kitabchi (22) previously noted that ascorbic acid inhibited steroidogenesis in isolated adrenal cells from tocopherol-deficient rats, but not in those from controls, and suggested that peroxidative damage occurred in the tocopherol-depleted cells. Thus, α-tocopherol seems to diminish the prooxidant effects of ascorbic acid and may serve to protect the adrenocortical cell from ascorbic acid-induced LP as well as other types of oxidative damage.

Although LP has been demonstrated in a variety of adrenal preparations *in vitro*, its significance *in vivo* remains unclear. Ito (25) found large amounts of lipofuscin, a pigment thought to be an end product of LP (26), concentrated in the zona reticularis of the guinea pig adrenal cortex. The site of lipofuscin deposition *in vivo*, therefore, coincides with the region of greatest peroxidative activity *in vitro*. In rats, α-tocopherol deficiency has been found to increase adrenal lipofuscin content, also suggesting the occurrence of LP *in vivo* (27). Our observations provide the most direct evidence to date for *in vivo* adrenal LP. The decreases in linoleic acid (18:2) and arachidonic acid (20:4) in microsomes from tocopherol-deficient guinea pigs strongly suggest that peroxidative losses occurred *in vivo*. The initiation of LP in adrenal microsomes *in vitro* decreases the concentrations of the same two fatty acids (28). These observations indicate that at least under conditions of tocopherol deficiency, LP occurs in the adrenal cortex. The data also emphasize the importance of α-tocopherol as an adrenal antioxidant; adrenal ascorbic acid levels were unaltered by tocopherol deficiency, but were apparently

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TABLE 2. FFA concentrations in adrenocortical inner and outer zone microsomes from control and tocopherol (E)-deficient animals

C:DB[a]	Fatty acid conc. (μg/g tissue)			
	Inner zone		Outer zone	
	Control	E-deficient	Control	E-deficient
14:0	0.6 ± 0.1 (1)	0.3 ± 0.1 (<1)	0.5 ± 0.1 (1)	0.6 ± 0.1 (1)
16:0	16.3 ± 2.1 (22)	12.1 ± 1.8 (22)	21.4 ± 3.1 (30)	16.7 = 1.2 (29)
16:1	1.1 ± 0.2 (1)	0.8 ± 0.2 (1)	1.1 ± 0.2 (2)	1.4 ± 0.2 (2)
18:0	10.8 ± 0.9 (14)	8.2 ± 1.3 (15)	8.4 ± 1.1 (12)	8.7 ± 0.3 (15)
18:1	23.8 ± 2.0 (32)	22.4 ± 3.9 (41)	18.3 ± 2.8 (25)	17.8 ± 0.9 (31)
18:2	6.3 ± 0.9 (8)	0.7 ± 0.2 (1)[b]	6.9 ± 1.1 (10)	1.7 ± 0.2 (3)[b]
18:3	0.9 ± 0.2 (1)	ND	0.3 ± 0.1 (<1)	0.1 ± 0.0 (<1)
20:4	15.3 ± 0.5 (20)	9.1 ± 2.1 (17)[b]	14.9 ± 1.6 (21)	10.3 ± 0.8 (18)[b]

Values are expressed as the mean ± SE of eight experiments; numbers in parentheses indicate the percentage of the total fatty acid content. ND, Not detectable (<50 ng).

[a] C, Number of carbon atoms; DB, number of double bonds in fatty acid chain.

[b] $P < 0.05$ *vs.* corresponding control value.

TABLE 3. Monooxygenase activities in inner and outer zone microsomes from control and tocopherol (E)-deficient guinea pigs

Enzyme activities (nmol/min·mg protein)	Inner zone		Outer zone	
	Control	E-deficient	Control	E-deficient
21-Hydroxylase	5.2 ± 0.5	4.6 ± 0.3	1.8 ± 0.2[a]	2.5 ± 0.5[a]
17α-Hydroxylase	4.0 ± 0.5	4.5 ± 0.3	12.2 ± 2.0[a]	10.5 ± 1.0[a]
C-17,20 Lyase	1.5 ± 0.1	1.6 ± 0.2	4.1 ± 0.3[a]	2.1 ± 0.3[b]

Values are expressed as the mean ± SE of four to six animals.

[a] $P < 0.05$ *vs.* corresponding inner zone value.

[b] $P < 0.05$ *vs.* corresponding control value.

insufficient to prevent the peroxidative loss of microsomal fatty acids. In fact, as noted above, in the absence of α-tocopherol, ascorbic acid might exert prooxidant effects and contribute to the initiation of LP.

The functional significance of adrenal LP remains to be resolved. A number of investigators have demonstrated that LP can effect the degradation of several steroidogenic enzymes, apparently by destruction of cytochrome P-450 (5, 29–32). In the guinea pig adrenal cortex the region of high *in vitro* peroxidative activity (inner zone) is associated with low activities of those enzymes most susceptible to LP (13, 15), including C-17,20 lyase, and very little steroid secretion (33, 34). Interestingly, tocopherol deficiency decreased C-17,20 lyase activity in the outer zone, eliminating the zonal differences in enzyme activity. The results suggest that LP *in vivo* can influence steroidogenesis and that regional differences in LP may contribute to some of the functional differences between the different zones of the adrenal cortex, as proposed by Hornsby *et al.* (7, 9). However, further investigations are clearly needed to determine the extent to which LP occurs in the normal (tocopherol-sufficient) adrenal cortex *in vivo* and the factors affecting adrenal LP. Such studies should help to establish the role, if any, of LP in the overall modulation of adrenocortical function.

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