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Effects of Vitamin A Deficiency on Selected Xenobiotic-metabolizing Enzymes and Defenses against Oxidative Stress in Mouse Liver

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ABSTRACT. Male and female C57Bl/6 mice were rendered vitamin A-deficient, and the effects of this deficiency on certain xenobiotic-metabolizing enzymes and defenses against oxidative stress were examined. Vitamin A deficiency significantly increased the levels of DT-diaphorase, glutathione transferase, and catalase in the hepatic cytosolic fraction from male mice (5.2-, 1.6-, and 3.5-fold, respectively), as well as from female mice (4.8-, 3.3-, and 2.4-fold, respectively). In the hepatic mitochondrial fraction (containing peroxisomes) from male animals, the activities of urate oxidase and catalase were increased 3.4- and 1.7-fold, respectively. The activity of catalase in the mitochondrial fraction from female mice was not affected by vitamin A deficiency, whereas the activity of peroxisomal urate oxidase was increased 2.9-fold. The hepatic level of ubiquinone was increased somewhat. The significance of the increases observed here is presently unclear, but it may be speculated that vitamin A and/or its metabolites are somehow involved in the down-regulation of these proteins. Another possibility is that these enzymes are increased as a result of hepatic oxidative stress caused by vitamin A deficiency. However, vitamin A deficiency had no effect on the activity of superoxide dismutase in this study, whereas the activity of glutathione peroxidase was slightly decreased (27%) in the hepatic cytosolic fraction from male mice. In addition, the hepatic level of α-tocopherol was decreased dramatically in the vitamin A-deficient animals. BIOCHEM PHARMACOL **59**;4:377–383, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. vitamin A deficiency; mouse liver; xenobiotic-metabolizing enzymes; antioxidant defense; oxidative stress

Vitamin A and its metabolites play important roles in cell differentiation, growth, reproduction, and vision [1, 2]. It has become clear that retinoids mediate their effects through nuclear receptors [3]. The RXR§ (α, β, and γ) and the RAR are involved in genetic regulation of various cellular processes [3–5]. Studies on the action of these receptors have been performed primarily using molecular biological and other *in vitro* systems. Such studies have demonstrated that RXRα can interact not only with RAR, but also with the vitamin D_3 receptor, the thyroid hormone receptor, and the PPAR. It has, for example, been shown that a heterodimer of PPAR and RXRα is involved in activation of the peroxisomal acyl-CoA oxidase gene [6, 7]. This latter finding has been corroborated *in vivo* in our laboratory using vitamin A-deficient mice [8]. This same study also revealed that vitamin A deficiency itself causes increases in the activities of hepatic catalase and cytochrome P-4504A. To further document the effects of vitamin A deficiency, the present study concentrates on selected xenobiotic-metabolizing enzymes and defenses against oxidative stress in mouse liver.

Glutathione transferases catalyze the conjugation of glutathione to an electrophilic compound, thereby generally rendering the electrophile considerably less harmful and more water-soluble (for a review, see Ref. 9). Glutathione reductase catalyzes the reduction of oxidized glutathione and thereby helps maintain the cellular level of reduced glutathione, which is important for the general redox state of the cell and, in particular, xenobiotic metabolism [10]. Superoxide dismutase, catalase, and glutathione peroxidase are enzymes that protect the cell from oxidative damage. The dismutation of the highly reactive superoxide anion to O_2 and H_2O_2 is catalyzed by superoxide dismutases (for a review, see Ref. 11). Catalase is involved in the inactivation of hydrogen peroxide. Degradation of hydroperoxides is catalyzed by glutathione peroxidase (for a review, see Ref. 12).

Oxidative stress may give rise to, among other things, quinones derived from xeno- and/or endobiotics. Many such quinones are reduced in a 2-electron step by DT-diaphorase [13] and thereafter conjugated for excretion

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§ *Abbreviations:* RXR, retinoid X receptor; RAR, retinoic acid receptor; PPAR, peroxisome proliferator-activated receptor.

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TABLE 1. Hepatic vitamin A levels in mice fed a normal or vitamin A-deficient diet

Diet	Hepatic vitamin A (μg/g liver)
Normal (24,000 IU vitamin A acetate/kg)	307 ± 33*
Vitamin A-deficient	<5†

*Mean value ± standard deviation for four animals.
†Not detectable in 4 vitamin A-deficient animals.

from the cell. Thus, DT-diaphorase prevents quinones from undergoing redox cycling, a process which gives rise to reactive oxygen species. Urate oxidase is a peroxisomal enzyme that catalyzes the oxidation of allantoin (for a review, see Ref. 14). This enzyme is, however, not present in peroxisomes from all tissues and species [15]. Urate oxidase is involved in hydrogen peroxide production. This may seem strange, since hydrogen peroxide is a reactive species of oxygen and can give rise to even more reactive species. However, peroxisomes are rich in catalase, which inactivates hydrogen peroxide. Tocopherol and ubiquinone in reduced form are the most effective lipid-soluble antioxidants protecting cellular membranes from oxidative damage [16]. Tocopherols are dietary antioxidants, whereas ubiquinone is an endogenous antioxidant synthesized by animal cells *de novo*. Tissue levels of ubiquinone are regulated under conditions of oxidative stress [17].

In this present study, the effects of vitamin A deficiency on the hepatic levels of these enzymes and on tocopherols and ubiquinone were investigated. Vitamin A-deficient mice had not previously been subjected to such a study.

MATERIALS AND METHODS

Chemicals

Menadione, 1-chloro-2,4-dinitrobenzene, and hydrogen peroxide (30%) were purchased from Merck; cytochrome *c*, dicoumarol, glutathione reductase, glutathione, *N*-ethylmaleimide, NAD^+, and BSA from Sigma; and NADPH from Boehringer Mannheim. All other chemicals were of reagent grade and were also procured from common commercial sources.

Animals and Treatment

Male C57Bl/6 mice (ALAB) were rendered vitamin A-deficient according to Smith and co-workers [18] by supplying pregnant mice with a vitamin A-deficient diet during the last two weeks of pregnancy and feeding the offspring this same diet after weaning. The vitamin A-deficient offspring were utilized for studies at 10–12 weeks of age, at which time the vitamin A level in the livers was less than 1.6% of the control value (Table 1) [8]. After longer periods of vitamin A deficiency, mice begin to show signs of toxicity [18]. All mice were housed in steel cages with a 12-hour light–dark cycle at 25°. They were given free access to a laboratory vitamin A-deficient diet (Vitamin A test diet) from USB or a vitamin A-adequate diet (Beekay rat and mouse standard diet, ALAB). The diets were supplied as pellets (the chow was mixed with water, baked, and thereafter dried overnight in a ventilated hood).

Subcellular Fractionation

At the age of 10–12 weeks, the mice were killed by cervical dislocation and the livers removed, freed from the gallbladder, and then rinsed in ice-cold 0.25 M sucrose solution. The livers were weighed and homogenized individually at 440 rpm in ice-cold 0.25 M sucrose using 4 up-and-down strokes of a Potter-Elvehjem homogenizer. More sucrose was added to give a 20% homogenate, which was subsequently centrifuged at 600 g_{av} for 10 min. The resulting supernatant was centrifuged at 10,000 g_{av} for 10 min to give a "mitochondrial" pellet containing primarily mitochondria, peroxisomes, and lysosomes. This pellet was resuspended and washed twice by centrifugation in sucrose. Finally, this fraction (referred to as the mitochondrial fraction) was resuspended in sucrose to give a volume of 2.0 mL. The supernatant from the original 10,000 g_{av} centrifugation was further centrifuged at 105,000 g_{av} for 60 min and the high-speed supernatant (cytosol, approx. 4 mL) saved. Studies on marker enzymes have shown that mitochondrial fraction prepared in this manner contains 50% of the peroxisomes, microsomal fraction 67% of the endoplasmic reticulum, and high-speed supernatant 92% of the cytosol [19].

Assays

The enzyme assays were performed with saturating substrate concentrations and conditions of linearity with time and protein [with the exception of catalase, which cannot be saturated under reasonable conditions (12)]. The activity of *catalase* was monitored spectrophotometrically at 240 nm with hydrogen peroxide as substrate [20]. *DT-diaphorase* was measured spectrophotometrically at 550 nm by following the NADPH-dependent, dicoumarol-sensitive reduction of cytochrome *c*, which continuously reoxidizes menadione [13]. *Glutathione reductase* was quantitated spectrophotometrically by following the disappearance of NADPH at 340 nm [10]. *Cytosolic glutathione transferase* was measured spectrophotometrically at 340 nm with 1-chloro-2,4-dinitrobenzene as substrate [21]. *Microsomal gluthathione transferase* was assayed in the same manner, but with *N*-ethylmaleimide as activator [22]. *Superoxide dismutase* was measured by fluorimetric determination of the hydrogen peroxide produced [23]. *Glutathione peroxidase* was quantified spectrophotometrically at 340 nm as the disappearance of NADPH in a glutathione reductase-coupled reaction [24]. Protein concentrations were determined by the method of Lowry *et al.* [25] with BSA as standard.

Tocopherol and Ubiquinone Analysis

Samples of homogenate or subfractions (400 μL) were supplemented with internal standards (0.8 nmol δ-tocoph-

TABLE 2. Effects of vitamin A deficiency on various enzymes in male C57Bl/6 mouse liver

	Vitamin A-adequate	Vitamin A-deficient (% of control)	
Cytosolic fraction			
DT-diaphorase*	17.8 ± 2	92 ± 27	(517)§
Catalase†	1.68 ± 0.5	5.89 ± 1.0	(351)‖
Glutathione transferase*	205 ± 19	331 ± 68	(161)¶
Glutathione reductase*	2.70 ± 0.33	2.47 ± 0.28	(91)
Superoxide dismutase‡	0.203 ± 0.025	0.165 ± 0.01	(81)
Glutathione peroxidase*	34.4 ± 5.8	25.1 ± 2.2	(73)¶
Mitochondrial fraction			
Urate oxidase‡	0.596 ± 0.06	2.05 ± 0.58	(344)§
Catalase†	15.3 ± 1.6	25.7 ± 4.3	(168)§
Superoxide dismutase‡	0.022 ± 0.005	0.016 ± 0.004	(73)
Microsomal fraction			
Glutathione transferase‡	145 ± 42	97 ± 48	(67)

The vitamin A-adequate values are means ± SD for three animals and the vitamin A-deficient values are means ± SD for four animals.
*μmol per min and g liver, †mmol per min and g liver, ‡nmol per min and g liver §$P < 0.01$, ¶$P < 0.05$, ‖$P < 0.001$ compared to the values for vitamin A-adequate mice.

erol and 0.4 nmol ubiquinone-6) and extracted with a mixture of 3.6 mL methanol and 2.4 mL petroleum ether (boiling point 40–60°) [26]. After phase separation, the upper solvent phase was transferred to another tube and evaporated under N_2. The residue was dissolved in 50 μL chloroform/methanol (2:1) and injected onto an HPLC. Ubiquinones and tocopherols were separated using a reversed-phase Microsorb C-18 column (3 μm, 4.6 mm × 10 cm, Rainin Instrument Co.) [27]. A binary convex gradient with a flow rate of 1.5 mL/min from the initial 90% methanol/water (9:1) in pump A to 100% methanol/2-propanol/hexane (2:1:1) in pump B was employed. Both solvent systems contained 20 mM lithium perchlorate as electrolyte. The lipids were monitored both with an electrochemical detector (applying a potential of +0.7 V vs Ag/AgCl) and with an UV detector (at 210 nm).

Hepatic Levels of Vitamin A

Liver samples were sent to the Swedish Bureau of Veterinary Medicine (SVA), Uppsala, Sweden, for vitamin A analysis. After alkaline hydrolosis followed by extraction with hexane, retinoids were separated by HPLC on a straight-phase silica column. The mobile phase used was hexane: 1,4-dioxane, 49:1 (v/v). Retionol served as an external standard.

Statistical Analysis

Data are given as means ± standard deviations and the results of the Student's *t*-test are presented where appropriate.

RESULTS

Liver Somatic Index and Protein Concentrations

No significant differences in liver and body weights were observed in mice administered the vitamin A-deficient diet compared with normal mice (data not shown). Accordingly, the liver somatic index (liver weight/body weight) was not affected by vitamin A deficiency. Vitamin A deficiency had no significant effect on the protein concentrations in any of the subcellular fractions examined here (data not shown).

Hepatic Enzyme Activities in Vitamin A-deficient C57Bl/6 Male Mice

Cytosolic DT-diaphorase, catalase, and glutathione transferase, as well as peroxisomal urate oxidase and catalase were all significantly increased to different extents (1.6- to 5.2-fold) in the livers of vitamin A-deficient mice (Table 2). On the other hand, cytosolic glutathione reductase and superoxide dismutase, mitochondrial superoxide dismutase and microsomal glutathione transferase in mouse liver were not significantly affected by vitamin A deficiency (Table 2). The activity of glutathione peroxidase in the hepatic cytosolic fraction from male mice was slightly decreased (27%) by vitamin A deficiency.

Effects of Vitamin A Deficiency on Enzymes in the Livers of Female C57Bl/6 Mice

Experiments employing female vitamin A-deficient mice demonstrated inductions of the same enzymes as for male mice (Fig. 1), with the exception of peroxisomal catalase, which was not increased in the female animals.

Effects of Vitamin A Deficiency on Tocopherol and Ubiquinol Levels in the Livers of C57Bl/6 Male Mice

The lipid-soluble antioxidants α- and γ-tocopherol, as well as the reduced forms of ubiquinone-9 and -10, were well separated on the HPLC chromatogram using an electrochemical detector, as shown in Fig. 2. In the liver homogenate from vitamin A-deficient mice, the α-tocopherol peak was almost totally eliminated, whereas the level of γ-tocopherol was unchanged. The levels of both reduced

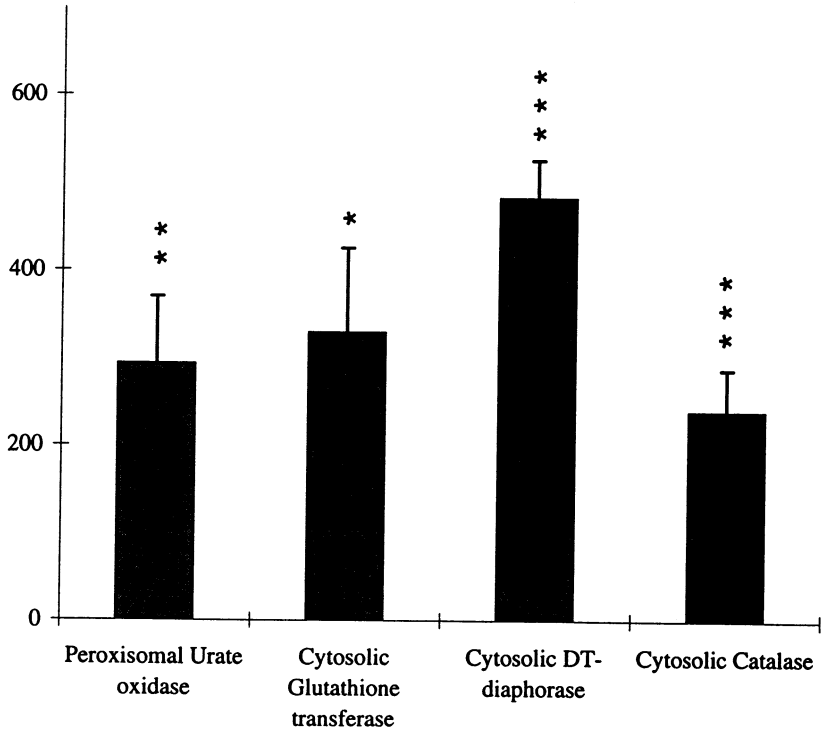

FIG. 1. Effects of vitamin A deficiency on urate oxidase in the mitochondrial fraction and on glutathione transferase, DT-diaphorase, and catalase in the cytosolic fraction from female mouse liver. The control activities (i.e. 100%) were 0.879 ± 0.132 nmol, 76.2 ± 16.8 μmol, 27.8 ± 5.8 μmol, and 2.25 ± 0.34 mmol per min and g liver, respectively. The vitamin A-adequate values are means ± SD for three animals, except for catalase, which is the mean ± SD for seven animals. The vitamin A–deficient values are means ± SD for four animals, except for catalase, which is the mean ± SD for five animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the values for vitamin A-adequate mice.

ubiquinone-9 and ubiquinone-10 were increased. No differences in the amounts of the oxidized forms of ubiquinone were observed by simultaneous monitoring using a UV detector (not shown). The amount of ubiquinone (ubiquinone-9 + ubiquinone-10) in the homogenate and in the mitochondrial fraction from livers of vitamin A-deficient mice was not significantly increased, while in the microsomal fraction the content of ubiquinone was highly elevated (3.4-fold) (Table 3). A dramatic decrease in α-tocopherol was detected in the nuclear and mitochondrial fractions and in the homogenate, while no change was observed in the microsomal fraction (Table 4). No change in the γ-tocopherol amount was found in any fraction. In the microsomal fraction, the level of this antioxidant was below the limit of detection.

DISCUSSION

The major ligand for the RXR is 9-*cis*-retinoic acid [28]. The RAR is activated mainly by all-*trans*-retinoic acid [29, 30]. These ligands are naturally occurring forms of vitamin A and play important roles in regulation of many genes. However, the involvement of the retinoic acids, RXR and RAR in gene regulation seems to be rather complex. It has, for example, been reported that RXR interacts with the thyroid hormone receptor and with the vitamin D_3 receptor, as well as with PPAR [5, 6]. No reports on the effects of vitamin A deficiency on xenobiotic-metabolizing enzymes and oxidative defenses in mouse liver have been reported previously. In rat, only a few such studies have been presented [31–33]. The activities of superoxide dismutase and glutathione reductase in the rat liver have been reported to be unaffected by vitamin A deficiency [31, 32]. Gupta *et al.* [33] reported a significant increase in glutathione *S*-transferase in the livers of vitamin A-deficient rats, whereas Ayalogu *et al.* [32] observed no effect on this same enzyme. Catalase activity was previously reported to be reduced in the livers of vitamin A-deficient rats [31].

The present study reveals that vitamin A deficiency significantly increased levels of DT-diaphorase, glutathione transferase, and catalase in the hepatic cytosolic fraction from both male and female mice. In the hepatic mitochondrial fraction from male mice, the activities of urate oxidase and catalase were increased. The activity of catalase in the mitochondrial fraction from female mice was not affected by vitamin A deficiency, whereas peroxisomal urate oxidase was increased. The significance of the increases observed here is presently unclear, but it may be speculated that vitamin A and/or its metabolites are somehow involved in the down-regulation of these proteins. Another possibility is that vitamin A deficiency results in oxidative stress. The induction of certain antioxidant enzymes observed in the livers of vitamin A-deficient mice may, thus, be a result of increased levels of reactive oxygen species. However, vita-

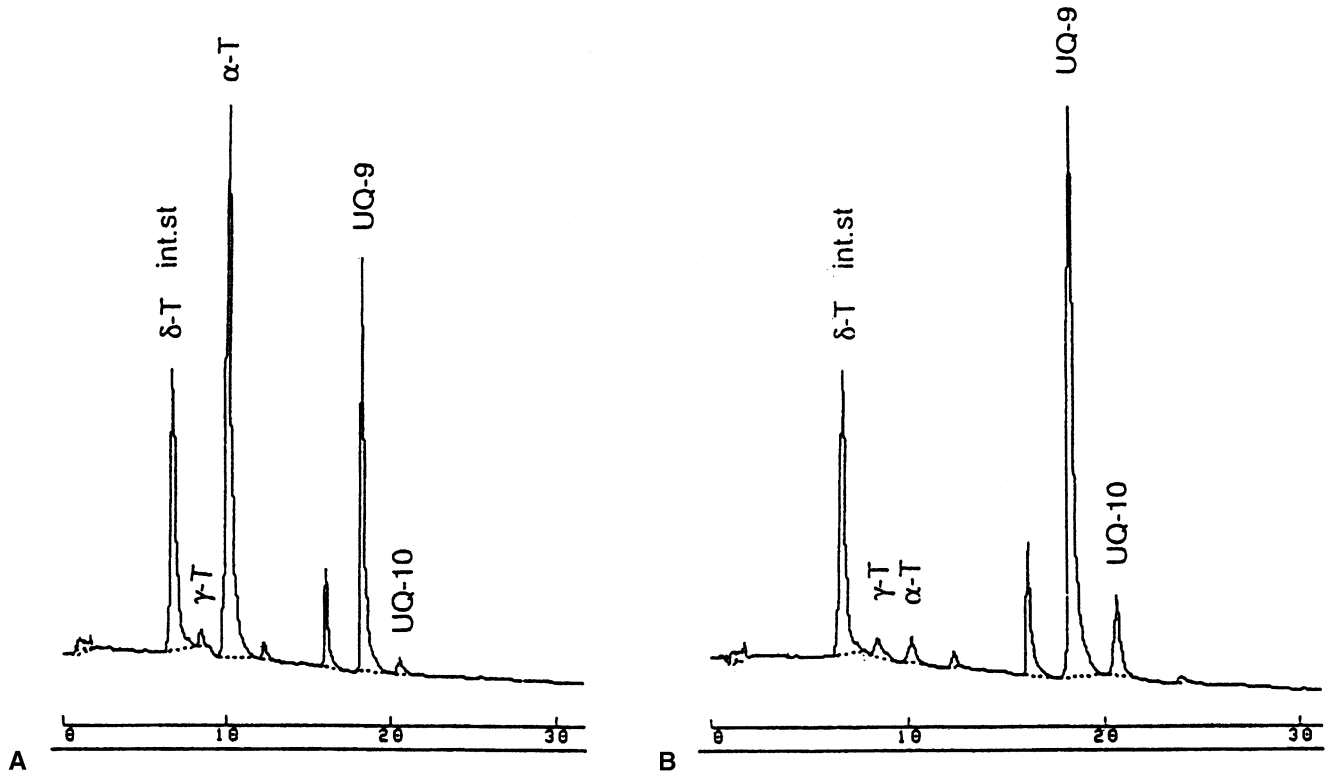

FIG. 2. HPLC chromatogram of the lipid-soluble antioxidants α- and γ-tochopherol (α-T and γ-T) and the reduced forms of ubiquinone-9 and -10 (UQ-9 and UQ-10) in homogenate from A) vitamin A-adequate and B) vitamin A-deficient mouse liver. Int.st, internal standard.

min A deficiency had no effect on the activity of superoxide dismutase in this study, and the activity of glutathione peroxidase in the hepatic cytosolic fraction from the male mice was slightly decreased by vitamin A deficiency.

The antioxidant form (reduced form) of ubiquinone was also increased in the microsomal fraction of vitamin A-deficient mouse liver. This could be the result of increased levels of reactive oxygen species. Other studies have shown that treatment of rats with peroxisome proliferators results in elevated tissue levels of ubiquinone [34–36]. The antioxidant level of α-tocopherol is 10 times higher than that of γ-tocopherol. In our mice, the level of γ-tocopherol was unchanged by vitamin A deficiency, whereas α-tocopherol was almost totally depleted in total homogenate and in the nuclear and microsomal fractions. It can be speculated that the α-tocopherol taken up from the diet is consumed as an antioxidant at a high rate in vitamin A-deficient mouse liver. However, in contrast to the case in mouse, the level of α-tocopherol has been reported to be strongly enhanced in vitamin A-deficient rat [37]. This discrepancy may reflect species differences or, possibly, difficulties in rendering experimental rodents truly vitamin A-deficient [8]. Obviously, this striking difference remains to be explained, as does the increase in catalase activity in vitamin A-deficient mice, in contrast to a corresponding decrease in rats. In order to establish whether or not vitamin A deficiency results in oxidative stress, studies on lipid peroxidation and DNA damage in vitamin A-deficient mouse are required.

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TABLE 3. Effect of vitamin A deficiency on the level of ubiquinone in male C57Bl/6 mouse liver

	Vitamin A-adequate	Vitamin A-deficient (% of adequate)	
	(nmol/g liver)		
Homogenate	66.0 ± 10.2	87.5 ± 32.6	(133)
Mitochondrial fraction	11.4 ± 3.63	16.1 ± 2.38	(141)
Microsomal fraction	3.20 ± 0.45	10.7 ± 4.33*	(336)

The values are means ± SD for three animals.
*$P < 0.05$ compared to the values for vitamin A-adequate mice.

TABLE 4. Effects of vitamin A deficiency on the levels of α- and γ-tocopherol in male C57Bl/6 mouse liver

	Vitamin A-adequate	Vitamin A-deficient (% of adequate) (nmol/g liver)	
Mitochondrial fraction			
α-Tocopherol	0.187 ± 0.047	0.019 ± 0.006*	(10)
γ-Tocopherol	0.127 ± 0.015	0.150 ± 0.056	(118)
Microsomal fraction			
α-Tocopherol	0.0080 ± 0.0057	0.0115 ± 0.0041	(145)
γ-Tocopherol	ND	ND	
Nuclear fraction			
α-Tocopherol	6.38 ± 2.55	1.53 ± 0.98†	(24)
γ-Tocopherol	0.41 ± 0.20	0.52 ± 0.28	(127)
Homogenate			
α-Tocopherol	25.4 ± 6.09	2.09 ± 0.42*	(8)
γ-Tocopherol	1.29 ± 0.07	1.48 ± 0.18	(115)

The values are means ± SD for three animals.
*$P < 0.01$ compared to the values for vitamin A-adequate mice; †$P < 0.05$; ND = not detectable.

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