Effect of Dietary Deficiency and Supplementation with all-rac-α-Tocopherol on Hepatic Content in Rats

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ABSTRACT We studied the effect of dietary deficiency and supplementation with vitamin E in the form of all-rac-α-tocopherol in relation to the content of α-tocopherol in parenchymal and nonparenchymal liver cells. The cells were isolated by centrifugal elutriation from rats fed diets containing normal, low or high amounts of vitamin E. The parenchymal cells contained about 90% of total hepatic α-tocopherol content in rats fed a nonpurified diet (reference group). However, the Kupffer and the endothelial/stellate cells contained four and two times more α-tocopherol, respectively, than the parenchymal cells per milligram of cell protein. When the rats were deprived of vitamin E for 8 wk, the content of α-tocopherol in parenchymal cells was reduced to 30% of values obtained from rats fed the nonpurified diet, and nonparenchymal cells contained very low levels of α-tocopherol (< 5% of reference values). A diet enriched in vitamin E resulted in a small but significant increase in the content of α-tocopherol in parenchymal cells but in small changes in nonparenchymal cells compared to the reference diet. Accordingly, the parenchymal cells may have storage capacity for α-tocopherol. The light mitochondrial and microsomal fractions contained high amounts of α-tocopherol. These fractions were subdivided by density gradient centrifugation to examine the α-tocopherol content in different cell organelles. The lysosomes and the Golgi apparatus were found to contain high levels of α-tocopherol, whereas peroxisomes contained small amounts. J. Nutr. 121: 1208-1213, 1991.

INDEXING KEY WORDS:
- all-rac-α-tocopherol
- elutriation
- diet
- subcellular fractionation
- rats

effect of therapeutic supplementation with vitamin E is well documented for several malabsorption states and in premature infants [5].

In serum, α-tocopherol is evenly distributed between VLDL and HDL in nonfasted rats. Newly absorbed α-tocopherol is transported in association with chylomicrons in lymph and blood [6, 7] before a significant hepatic uptake of α-tocopherol occurs. Most α-tocopherol is taken up by the parenchymal cells, but nonparenchymal cells also have the ability to accumulate α-tocopherol [7, 8]. In a previous study we observed that the amount of α-tocopherol was reduced in the hepatocytes of rats fed ethanol chronically, possibly because of increased oxidative stress from the ethanol [9]. The distribution of α-tocopherol among different types of liver cells as a function of varying intake has so far not been studied.

In this paper we present data on the content of α-tocopherol in different types of liver cells isolated by elutriation from rats fed nonpurified diet, a diet deficient in vitamin E or a diet enriched with vitamin E. Furthermore, the subcellular distribution of α-tocopherol in the liver of rats fed a nonpurified diet as well as marker enzymes is reported. The crude microsomal and light mitochondrial fractions were further separated by density gradient centrifugation to obtain the Golgi apparatus, lysosomes and peroxisomes. To date, the amount of α-tocopherol in these cell organelles has only been studied in rats receiving about fivefold greater amount of vitamin E than in the current study.

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2Fellow of the Norwegian Council on Cardiovascular Disease.
MATERIALS AND METHODS

Chemicals. All-rac-α-Tocopherol, UDP-galactose, NADH (sodium salt) and Piske-Subbarow reducer reagents were obtained from Sigma (St. Louis, MO). UDP-[14C]galactose was provided by New England Nuclear Products (E. I. Du Pont, Dreieich, Germany). [7-14C]Tyramine-HCl was provided by Amersham International (Buckinghamshire, England), and tyramine was obtained from Fluuka AG (Switzerland). All other chemicals were of high purity commercial grade.

Animals. Male albino Wistar rats were purchased from Mellegaard Hansens Avilaboratorium (Ejby, Denmark). The rats (270–340 g) were used in studies of subcellular localization of α-tocopherol and as controls in the elutriation experiments. They were allowed ad libitum access to a nonpurified, pelleted diet (reference diet) containing ~30 mg vitamin E/kg (Table 1) (Arex Mellesentralen, Oslo, Norway) and water until they were killed by decapitation about 10 d after arrival. The rats fed the reference diet received ~0.5 mg vitamin E per day during this 10-d period. The vitamin E–depleted rats received a vitamin E–deficient diet providing ~0.1 mg vitamin E per day (Arex Mellesentralen) during the 8 wk prior to the experiments. The vitamin E–deficient diet had the same composition as the reference diet. The animals in the vitamin E–enriched group were fed the reference diet supplemented with 30 mg vitamin E per day for 5 d before termination of the experiment. The vitamin supplement was given daily in 300 μL soybean oil via duodenal tube.

Preparation of liver subcellular fractions. After the rats were killed, their livers were quickly removed, finely minced and homogenized in ice-cold buffer (pH 6.5) containing 0.25 mol/L sucrose and 1 mM EDTA, using a Potter-Elvehjem homogenizer with a loose-fitting pestle to avoid damage to the fragile subcellular organelles (e.g., peroxisomes). The subcellular fractionation was performed as previously described (10). The light mitochondrial fraction was subfractionated on a linear Nycodeenz gradient, and the microsomes were fractionated on a discontinuous density sucrose gradient (10).

Preparation of liver cells. Total liver cell suspensions were prepared by a modified collagenase perfusion technique (7). Parenchymal cells were isolated from the total liver cell suspension by differential centrifugation and elutriation in a JE-6 elutriator rotor (Beckman Instruments, Palo Alto, CA) at 200 × g at a flow rate of 20–45 mL/min (11). Nonparenchymal liver cells were separated from the total liver cell suspension after incubation with 0.5% (wt/v) pronase for 1 h at 37°C. This treatment selectively destroys parenchymal cells. The nonparenchymal cell suspension was separated further by centrifuge elutriation at 200 × g with a flow rate of 20 mL/min. The combined fraction of endothelial and stellate cells was elutriated at 740 × g with a flow rate of 11.3–22.7 mL/min, and the Kupffer cells were elutriated at a flow rate of 22.7–53.0 mL/min at 740 × g (12). Cells were then concentrated and washed twice in phosphate-buffered saline. The Kupffer cells were identified cytochemically by a positive peroxidase reaction. The majority of cells in the combined fraction of endothelial/stellate cells were endothelial cells as determined by fluorescence microscopy, whereas only 4 ± 2% of the cells were stellate cells in the fractions isolated from livers of rats fed the reference diet.

Analytical procedures. Extraction of α-tocopherol was performed as previously described (7), then determined by HPLC combined with fluorometry (7). Protein content of the samples was determined as described by Lowry et al. (13), using bovine serum albumin as a standard.

Catalase (EC 1.11.1.6, marker enzyme for peroxisomes, L-fraction) was monitored spectrophotometrically by measuring the breakdown of H2O2 during incubation at 0°C. The amount of H2O2 was measured by its reaction with titaniumoxysulphate-forming peroxytitaniumsulphate (14). Esterase (EC 3.1.1.6, marker for microsomes, P-fraction) was assayed spectrophotometrically by following hydrolysis of α-nitrophenyl acetate (15). Monoamine oxidase (EC 1.4.3.4, marker for mitochondrial outer membrane, M-fraction) was measured as previously described (16), with modifications (17). Acid phosphatase (EC 3.1.3.2, marker for lysosomes, L-fraction) was assayed (18) as the amount of inorganic phosphate formed (19) after incubation with β-glycerophosphate. UDP-galactosyltransferase (EC 2.4.1.38, marker for Golgi-apparatus, P-fraction) was measured as transfer of [14C]galactose to ovomucoid (20, 21). Radioactivity was determined in a LKB Wallace liquid scintillation counter, model Rack-beta 1215 (Turku, Finland).

Statistics. Data are presented as means ± SD of results for five rats in each group. Wilcoxon two-sample test (one-tailed) was used to evaluate differences between the groups.

RESULTS

The amount of α-tocopherol found in different rat liver cells is presented in Table 2. Kupffer cells had the highest content of α-tocopherol per milligram of
protein of cells elutriated from rats fed the reference diet. Calculated per milligram of protein, Kupffer cells contained four times more α-tocopherol than parenchymal cells and nearly twice the amount in the fraction of endothelial/stellate cells. In a preliminary experiment separating endothelial and stellate cells, we found that stellate cells contained less than 100 pmol α-tocopherol per milligram of protein. When rats were fed a vitamin E-deficient diet, we observed a striking reduction in the content of α-tocopherol in nonparenchymal cells. In endothelial/stellate cells, α-tocopherol was not detectable, whereas the content in Kupffer cells was reduced to less than 5% of the control value. The content of α-tocopherol in hepatocytes was reduced to about 30% of the initial value. Supplementing α-tocopherol to reference rats resulted in a sixfold increase in the amount of α-tocopherol in the parenchymal cells, but the content of α-tocopherol in nonparenchymal cells was similar to the values obtained with the reference diet.

Based on the assumption that parenchymal cells, Kupffer cells, endothelial and stellate cells constitute 65, 10, 19 and 6% of hepatic cells [12, 22, 23] and that their protein content per 106 cells is 1.6, 0.118, 0.047 and 0.072 mg [23, 25], respectively, one may calculate that the parenchymal cells of rats fed the reference diet contain approximately 90% of hepatic α-tocopherol.

The cross-contamination within fractions of nonparenchymal cells was limited (Table 3), and the observed content of α-tocopherol in endothelial/stellate cells thus could not be explained by contamination of Kupffer cells. No parenchymal cells were detected in the nonparenchymal cell fractions, with the exception of a 7% contamination of cells isolated from rats receiving the vitamin E-enriched diet.

The subcellular distribution of α-tocopherol is outlined in Table 4. Light mitochondria and microsomes had the highest content of α-tocopherol, whereas the cytosolic fraction contained only small amounts of α-tocopherol calculated per milligram of protein. Relatively high levels of α-tocopherol were also recovered in the heavy mitochondrial fraction. The subcellular

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**TABLE 2**

Distribution of α-tocopherol in livers of rats fed vitamin E diets

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Normal [reference]</th>
<th>Deficient [supplemented]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>Parenchymal cells</td>
<td>121 ± 22 (93)</td>
<td>39 ± 10* (&gt;99)</td>
</tr>
<tr>
<td>Endothelial/stellate cells</td>
<td>281 ± 135* [3]</td>
<td>ND*</td>
</tr>
<tr>
<td>Kupffer cells</td>
<td>517 ± 288* [4]</td>
<td>14 ± 5* [&lt;1]</td>
</tr>
</tbody>
</table>

*Data are presented as means ± SD of values obtained from five rats in each group. Figures in parentheses give the relative distribution [%] of α-tocopherol among the cell types calculated for whole liver. Data were analyzed by the Wilcoxon two-sample test (one-tailed). ND, not detectable (< 5 pmol/mg protein). *Significantly higher (P < 0.01) than value for parenchymal cells of reference rats. †Significantly lower (P < 0.01) than the corresponding values of reference rats.

**TABLE 3**

Contamination of centrifugally elutriated nonparenchymal cell-fractions with other cell types from rats fed diets with different vitamin E content

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Dietary content of vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal [reference]</td>
</tr>
<tr>
<td></td>
<td>% of cells</td>
</tr>
<tr>
<td>Kupffer cells in fraction of</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>endothelial/stellate cells</td>
<td>19 ± 10</td>
</tr>
<tr>
<td>Endothelial/stellate cells in</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>fraction of Kupffer cells</td>
<td>11 ± 12</td>
</tr>
</tbody>
</table>

*Data are means ± SD of results obtained from five rats on each diet.

**TABLE 4**

Subcellular distribution of α-tocopherol in livers of reference diet-fed rats

<table>
<thead>
<tr>
<th>Fraction</th>
<th>pmol/mg protein</th>
</tr>
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<tbody>
<tr>
<td>Mitochondrial</td>
<td>103.3 ± 23.9</td>
</tr>
<tr>
<td>Light mitochondrial</td>
<td>210.1 ± 50.6</td>
</tr>
<tr>
<td>Microsomal</td>
<td>179.2 ± 32.5</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>23.5 ± 2.3</td>
</tr>
</tbody>
</table>

*Data are presented as picomoles α-tocopherol per milligram protein in the fractions. Data are means ± SD for five rats on each diet.
distribution of marker enzymes is shown in Table 5 with the relative specific activity of marker enzymes in parentheses. The data reveal that all subcellular fractions contained their respective marker enzymes in highest relative specific activity.

In subfractions of light mitochondria (L-fraction), most a-tocopherol was recovered in the lysosomes, which are located in the top (highest fraction number). Very little a-tocopherol was found in the bottom of the gradient, where peroxisomes are located (Fig. 1).

After subfractionation of the microsomes (P-fraction), the highest specific activity of a-tocopherol was found in the top of the gradient, corresponding to the Golgi fraction (Fig. 2). The concentration of phospholipids was measured in the different L- and P-subfractions and revealed that phospholipids were distributed throughout the gradients. The enrichment of a-tocopherol on top of the gradients is, therefore, not due to lipid-laying on top of the gradients.

**DISCUSSION**

Vitamin E therapy may be indicated in different malabsorption syndromes and for premature children. Alcoholics have reduced levels of vitamin E in serum [26] and liver [unpublished observations] and may also profit from a supplement of vitamin E. It is also well established that even subjects consuming high doses of vitamin E do not show any overt sign of toxicity. The liver as well as muscle and adipose tissue may also have a large storage capacity for a-tocopherol [27]. The hepatic metabolism of a-tocopherol as a function of different levels of intake has not yet been studied. In this study we found that about 90% of hepatic a-tocopherol was recovered in the parenchymal cells of rats fed a nonpurified diet. This is somewhat higher than earlier reported [7]. In the previous study, however, we examined the distribution of a-tocopherol in parenchymal and nonparenchymal rat liver cells isolated by differential centrifugation only. Centrifugal elutriation used in this study makes it possible to obtain a better separation of the different liver cell types. Furthermore, the rats in the two studies received different amounts of vitamin E in the diet prior to the studies. The distribution of a-tocopherol in the different liver cells is dependent on the level of intake as illustrated by the different values obtained for rats receiving different amounts of vitamin E. After supplementation with large amounts of vitamin E, the parenchymal cells had the highest relative increase in a-tocopherol content, whereas the content of a-tocopherol in nonparenchymal cells was unchanged. This finding suggests that parenchymal cells have storage capacity for a dietary surplus of a-tocopherol. When rats were deprived of a-tocopherol,
the parenchymal cells had the least reduction in the amount of α-tocopherol, whereas the nonparenchymal cell fractions lost most (>95%) of their α-tocopherol. This indicates that parenchymal cells have the ability to preserve some of their α-tocopherol when the supply of vitamin E is limited, possibly by mobilization of α-tocopherol from other tissues. Some cell types in the liver may, accordingly, be deficient in vitamin E without this being clearly reflected in total vitamin E content. Total hepatic vitamin E content might, therefore, not be an ideal indicator of the vitamin E status.

Kupffer cells had the highest content of α-tocopherol per milligram of protein in rats fed the reference diet. These cells have phagocytic capacity and are rich in lysosomes. One mechanism whereby phagocytic cells inactivate the phagocytosed material is by generation of free radicals and peroxides [28]. Accordingly, it is possible that these cells are in need of high levels of α-tocopherol for protection from cellular injury caused by these reactive compounds.

Marker enzymes of cell organelles were measured in all subcellular fractions and demonstrated a satisfactory separation. Most α-tocopherol was recovered in membrane-rich fractions; and the light mitochondrial fraction, containing lysosomes, had the highest content per milligram of protein. The content and relative distribution of α-tocopherol in the subcellular fractions were largely of the same order of magnitude as previously reported [10, 29–31]. However, in this study the content of α-tocopherol was somewhat lower in the mitochondrial fraction. The distribution of marker enzymes indicates that we have obtained a satisfactory purification of the mitochondrial fraction with minor cross-contamination to other subcellular fractions. In spite of the fact that a binding protein for α-tocopherol has been described in cytosol [32, 33], the cytosolic content of α-tocopherol was low.

Lysosomes, peroxisomes and Golgi apparatus were enriched by density gradient centrifugation. The content of α-tocopherol in the cell organelles of animals receiving a nonpurified diet has, to our knowledge, not been examined before. We studied the distribution of α-tocopherol in the organelles of chronic ethanol-fed rats receiving about five times more vitamin E per day [10]. The relative distribution of α-tocopherol between these cell organelles are similar to the two studies. The relatively high levels of α-tocopherol found in the lysosomal and Golgi fractions may be responsible for a major part of the antioxidant protection in these subcellular compartments.

Parenchymal liver cells have storage capacity for α-tocopherol. This finding is in contrast to what has been observed for retinoids, where stellate cells are the major storage cells [34]. The Kupffer cells from rats receiving a normal level of α-tocopherol in the diet contained more α-tocopherol than the parenchymal and endothelial/stellate cells per milligram of protein. This finding may possibly illustrate the physiological role of α-tocopherol as an antioxidant. These phagocytosing cells may generate high levels of free radicals. α-Tocopherol is the most important lipid-soluble antioxidant and may accordingly be of particular importance in these cells.

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**LITERATURE CITED**