Free Rad. Res. Comms., Vol. 11, Nos. 1-3, pp. 99-107
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APPLICATION OF DEUTERATED α-TOCOPHEROLS TO THE BIOKINETICS AND BIOAVAILABILITY OF VITAMIN E

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(Received, December 15, 1989; in final form, April 10, 1990)

α-Tocopherol, a superior chain-breaking, peroxyl radical-trapping antioxidant and the most active component of vitamin E, is elevated in liver tumor cells, contributing to their greater resistance towards lipid peroxidation compared to cells from normal tissues. Also, in regenerating rat liver the level of vitamin E has been found to fluctuate in phase with the rate of cell division. In order to study the biokinetcis and mechanisms of the distribution of vitamin E in organs and within tissues of animals, deuterated forms of α-tocopherol have been synthesized and their uptake into blood and tissues has been measured by gas chromatography-mass spectrometry. Measurement of the competitive uptake from a mixture of the *RRR*- and *SRR*-α-tocopherol stereoisomers labelled with different amounts of deuterium shows that the liver exerts a strong preference for secretion of the natural (*RRR*) stereoisomer into the plasma. It is suggested that a tocopherol-binding protein plays a key role in this process.

KEY WORDS: Deuterated α-tocopherol, biokinetics, bioavailability, antioxidant, liver tocopherol-binding protein.

In liver tumor cells the levels of α-tocopherol (α-TOH), the most potent form of vitamin E,[1,2] are higher than found in normal tissues and this is a major contributing factor to the resistance of these cells to lipid peroxidation.[3] Studies conducted using the regenerating liver model in partially hepatectomized rats suggest the existence of a fundamental relationship between the level of vitamin E and the rate of cell division.[4] Thus, it has been found that in suitably entrained, partially hepatectomized rats the level of α-TOH cycles over a 24 h period (Figure 1), as does the rate of DNA synthesis, with the peak in the vitamin E level coinciding with the maximum in the rate of cell division.[4]

We have shown that the natural form of vitamin E, 2*R*,4′*R*,8′*R*-α-tocopherol (*RRR*-α-TOH; Figure 2) is the major and probably the only lipid-soluble, radical-trapping antioxidant present in human blood (excluding the small amount of γ-tocopherol that is sometimes present),[5-7] as well as in various normal and cancerous animal tissues.[8] *In vitro* studies have shown that among phenols, α-TOH possesses superior chain-breaking, peroxyl radical-trapping reactivity which is controlled almost completely by the chroman head group.[9-12] However, the overall effectiveness of α-TOH as a lipid peroxidation antioxidant *in vivo* is determined not only by its inherent ability to react with and trap peroxyl radicals (ROO•; reactions 1 and 2), but is determined also by its ability to undergo transfer between and be retained within

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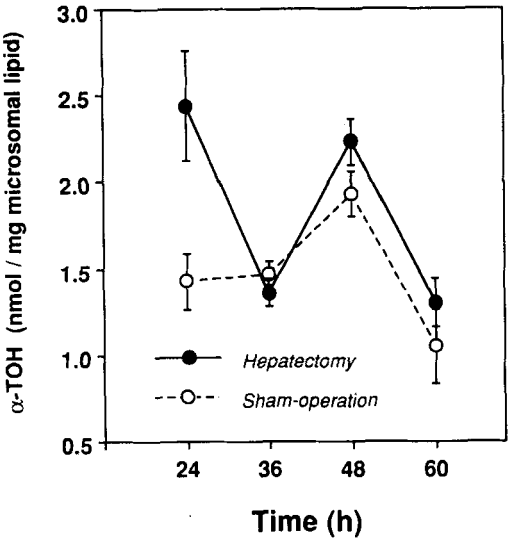


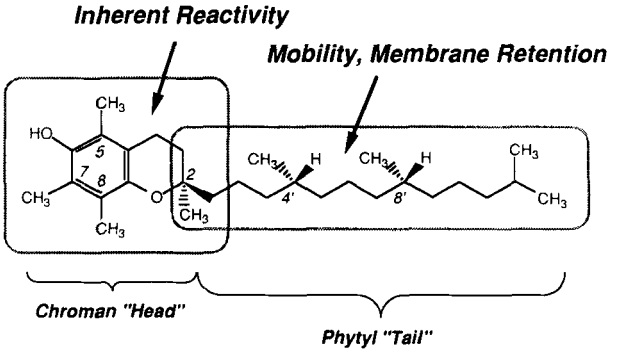
FIGURE 1 Plot showing the variation of α-TOH levels in regenerating livers of rats after partial hepatectomy and in livers of sham-operted rats (Prepared from data in ref. [4]).

$$\alpha\text{-TOH} + \text{ROO}\cdot \rightarrow \alpha\text{-TO}\cdot + \text{ROOH} \quad (1)$$

$$\alpha\text{-TO}\cdot + \text{ROO}\cdot \rightarrow \text{inactive products} \quad (2)$$

the lipids of cellular and subcellular membranes. In model studies it has been shown that inter-membrane mobility and membrane retention are largely controlled by the phytyl tail.[13,14]

In view of vitamin E's ability to control lipid peroxidation *in vivo*, it is important to know more about the dynamics of its absorption, transport and distribution within tissues. Until recently, little was known about the biokinetics of vitamin E, largely



Natural (2*R*,4'*R*,8'*R*) α-Tocopherol (*RRR*-α-TOH)

FIGURE 2 Structure of natural *RRR*-α-tocopherol.

because of the limitations and inconvenience of using radiolabelled vitamin E. Therefore, in response to the need to obtain biokinetic information directly and more conveniently, we have developed and applied a new approach. We have synthesized multi-gram quantities of natural and unnatural stereoisomers of α-TOH labelled with deuterium in metabolically inactive regions {e.g., 5-CD_3-α-TOH (d_3-α-TOH); 5,7-$(CD_3)_2$-α-TOH (d_6-α-TOH); 5,7,8-$(CD_3)_3$-α-TOH (d_0-α-TOH), etc.}.[15] These compounds have been used in both long-term feeding studies in laboratory animals[16,17] and in single or multiple dose studies in humans.[18–21] Extraction of the lipids from fluids and tissues (after the addition of d_9-α-TOH as an internal standard) followed by quantitative analysis for the residual, unlabeled and the newly absorbed, deuterated α-TOH's using gas chromatography-mass spectrometry (GC-MS) has subsequently provided the first reliable biokinetic data for the uptake and net retention of vitamin E in humans and other animals.

Biokinetic Studies

A unique application of deuterated α-TOH has been to use it as the sole dietary source of vitamin E for laboratory animals and to measure the long-term rate of its uptake into blood and tissues. Typically, young rats or guinea pigs are placed on a diet containing a known and fixed amount of unlabeled *RRR*-α-tocopheryl acetate (d_0-*RRR*-α-TAc; usually 36 mg/kg diet) for a lead-in period of 2–4 weeks and then switched to an identical diet containing the same amount of the deuterated form (d_3- or d_6-*RRR*-α-TAc; Figure 3). Determination of the amount of "old" d_0-*RRR*-α-TOH remaining and the amount of "new" d_3- or d_6-*RRR*-α-TOH taken up into tissues obtained from animals sacrificed at appropriate time intervals provides an estimate of the turnover rates in the different tissues. In this way it has been found that turnover in rats and guinea pigs is most rapid in plasma, liver and lung and is slowest in brain and neural tissues.[16,17]

Very recently, we have shown by means of this technique that the rate of turnover of α-TOH in plasma and tissues of guinea pigs is unaffected by dietary modulation of their vitamin C status, even when the level of vitamin E is declining in the animals.[17] This is a surprising result. It has been clearly demonstrated *in vitro* that vitamin C is able to "spare" or regenerate vitamin E.[22–24] Thus, if the effect were important *in vivo* we would expect to see a decreased turnover of vitamin E. One possible reason for the absence of any perceptible effect under normal physiological conditions in guinea pigs is that the flux of peroxyl radicals which enters the lipids of a healthy animal is not as large as has been supposed. Thus, for oxidatively unstressed animals only a small

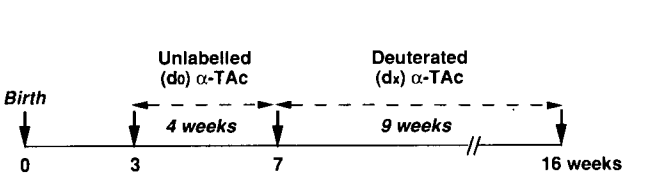


FIGURE 3 Illustration of protocol for conduct of long-term uptake and turnover studies of vitamin E in young rats or guinea pigs using a constant dietary intake of unlabeled and then deuterated (d_3- and/or d_6-) α-TAc.

fraction of the available vitamin E would be destroyed by trapping of peroxyl radicals. Support for this comes from Tappel's finding that expired pentane levels from animals are extremely low, even for animals that are receiving inadequate or no vitamin E, relative to the levels reached for animals that are oxidatively stressed in various ways.[25–28] Another possibility is that the tocopheroxyl radical is formed extensively, even in healthy animals, but it is reduced *in vivo* to α-TOH, not by ascorbate, but by some other, possibly enzymic, process. Indeed, there is considerable evidence that rat liver microsomes and other organelles and tissues contain a membrane-bound, heat-labile, glutathione-dependent, free radical reductase which probably acts by converting α-TO· to α-TOH.[29–39] Very recently, Packer and co-workers[40] have shown directly that mitochondria and microsomal membranes have a free radical reductase activity that prevents chromanoxyl radical accumulation and which may also be NADPH dependent.

Bioavailability Studies using the Competitive Uptake Method

A very useful feature of the deuterated vitamin E technique is the ability to make use of α-tocopherols substituted with different amounts of deuterium (i.e., d_3, d_6, etc.) and the ease with which they can be analyzed simultaneously to measure, within the same human subject or animal, the simultaneous, competitive uptake of two (or even three) differently deuterated forms of vitamin E. This competitive uptake technique eliminates a lot of the individual statistical variability, inevitable when different compounds are compared between groups in the conventional manner, because the subject or animal, in effect, acts as its own control.

An illustration of how competitive uptake can be used to measure relative bioavailability has been provided in a study of α-TOH versus α-TAc.[18] The relative bioavailabilities of these two forms of vitamin E are of interest because the free form occurs naturally in food, whereas the ester, which is much more air-stable than the phenol, is the form most commonly used in vitamin E supplements. α-TAc is inactive, both biologically and as an antioxidant, and first must be hydrolyzed to the phenolic

TABLE I

OH/"Ac" (d_6-α-TOH/d_3-α-TOH) molar ratios in plasma and various tissues in rats after oral dosing of a 1:1 mixture of deuterated free and acetylated natural α-tocopherol[a]

	Traditional[b]	With Food[c]
Plasma	0.55 ± 0.07	0.96 ± 0.04
Liver	0.44 ± 0.06	0.97 ± 0.02
Spleen	0.50 ± 0.12	1.01 ± 0.02
Lung	0.54 ± 0.14	1.10 ± 0.11
Heart	0.60 ± 0.05	1.28 ± 0.32

[a] From data in reference [18].

[b] Two vitamin E sufficient and two vitamin E deficient male Sprague-Dawley rats were dosed with vitamin E in a manner similar to that used in the traditional fetal gestation-resorption vitamin E bioassay. Thus, each animal was given four doses, once daily (2 mg/kg body weight/day), of an equimolar mixture of d_6-*RRR*-α-TOH and d_3-*RRR*-α-TAc dissolved in ca. 100 μL of tocopherol-stripped corn oil. Tisues were obtained one day after the last dose and the levels of d_3- and d_6-α-TOH were determined by GC-MS. The results for the deficient and sufficient animals have been combined because no differences between the two groups were apparent.

[c] Five male Sprague-Dawley rats were intubated with a mixture (4 mg) of d_6-*RRR*-α-TOH and d_3-*RRR*-α-TAc mixed in an aqueous bolus (5 mL) of laboratory diet (3.8 g dry weight). Tissues and blood were obtained 24 h later and the deuterated tocopherols analyzed in the usual way.

form, α-TOH, in the gut before absorption of the vitamin can occur. Previously, the phenol has been reported to be only about half as potent as the acetate in the rat fetal gestation-resorption assay.[41,42] We found this result to be surprising. We therefore began a competitive uptake study by administering an equimolar mixture of d_6-*RRR*-α-TOH and d_3-*RRR*-α-TAc to two vitamin E deficient and two normal male rats under the same conditions that were used in the resorption bioassay (i.e., four consecutive, daily oral doses in tocopherol-stripped corn oil) and we have measured the relative amounts of deuterated *RRR*-α-TOH taken up from the two vitamin E forms by each rat.[18] The phenol/"acetate" (OH/"Ac") ratios in the blood and the tissues after sacrifice on the fifth day were in good agreement with the bioassay result, and they were remarkbly similar for all four animals (mean OH/"Ac" ratio = 0.49 ± 0.05; see Table I), whether or not they were vitamin E deficient, indicating that vitamin E status is not important for determining relative absorption and transport of the two forms.

In contrast to the traditional fetal resorption assay technique, in which the vitamin is administered in tocopherol-stripped corn oil or some other similar vehicle, when the deuterated tocopherol mixture was administered in an aqueous bolus of laboratory food to five rats, the mean OH/"Ac" ratio in the blood and tissues 24 h later was 1.06 ± 0.11 (see Table I). The relative bioavailability therefore shows a strong dependence upon the vehicle in which the vitamin E is delivered. These experiments suggest that the relative biopotency measured by the resorption assay would yield a value close to one if the compounds were administered in an aqueous bolus of food, rather than in oil.

When five adult humans were fed a single oral dose of an equimolar mixture of the same deuterated phenol-acetate pair (ca. 50 mg of each) with their evening meal, the mean OH/"Ac" ratio measured in the plasma (see Table II) and red cells over the ensuing two days was also very close to 1.0, indicating that both α-TOH and α-TAc are absorbed equally well under normal dietary conditions.

The competitive uptake method has proven especially useful when the relative uptake into blood and tissues of an orally ingested 1:1 mixture of a pair of differently deuterated α-tocopherol diastereoisomers is investigated. In particular, use of a hexadeuterated form of the naturally occurring *RRR* stereoisomer (d_6-*RRR*-α-TAc) and a trideuterated form of its 2-epimer, the 2*S*,4′*R*,8′*R* (*SRR*) stereoisomer (d_3-*SRR*-α-TAc), in which the configuration of the phytyl tail and chroman ring is reversed, has provided (1) a direct measure of the influence of stereoisomerism in biodiscrimination, which is important when considering differences between natural vitamin E (one stereoisomer) and synthetic vitamin E (a mixture of all eight possible stereoisomers)

TABLE II

OH/"Ac" (d_3-*RRR*-α-TOH/d_6-*RRR*-α-TOH) molar ratios in plasma of five adult humans after ingestion of 100 mg of a mixture of d_3-*RRR*-α-TOH and d_6-*RRR*-α-TAc with an evening meal[a].

Subject	15–21 h	42–44 h
HB	0.98	0.94
SL	0.99	0.99
DF	1.04	0.99
KI	1.17	
GB	1.09	

[a] From data in reference [18]

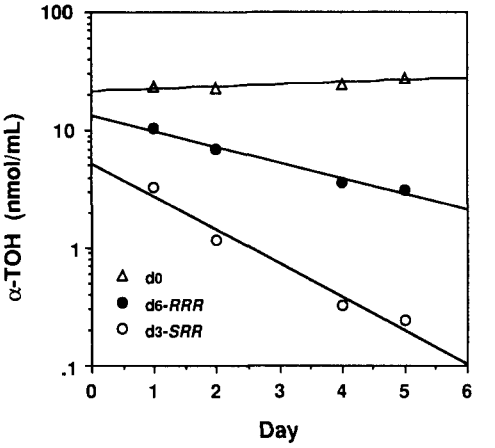


FIGURE 4 Semi-logarithmic plot of d_6-*RRR*-α-TOH and d_3-*SRR*-α-TOH in the plasma of a young man after ingestion, with an evening meal, of a single capsule containing a 1:1 mixture of 50 mg of each of the corresponding acetates.

and (2), a probe for studying the mechanism of absorption, transport, uptake and loss of vitamin E *in vivo*.

A long-term study of the uptake of d_3-*SRR*-α-TOH and d_6-*RRR*-α-TOH into the blood and tissues of rats continuously fed a mixture of the two acetates in their diets has shown that blood and all tissues, except liver, show a preference for the natural over the unnatural stereoisomer.[16] The liver initially accumulated twice as much *SRR*- as *RRR*-α-TOH but the level declined over the ensuing period of weeks to an amount slightly below that of the *RRR*-α-TOH. Although the discrimination may first occur, in principle, during absorption from the gut via the cholesterol esterase/bile salt mediated hydrolysis of the acetates, single dose studies conducted on rats with cannulated lymph ducts have shown that only slightly more *RRR*- than *SRR*-α-TOH is absorbed into the lymph.[43] Thus the initial preponderance of the unnatural form found in the liver points to the existence of a mechanism for selectively secreting the *RRR*-α-TOH into the plasma. We have hypothesized[16] that a cytosolic liver tocoph-

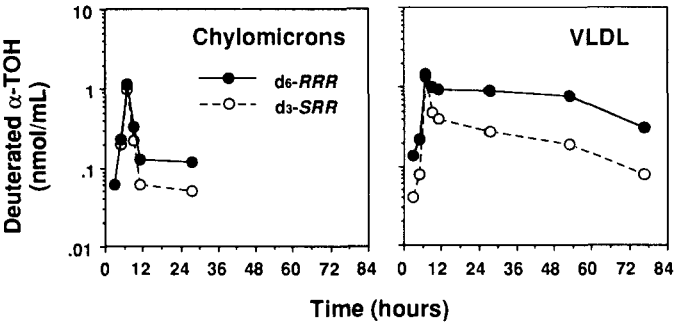


FIGURE 5 Concentrations of d_3-*SRR*-α-TOH and d_6 *RRR*-α-TOH in chylomicron and very low density lipoprotein (VLDL) fractions (nmol mL^{-1} plasma) of a 38 year old woman after ingestion with breakfast of a single dose of mixture of 75 mg each of d_3-*SRR*-α-TAc and d_6-*RRR*-α-TAc. (Prepared from data for subject #6 in ref. [21].)

erol-binding protein, identified earlier,[44] is responsible for the preferred secretion of the natural stereoisomer into plasma.

Humans also show a substantial preference for uptake of *RRR*- over *SRR*-α-tocopherols into plasma. Subjects given a single dose of a 1:1 mixture of d_3-*SRR*-α-TAc and d_6-*RRR*-α-TAc (50 mg of each) with an evening meal show within one day a substantial enrichment of the d_6-*RRR*-α-TOH over d_3-*SRR*-α-TOH (ratio $\geqslant 3$) in their plasma (Figure 4).[21] Analysis of lipoprotein fractions points to the liver as the major contributor to this discrimination.[21] Thus, the chylomicrons, which are formed in the gut and carry tocopherol from there to the liver via the lymph, were found to contain roughly equal amounts of both stereoisomers, whereas nine hours after the dose was taken the very low density lipoproteins (VLDL), which are synthesized in the liver, were substantially enriched with d_6-*RRR*-α-TOH (Figure 5).

Studies are now underway to determine if the tocopherol-binding protein does indeed control the preferential secretion of natural vitamin E from the liver. We also plan to determine the relationship, if any, of this protein to cell division and the cyclic variations of α-TOH observed in regenerating rat liver.

Acknowledgements

Financial support from the Association for International Cancer Reserch, the National Foundation for Cancer Research, Eastman Chemical Products, Inc., Eisai Co. Ltd., Henkel Corporation and the Natural Source Vitamin E Association is gratefully acknowledged.

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Accepted by Prof. H. Sies