# 10

# α-Tocopherol Stereoisomers

Søren Krogh Jensen and
Charlotte Lauridsen

*Department of Animal Health, Welfare and Nutrition, Faculty of Agricultural Sciences*
*University of Aarhus, DK-8830 Tjele, Denmark*



*Vitamins and Hormones, Volume 76*


0083-6729/07 $35.00
DOI: 10.1016/S0083-6729(07)76010-7

Vitamin E comprises a group of compounds possessing vitamin E activity. α-Tocopherol is the compound demonstrating the highest vitamin E activity, which is available both in its natural form as *RRR*-α-tocopherol isolated from plant sources, but more common as synthetically manufactured all-*rac*-α-tocopherol. Synthetic all-*rac*-α-tocopherol consists of a racemic mixture of all eight possible stereoisomers. Assessing the correct biological activity in form of bioavailability and biopotency has been a great challenge during many years as it is difficult to measure clinical endpoints in larger animals than rats and poultry. Thus, the biological effects in focus are resorption of fetuses, testicular degeneration, muscle dystrophy, anemia, encephalomalacia, and in recent years the influence of vitamin E on the immune system are the most important clinical markers of interest. For humans and animals, only different biomarkers or surrogate markers of bioactivity have been measured. In studies with rats, a good consistency between the classical resorption–gestation test and the bioavailability of the individual stereoisomers in fluids and tissues has been shown. For humans and other animals, only different biomarkers or surrogate markers of bioactivity have been measured, and due to the lack of good biological markers for bioactivities, bioavailability is often used as one of the surrogate markers for bioactivities with those limitations this must give. Therefore, a relatively simple analytical method, which allows analysis of the individual stereoisomers of α-tocopherol, is an important tool in order to quantify relative bioavailability of the individual stereoisomers. The analytical method presented here allows the quantification of total tocopherol content and composition by normal phase HPLC and subsequent separation of the stereoisomers of α-tocopherol as methyl ethers by chiral HPLC. Using this method, the α-tocopherol stereoisomers are separated into five peaks. The first peak consists of the four 2*S* isomers (*SSS*-, *SSR*-, *SRR*-, *SRS*-), the second peak consists of *RSS*-, the third peak consists of *RRS*-, the fourth peak consists of *RRR*-, and the fifth peak consists of *RSR*-α-tocopherol. The discussion on the bioavailability of *RRR*- and all-*rac*-α-tocopheryl acetate has primarily been based on human and animal studies using deuterium-labeled forms, whereby a higher biopotency of 2:1 (of *RRR*: all-*rac*) has been demonstrated, differing from the accepted biopotency ratio of 1.36:1. In agreement with previous studies, the 2*S*-forms exert very little importance for the vitamin E activity due to their limited bioavailability. We find notable differences between animal species with regard to the biodiscrimination between the 2*R*-forms. Especially, cows preferentially transfer *RRR*-α-tocopherol into the milk and blood system. The distribution of the stereoisomer forms varies from tissue to tissue, and in some cases, higher levels of the synthetic 2*R*-forms than of the *RRR*-form are obtained, for example, for rats. However, the biodiscrimination of the stereoisomers forms is influenced by other factors such as age, dietary levels, and time after dosage. More focus should be given on the bioactivity of the individual 2*R*-forms rather than just the comparison between *RRR*- and all-rac-α-tocopheryl acetate.

## I. INTRODUCTION

Vitamin E is the exception to the paradigm that natural and synthetic vitamins are equivalent because their molecular structures are identical. Natural α-tocopherol (*RRR*-α-tocopherol) is a single stereoisomer. Plants and other oxygenic, photosynthetic organisms are the only organisms able to synthesize tocopherols (DellaPenna, 2005), and since this synthesis is facilitated by stereo-specific enzymes, the resulting tocopherols always posses the same stereochemical structure, namely the *RRR*-structure (Fig. 1).

Synthetic α-tocopherol (all-*rac*-α-tocopherol) is produced commercially by a chemical reaction of tetramethylhydroquinone (TMHQ) with racemic isophytol (VERIS Research summary, 1999). Racemic isophytol is synthesized from isoprenoid units and since isophytol has three chiral centres the resulting α-tocopherol has $2^3$ possible conformations and thus yields a racemic mixture of all eight possible stereoisomers.

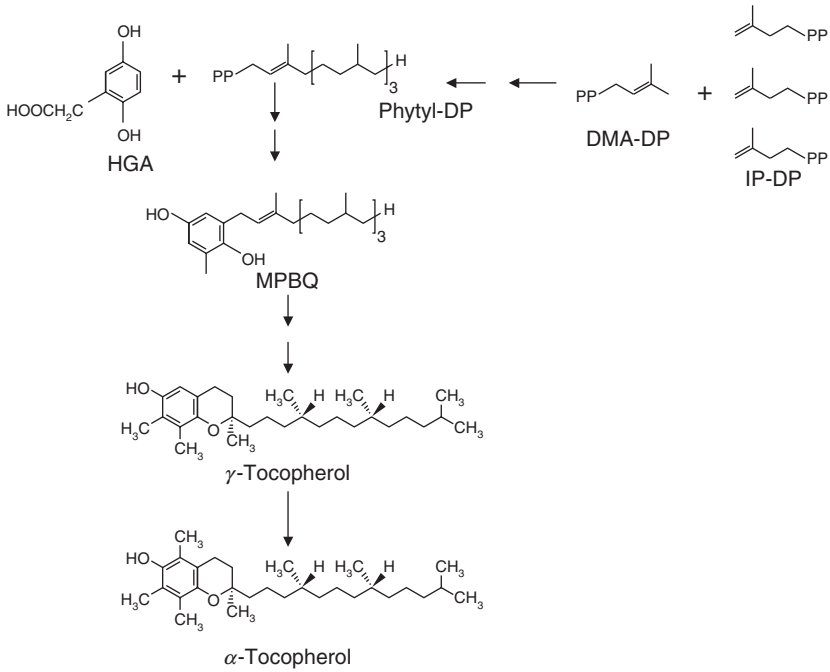


FIGURE 1. Synthesis of α-tocopherol in higher plants. (DMA-DP—dimethylallyl diphosphate; IPDP—isopentenyl diphosphate; HGA—homogentisic acid; Phytyl DP—phytyl diphosphate; MPBQ—2-methyl-6-phytyl-1,4-benzoquinone) (modified after DellaPenna, 2005).

## II. SOURCES OF TOCOPHEROL, NOMENCLATURE, AND BIOACTIVITY

### A. PRESENCE IN FOOD/FEED INGREDIENTS

Commercial vitamin E supplements can be classified into several distinct categories: fully synthetic vitamin E (all-*rac*-α-tocopherol), the most inexpensive, most commonly sold supplement forms usually as the acetate ester.

Most natural vitamin E is derived during refining of vegetable oils, mainly soybean oil, sunflower oil, and canola/rapeseed oil.

The natural sources of vitamin E can be divided into truly natural *RRR*-α-tocopherol, where *RRR*-α-tocopherol is extracted and isolated directly from a vegetable source without any modifications. Semisynthetic natural source α-tocopherol is *RRR*-α-tocopherol produced from a vegetable source, but where the manufacturer has converted the common natural $\beta$, $\gamma$, and $\delta$-tocopherol isomers into esters using acetic or succinic acid and eventually added methyl groups to yield *RRR*-α-tocopheryl esters such as *RRR*-α-tocopheryl acetate or *RRR*-α-tocopheryl succinate.

The most abundant sources of vitamin E are vegetable oils such as wheat germ oil, sunflower, canola/rapeseed oil, maize/corn, soybean, palm oil, and olive oil. Nuts, sunflower seeds, sea buckthorn berries, and wheat germ are also good sources. Other sources of vitamin E are whole grains, fish, peanut butter, and green leafy vegetables. In general, green photosynthesizing parts of the plants contain the highest concentration of α-tocopherol (DellaPenna, 2005) and the green parts of the plants are the major source of natural vitamin E for domestic animals.

### B. NOMENCLATURE

Vitamin E was discovered by Evans and Bishop, 1923, who observed that its deficiency caused fetal resorption in the rat. An active substance was isolated from wheat germ oil in 1936 and named "tocopherol" from the Greek words *tokos* (childbirth) and *pherein* (to carry) plus the *-ol* suffix designating phenol or alcohol (Packer, 1994). In 1938, the structure of natural α-tocopherol was elucidated and the same time the first synthesis of a biologically active product, consisting of a mixture of the natural diastereoisomer and its two-epimer, was reported. Studies in the following years revealed the existence in nature of a whole family of structurally related compounds with qualitatively identical biological action (IUPAC-IUB, 1981).

Recommendations for the nomenclature of the vitamins, including the tocopherols, were published in 1966 and in 1981 IUPAC-IUB recommended the following nomenclature. The term tocol is the trivial designation for 2-methyl-2-(4,8,12-trimethyldecyl)chroman-6-ol. The term tocopherol(s) should be used as a generic descriptor for the methylated tocols, and although

the term tocopherol is not equivalent to the term vitamin E, it include all mono- (δ-tocopherol), di- (β- and γ-tocopherol), and trimethyltocols (α-tocopherol).

Chemical species are said to possess the property of chirality if a molecule and its mirror image of that molecule are distinguishable. In such situation, the molecule and its mirror image are described as enantiomers or stereoisomers. The fact that isolated enantiomers rotate the plane of polarized light has given rise to the expression optical isomers.

The only naturally occurring stereoisomer of α-tocopherol found in nature has the configuration 2*R*,4′*R*,8′*R* according to the sequence rule system. Its semisystematic name is therefore (2*R*,4′*R*,8′*R*)-α-tocopherol and also known as D-α-tocopherol. Today the short term *RRR*-α-tocopherol has been well adopted and the same system is used for all other individual stereoisomers of tocopherols. It is important to notice that today the *RS* system with respect to stereochemical configuration has replaced the dl system.

The diastereoisomer of *RRR*-α-tocopherol, formerly known as L-α-tocopherol, being the epimer of *RRR*-α-tocopherol at C2 with the configuration *SRR* should be called 2-*epi*-α-tocopherol.

As mentioned above the first synthesis of α-tocopherol was an equimolar mixture of *RRR*- and *SRR*-α-tocopherol, this compound is called 2-*ambo*-α-tocopherol (*R*/*SRR*-α-tocopherol) and formerly known as DL-α-tocopherol until the optical activity of phytol was recognized, when DL-α-tocopherol was restricted to all-*rac*-α-tocopherol. The acetate of 2-*ambo*-α-tocopheryl acetate was the former international standard vitamin E activity.

All-*rac*-α-tocopheryl acetate is the international standard vitamin E compound today with an activity of 1 International Unit (IU)/mg (Blatt *et al.*, 2004; VERIS Research summary, 1999). However, since all-*rac*-α-tocopheryl acetate consists of an equimolar mixture of all eight possible stereoisomers (*RRR, RRS, RSS, RSR, SRR, SSR, SRS*, and *SSS*), each of them has its own biological activity.

### C. BIOACTIVITY AND BIOAVAILABILITY

"Biologic activity," "bioactivity," or "biopotency" are expressions describing the biological effect of a nutrient on the living organism. Assays of biological activity measure effects on clinical endpoints, biomarkers, or surrogate markers of clinical endpoints (Derendorf *et al.*, 2000). For substances with vitamin E activity, the biological effects in focus are resorption of fetuses, testicular degeneration, muscle dystrophy, anemia, encephalomalacia, and in recent years the influence of vitamin E on the immune system has become more and more important (Scherf *et al.*, 1996). Measures of bioavailability, however, are plasma or tissue concentrations of tocopherols and their metabolites, which are not measures of activity. According to the pharmacology, bioavailability is defined as the plasma concentration of a water-soluble drug

given orally compared with the concentration when the drug is given intravenously (Traber, 2000). However, for fat-soluble nutrients, bioavailability is difficult to assess because the nutrient cannot be given intravenously, so relative bioavailability is often measured (Traber, 2000). From a nutritional point of view, the bioavailability may be defined as the proportion of vitamin E ingested that undergoes intestinal absorption and utilization by the body, and this definition therefore encompasses the processes of vitamin E absorption, transport, distribution, and metabolism (Bramley *et al.*, 2000). The relative bioavailability of the different forms of vitamin E varies between tissues as well as with dose, time after dosing, and duration of dosing as demonstrated in an excellent review by Blatt *et al.* (2004). This nonconstant relative bioavailability predicts nonconstant relative activity (Blatt *et al.*, 2004), and preferably, both measures of bioavailability and bioactivity should be taken into account when assessing the biopotency of the different vitamin E forms.

Ideally, assays of bioactivity should measure clinical endpoints like resorption of fetuses, testicular degeneration, muscle dystrophy, anemia, encephalomalacia, and so on. However, these experiments are difficult and costly to conduct—like they often involve ethical considerations (Blatt *et al.*, 2004; Scherf *et al.*, 1996) and the tests are not applicable to larger animals or human subjects (Jensen *et al.*, 2006). Experiments involving clinical endpoints have only been performed with rats, chicks, and a single experiment with rabbits back in 1947 (Scherf *et al.*, 1996), and the relative bioactivity of the various vitamin E compounds used for all animals has been established from these experiments.

Clinical endpoints of vitamin E activity by definition involve only the prevention or resolution of vitamin E deficiency. There are no valid clinical assays of the relative vitamin E activity of the individual stereoisomers of α-tocopherol in humans and animals except rats and chickens because no clinical trials show their relative dose–effect relationships in preventing or treating vitamin E deficiency. For humans and other animals, only different biomarkers or surrogate markers of bioactivity have been measured (Blatt *et al.*, 2004).

It is important to distinguish between bioavailability and bioactivity as bioavailability only gives information about availability of the compound, but nothing about its bioactivity. Bioavailability is a prerequisite for bioactivity, and due to lack of satisfactory biomarkers to compensate for the lack of measurements of clinical endpoint, bioavailability is an often reported biomarker (Jensen *et al.*, 2006; Scherf *et al.*, 1996). In studies with rats, a good consistency between the classical resorption–gestation test and the bioavailability of the individual stereoisomers in fluids and tissues has been shown (Scherf *et al.*, 1996; Weiser *et al.*, 1996). However, this may have been a coincidence because the relative bioavailability of the stereoisomers is not

constant in humans and animals because their relative concentrations vary between tissues and vary with time after dosing, duration of dosing, and amount of each dose (Blatt *et al.*, 2004).

Harris and Ludwig (1949) established in a series of resorption–gestation test with rats a relative bioactivity of *RRR*-α-tocopheryl acetate against 2-*ambo*-α-tocopherylacetate of 1.36, which later has been accepted as the official bioactivity ratio between natural and synthetic α-tocopherol and esters thereof. This ratio has later been confirmed in several other experiments with rats, chickens, and a single experiment with rabbits. These experiments has comprised experiments where clinical endpoints as resorption–gestation, red blood cell hemolysis, and curative myopathy with rats. In chick, the experiments has comprised studies both involving clinical endpoints as muscular dystrophy, encephalomalacia, as well as different biomarkers as liver storage and plasma levels. With rabbits, one experiment with creatin–urea as biomarker has been performed. On average, these experiments have shown a bioactivity of *RRR*-α-tocopheryl acetate against 2-*ambo*-α-tocopheryl acetate (*R*/*SRR*-α-tocopheryl acetate) of 1.41 with variations from 1.22 to 1.68 (Scherf *et al.*, 1996).

The relative bioactivity ratio between *RRR*-α-tocopheryl acetate, 2-*ambo*-α-tocopheryl acetate, and all-*rac*-α-tocopheryl acetate was studied by Weiser and Vecchi (1981) in resorption–gestation tests with rats. These experiments showed a small discrepancy between the old standard 2-*ambo*-α-tocopheryl acetate and all-*rac*-α-tocopheryl acetate. Thus, in this experiment, the bioactivity of 2-*ambo*-α-tocopheryl acetate was on average 1.09 compared to all-*rac*-α-tocopheryl acetate. This discrepancy is likely caused by the fact that 2-*ambo*-α-tocopherol consist of 50% *RRR*-α-tocopherol and 50% SRR-α-tocopherol, whereas all-*rac*-α-tocopherol only consist of 12.5% α-tocopherol, 37.5% *RR*/*SR*/*S*-α-tocopherol and 50% *SR*/*SR*/*S*-α-tocopherol. Accordingly, the bioactivity of *RRR*-α-tocopheryl acetate compared to 2-*ambo*-α-tocopheryl acetate and all-*rac*-α-tocopheryl acetate was 1.32 and 1.50, respectively. The relative bioactivity of different stereoisomers of α-tocopheryl esters and mixtures thereof determined on basis of the rat resorption gestation test are shown in Table I.

There is also a need to clarify the relative bioavailability of natural and synthetic vitamin E, which is currently a subject of some controversy (Lodge, 2005). Previous studies using a competitive uptake approach have found bioavailability ratios of natural: synthetic vitamin E of 2:1, differing from the accepted biopotency ratio of 1.36:1. However, when using a noncompetitive uptake approach to compare the plasma biokinetics of *RRR*- and all-*rac*-α-tocopheryl acetate in smokers and nonsmokers, the relative bioavailability ratio was close to the currently accepted ratio of 1.36:1 (Lodge, 2005). However, the current debate on the bioavailability focuses mostly on the comparison of *RRR*- versus all-*rac*-; however, analysis of the individual stereoisomers will be of greater importance for the bioavailability subject.

TABLE I. Relative Biological Activities of Different Stereoisomers of α-Tocopherol and Mixtures Thereof Found Resorption–Gestation Test with Rats

| Compounds | References | Relative bioactivity |
|---|---|---|
| *S*/*RRR*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Harris and Ludwig, 1949 | 0.73 |
| *S*/*RRR*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1981 | 0.76 |
| All-*rac*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1981 | 0.67 |
| *S*/*RRR*-α-tocopheryl acetate relative to all-*rac*-α-tocopheryl acetate | Weiser and Vecchi, 1981 | 1.09 |
| *SSS*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Ames *et al.*, 1963 | 0.21 |
| All-*rac*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.77 |
| *RR*/*SR*/*S*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.92 |
| *RRS*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.90 |
| *RSS*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.73 |
| *RSR*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.57 |
| *SRR*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.31 |
| *SSR*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.21 |
| *SRS*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.37 |
| *SSS*-α-tocopheryl acetate relative to *RRR*-α-tocopheryl acetate | Weiser and Vecchi, 1982 | 0.60 |

## III. ANALYTICAL METHODS FOR SEPARATION OF α-TOCOPHEROL STEREOISOMERS

Analysis of the individual stereoisomers of α-tocopherol is an important tool in order to quantify relative bioavailability of the individual stereoisomers (Jensen *et al.*, 2006). Separations of the eight stereoisomers of α-tocopherol are a great challenge and until now no single method allow the separation of all eight stereoisomers in one single chromatographic run (Nelis *et al.*, 2000). The use of deuterium labeled α-tocopherol in conjunction with GC-MS (Ingold *et al.*, 1987) or HPLC-MS (Lauridsen *et al.*, 2001b) is

another important method to study bioavailability of different preparations of natural and synthetic vitamin E.

### A. GC AND LC METHODS

Neither GC or LC methods is alone capable of distinguishing between all eight α-tocopherol stereoisomers, but the two methods are complementary and hence, can be applied consecutively (Nelis *et al.*, 2000). The first reported chromatographic method that allowed separation of α-tocopherolstereoisomers was a GC method which allowed separation of the four pairs of diastereoisomers (*RSS*/*SRR*, *RSR*/*SRS*, *RRR*/*SSS*, and *RRS*/*SSR*) on a 115-m SP 2340 capillary column of trimethylsilyl (TMS) ethers of α-tocopherol stereoisomers. Weiser and Vecchi (1981) performed the same separation but used methyl ethers of α-tocopherol stereoisomers instead and Cohen *et al.* (1981) performed synthesis of all eight stereoisomers of α-tocopheryl acetate and determined their diastereisomeric and enantomeric purity by GC. These initial methods for separation of the stereoisomers were based on the principle that diastereoisomers possess different physicochemical properties and can be separated on conventional columns.

Chiral HPLC has proven to be one of the best methods for direct separation and analysis of enantiomers. It is more versatile than chiral GC because the compounds do not need to be volatile. Current chiral HPLC methods are either direct, which utilizes chiral stationary phases and chiral additives in the mobile phase, or indirect, which involves derivatization of samples.

The chiral HPLC methods published for separation of α-tocopherol stereoisomers until today is all based on chiral stationary phases and in the first published methods the columns were prepared in the research laboratories. Thus, Ueda *et al.* (1993) developed a HPLC method based on separation of stereoisomers of α-tocopheryl acetates on a Chiralpak OP(+) column ($250 \times 4.6\,mm^2$) with methanol–water (96:4 v/v) as mobile phase at a flow rate of 0.3 ml/min. This column consists of poly(diphenyl-2-pyridylmethyl methacrylate) and is capable of separating compounds possessing an aromatic group. This method allowed separation of stereoisomers of α-tocopheryl acetates into four distinct peaks (peak 1: consisted of the four 2*R*-stereoisomers, *RRR*/*RRS*/*RSS*/*RSR*; peak 2 consisted of *SSS*/*SSR*; peak 3 consisted of *SRR* and peak four consisted of *SRS*).

Weiser *et al.* (1996) further developed the method published by Vecchi *et al.* (1990) based on preparative chiral HPLC of stereoisomers of α-tocopheryl acetates followed by GC of the corresponding methyl ethers resulting in separation of all eight stereoisomers. The preparative chiral HPLC was performed on a Nucleosil 1000–5 Machery-Nagel $250 \times 4.6\,mm^2$ HPLC column coated with (+)poly(triphenylmethylmetacrylate), whereby four pairs of diastereoisomers were separated (*RSR*/*RSS*; *RRR*/*RRS*; *SSS*/*SSR*, and *SRS*/*SRR*). These four peaks were preparative collected two and two,

thus the first fraction contained the four 2*R*-stereoisomers and the second fraction contained the four 2*S*-stereoisomers. The four 2*R*-stereoisomers and the four 2*S*-stereoisomers were then transferred into their corresponding methyl ethers and subsequently separated individually on a 100-m glass capillary GC column with an internal diameter of 0.3 mm, dynamically coated with Silar 10C held isothermally at 165°C.

Chiral HPLC columns are made by immobilizing single enantiomers onto the stationary phase. Resolution relies on the formation of transient diastereoisomers on the surface of the column packing. The compound, which forms the most stable diastereoisomer, will be most retained, whereas the opposite enantiomer will form a less stable diastereoisomer and will elute first. To achieve discrimination between enantiomers, a minimum of three points of interaction to achieve chiral recognition are needed.

The fast development of commercial HPLC columns with chiral stationary phases mainly for the pharmaceutical industry has provided new challenges and possibilities for the separation of α-tocopherol stereoisomers.

One of the most widespread chiral HPLC column today is based on modified cellulose coated onto silica. The cellulose is further modified with different aromatic compounds. For separation of stereoisomers of tocopherols tris(3,5-dimethylphenylcarbamate)-cellulose from the Japanese manufacturer Daicel has proven to be excellent. The separations occur due to a multimode mechanism involving hydrogen bonding, π–π interactions, dipole stacking, and inclusion complexes. The phases contain chiral cavities or ravines, which have a high affinity for aromatic groups. For separation to occur, there must be a tight fit of part of the solute into the cavity, and at least one of the substituents on the chiral center needs to interact with the steric environment just outside the cavity. The forces that lead to this interaction are very weak and require careful optimization by adjustment of the mobile phase and temperature to maximize selectivity. Typically, a free energy of interaction difference of only 0.03 kJ/mol between the enantiomers and the stationary phase will lead to resolution. Obviously, the highly polar hydroxyl group of tocopherols give rise to nonstereoselctive binding between the tocopherol molecule and the carbamate site of the stationary phase. Thus, the derivatization into methyl ethers is essential and much more efficient than acetylation in order to block these unspecific associations and provides the tocopherol molecules with groups capable of interacting with the chiral cellulose stationary phase (Drotleff and Ternes, 2001). Another advantage of the methyl ethers over acetylated tocopherol is their considerable fluorescence properties of the methyl ethers that allow a very specific and sensitive detection.

The method published by Lauridsen and Jensen (2005) and Jensen *et al.* (2006) offers an easy and simple combination of analysis of both total tocopherol content and stereoisomer composition of α-tocopherol in biological samples. This method allows the quantification of total tocopherol

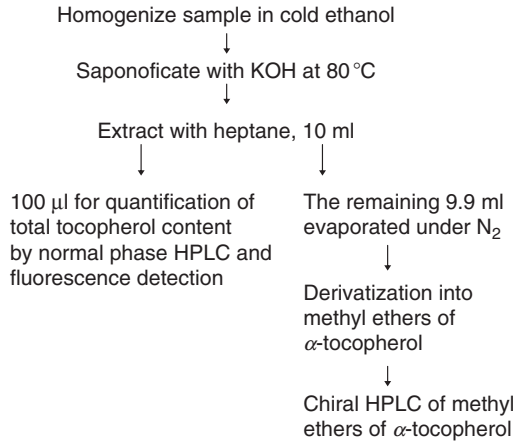


FIGURE 2. Scheme showing the clean up procedure, derivatization, and analytical steps for analysis of total tocopherol content and stereoisomer composition of α-tocopherol (Jensen *et al.*, 2006).

content and composition by normal phase HPLC after saponification and extraction into heptane (Jensen *et al.,* 1999) and subsequent separation of the stereoisomers of α-tocopherol as methyl ethers by chiral HPLC as described by Drotleff and Ternes (2001). By this method, the α-tocopherol stereoisomers are separated into five peaks. The first peak consists of the four 2*S* isomers (*SSS*-, *SSR*-, *SRR*-, *SRS*-), the second peak consists of *RSS*-, the third peak consists of *RRS*-, the fourth peak consists of *RRR*-, and the fifth peak consists of *RSR*-α-tocopherol. The analytical procedure is summarized in Fig. 2 and a HPLC chromatogram of the methyl ethers of α-tocopherol is shown in Fig. 3.

### B. DEUTERIUM-LABELING AND MASS SPECTROMETRY

The use of stable deuterium-labeled α-tocopherol has been described for the quantification of labeled tocopherols in blood and tissue after administration of deuterium-labeled vitamin E. The evaluation of samples by GC-MS enabled the investigators to distinguish tissue and blood bioavailability of all-*rac*- versus *RRR*-α-tocopherol (Burton *et al.*, 1998). The use of this technique provides several advantages. First, unlabeled endogenous as well as labeled exogenous vitamin E can be determined by single-ion monitoring by MS. Second, as a result of substituting different amounts of deuterium on the chromanol ring, it is possible to administer different forms of vitamin E simultaneously, allowing the subject to act as his/her own control. Hereby the necessity of a crossover protocol is eliminated and newly absorbed vitamin E

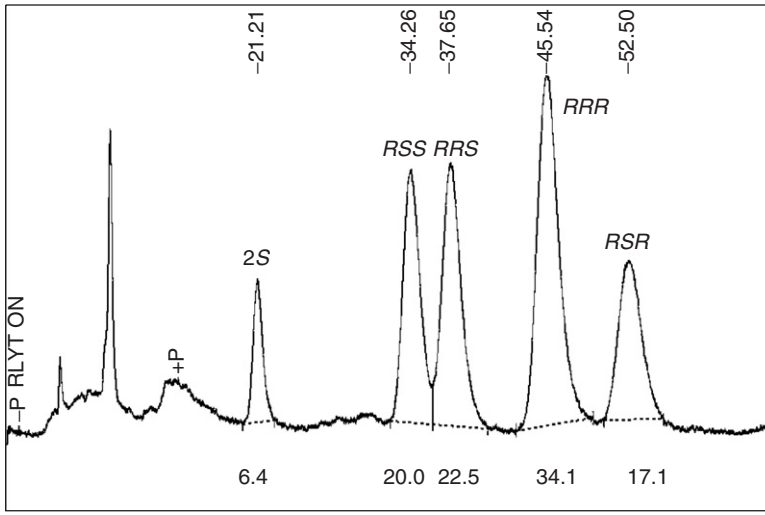


FIGURE 3. HPLC chromatogram of methyl ethers of α-tocopherol from sow plasma separated on a Chiralcel OD-H column. 2SR/SR/S denotes the mixture *SSS*-, *SRR*-, *SSR*-, *SRS*- α-tocopherol. *RSS, RRS, RRR*, and *RSR* denote *RSS*-, *RRS*-, *RRR*-, and *RSR*-α-tocopherol, respectively. The numbers below the baseline denote the relative distribution of the stereoisomers.

can be distinguished from the unlabeled vitamin E already present in the body. In addition, the deuterated vitamin E technique allows direct comparison of two distinctly labeled vitamin E forms in the same animal, whereby most of the variation attributable to differences between individuals, and factors that change with time can be eliminated (Lauridsen *et al.*, 2002a).

The deuterated technique is based on specific labeling of α-tocopherol with trimethyl deuterium ($CD_3$). α-Tocopherol can be labeled in either position 5, 7, or 8 or any combination thereof (Fig. 4). Hereby the tocopherol molecules are labeled with 3, 6, or 9 deuterium atoms located in the nonlabile aromatic methyl positions, making it possible to distinguish the silated ethers by GC-MS due to different parent ion masses. For labeling *RRR*-α-tocopherol, either γ- or δ-tocopherol are methylated with $CD_3$ labeled in position 5 or 5 and 7 on the chromanol ring yielding *RRR*-α-5-($CD_3$)-tocopherol ($d_3$) and *RRR*-α-5,7-$(CD_3)_2$-tocopherol ($d_6$), respectively (Burton and Traber, 1990; Fig. 4).

Although being very sensitive and specific, the GC-MS method of deuterated tocopherols is time-consuming and costly. The introduction of liquid chromatography tandem mass spectrometry (LC-MS/MS) is a convenient and sensitive technique for the analysis of vitamin E in extracts from biological samples (Lauridsen *et al.*, 2001b). The use of liquid chromatography instead of gas chromatography decreases preparation time and cost, as

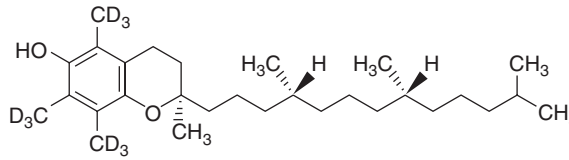


FIGURE 4. Possible positions of deuterium in deuterated α-tocopherol (modified after Burton and Traber, 1990).

the derivitization step is unneeded. Thus, the LC-MS/MS analysis has simplified the clean up procedure and shortened the analysis time when compared to the GC-MS measurement.

## IV. BIOAVAILABILITY AND SECRETION INTO MILK

As discussed above, bioavailability is an important part of the term bioactivity and is quantitative measurable in blood, tissue, and excreta. In addition, the lack of good biological markers for bioactivities, bioavailability is often used as one of the surrogate markers for bioactivities with those limitations this must give (Blatt *et al.*, 2004; Jensen *et al.*, 2006). Therefore, results of α-tocopherol stereoisomers in different animal species and humans are presented with the focus on bioavailability rather than bioactivity.

### A. RATS

Bioavailability of stereoisomers of α-tocopherol in rats has been studied both with the deuterium-labeling technique (Blatt *et al.*, 2004; Burton and Traber 1990; Ingold *et al.*, 1987) as well as chiral GC and LC techniques (Blatt *et al.*, 2004; Jensen *et al.*, 2006; Weiser *et al.*, 1996).

Ingold *et al.* (1987) studied the uptake and biodiscrimination of deuterated *RRR*-α-tocopherol versus SRR-α-tocopherol in male rats fed a diet containing 36-mg *RRR*-α-tocopheryl acetate/kg diet for 4 weeks and then 18-mg $d_6$*RRR*- and 18-mg $d_3$SRR-α-tocopheryl acetate/kg diet over a 5-month period. This experiment demonstrated the variation in bioavailability of different stereoisomers in blood and various tissues as well as changes in bioavailability over time. Thus, as shown in Fig. 5, there was a 1.4-fold enrichment of $d_6$*RRR*-α-tocopherol in plasma after the first day, and this enrichment increased to 2.5 over the 5-month duration of this experiment. In the liver, $d_3$*SRR*-α-tocopherol was retained twice as much as

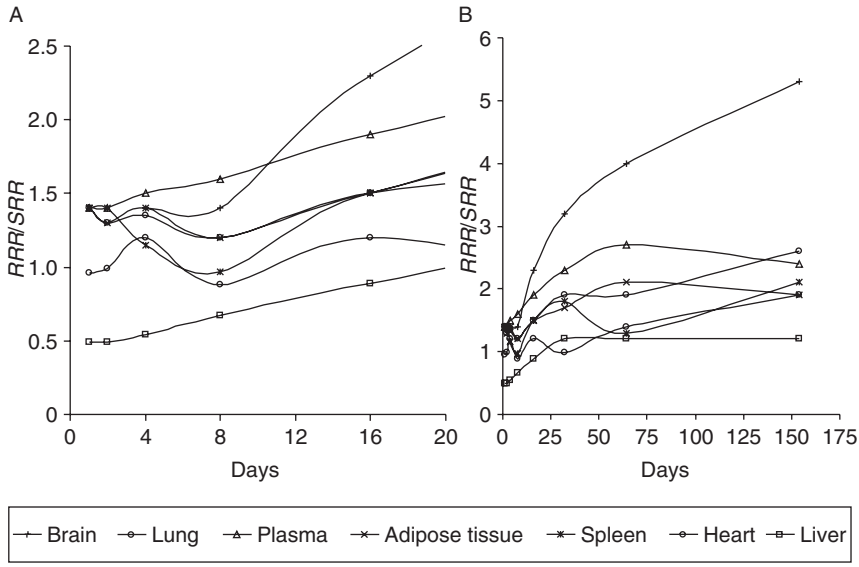


FIGURE 5. Time dependence of the discrimination in net uptake of deuterated *RRR*- and *SRR*-α-tocopherols in plasma and tissue of rats (after Ingold *et al.*, 1987). (A) Day 1–16 and (B) day 1–154.

$d_6$*RRR*- α-tocopherol during the first period and after 32 days the ratio between $d_6$*RRR*- and $d_3$SRR-α-tocopherols approached 1. The greatest discrimination in favor of $d_6$*RRR*-α-tocopherol was observed in the brain.

Weiser *et al.* (1996) studied the relative distribution of all eight stereoisomers of α-tocopherol in plasma and tissue from rats fed a daily dose of 0.82-mg all-*rac*-α-tocopheryl acetate for 90 days by a combination of chiral HPLC and GC, some of the results are summarized in Fig. 6. Generally, the sum of all 2*R*-α-tocopherol in the analyzed plasma and tissues comprised 74–88% of all the α-tocopherols with exception of the liver, which at day 8 comprised equal amount of the 2*R*- and 2*S*-α-tocopherols. At day 90, 70% of the α-tocopherol in the liver was 2*R*-α-tocopherol. Keeping in mind that *RRR*-α-tocopherol has the highest biological activity, it is interesting to notice that the three synthetic 2*R*-forms generally occurred in higher concentration than *RRR*-α-tocopherol. Among the 2*S* forms, *SRS*-α-tocopherol showed the highest relative concentration in plasma as well as in the analyzed tissues.

However, according to Blatt *et al.* (2004) only exist a weak and variable coherence between relative bioactivity assessed on the basis of the rat resorption–gestation test as used by Weiser and Vecchi (1982) and bioavailability assessed using comparable conditions in Weiser *et al.* (1996).

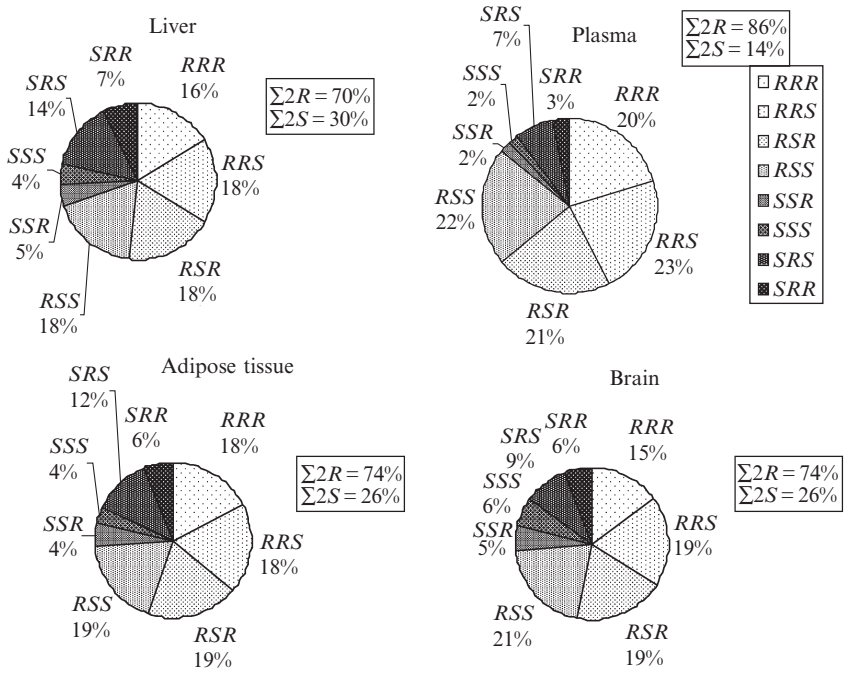


FIGURE 6. Relative distribution of all eight stereoisomers in liver, adipose tissue, brain, and plasma from rats fed at a daily dose of 0.82-mg all-*rac*-α-tocopheryl acetate for 90 days (after Weiser *et al.*, 1996).

Jensen *et al.* (2006) studied the effect of different dietary doses varying from 25 to 200 mg/kg diet of all-*rac*-α-tocopheryl acetate for 10 days on the relative distribution of stereoisomers in plasma and tissues. With increasing dietary content of all-*rac*-α-tocopheryl acetate in the feed, the proportion of *RRR*-α-tocopherol decreased in plasma (22–18%), liver (20–13%), lung (21–19%), and adipose tissue (25–20%). The three synthetic 2*R*-stereoisomers remained fairly constant within each of the tissues. *RRS*-α-tocopherol constituted the highest proportion of the four 2*R*-stereoisomers with exception of the liver, where the sum of the four 2*S*-stereoisomers constituted the highest proportion (29–33%). Thus, the relative bioavailability of *RRR*-α-tocopherol compared with all-*rac*-α-tocopherol varied from 1.07 to 2.02, *RRS*-α-tocopherol varied from 1.40 to 2.12, *RSS*-α-tocopherol varied from 1.35 to 2.00, *RSR*-α-tocopherol varied from 1.40 to 1.94, and the relative bioavailability of the sum of the four 2*S*-forms varied from 0.16 to 0.66. In summary for rats on the basis of the referred literature, a clear preference for the 2*R*-stereoisomers over the 2*S*-stereoisomers has been observed; however, between the four 2*R*-stereoisomers no significant biodiscrimination has been noticed.

## B. PIGS

There are three critical phases with regard to vitamin E status in pigs, and these are during reproduction, at birth, and after weaning. In this paragraph, we will focus on the α-tocopherol stereoisomers in relation to the periods in which vitamin E is essential for the development of growth and health. The studies with stereoisomers of α-tocopherol in pigs have been performed both with the stable isotope-labeled technique and chiral HPLC.

### 1. At Birth

Sows discriminate between *RRR*- and all-*rac*-α-tocopheryl acetate with a preference for *RRR*-α-tocopherol (Lauridsen *et al.*, 2002a,b). When using the stable isotope-labeled α-tocopherols, the natural and synthetic forms can be ingested simultaneously for intraindividual purposes. Pregnant sows were provided 150 mg each of $d_3$-*RRR*-α- and $d_6$-all-*rac*-α-tocopheryl acetate daily from 7 days before to 7 days after giving birth, and the α-tocopherol delivery to fetuses and to suckling piglets were followed (Lauridsen *et al.*, 2002a). Despite elevated sow plasma-deuterated α-tocopherol concentrations, no labeled α-tocopherol was detected in piglet plasma or tissues. Sow plasma and milk $d_3$-α- to $d_6$-α tocopherol were 2:1, leading to a 2:1 ratio in suckling piglet plasma and tissues. At day 7 after birth, most tissues of the piglets contained a tenfold increase in total α-tocopherol concentration compared with piglets at birth. The highest concentrations of deuterated vitamin E were in the liver, followed by the lung, heart, kidney, muscle, intestine, and brain (Lauridsen *et al.*, 2002a). It was concluded that the bioactivity of synthetic all-*rac*-α-tocopherol is roughly one-half of that of natural *RRR*-α-tocopherol during pregnancy and lactation in sows, resulting in a 2:1 ratio of the natural and the synthetic vitamin E forms in milk and the suckling progeny.

In a later experiment with sows, we studied the concentration of α-tocopherol and its stereoisomer distribution by using the HPLC-method, which separates the stereoisomers of α-tocopherol. Sows were provided with either 75 IU of all-*rac*-α-tocopheryl acetate/kg feed (synthetic) or 75 IU of *RRR*-α-tocopheryl acetate/kg feed (natural) 1 week prior to parturition and during lactation for 28 days. Milk samples were obtained from the sows during lactation (day 2, 16, and 28 after parturition), and plasma samples were obtained from the sows at 1 week before parturition (day 108 of gestation) and at day 2 and 16 of lactation. In addition, plasma samples were obtained from the suckling piglets at day 4 and 16 after birth. The results of the concentration of α-tocopherol and its composition of the stereoisomers in the milk and plasma samples are given in detail in Lauridsen and Jensen (2006a). In brief, the most predominant form of stereoisomer forms of α-tocopherol was the *RRR*-form irrespective of feeding natural or synthetic vitamin E, whereas the 2*S*-forms were only present in very limited proportions.

Among the sows fed the synthetic vitamin E, the *RRR*-form contributed with between 32 and 36% in milk, followed by 24–26% of the *RRS*-, 18–20% of the *RSR*-, and 17–18% of the *RSS*-, and 6–8% of the *SR*/*SR*/*S*- during the period from 2 to 28 days of lactation. For comparison, sows fed the natural form secreted between 69 and 91% of the *RRR*-form into the milk, followed by 2–12% of the *RRS*-, 5–10% of the *RSR*-, and 2–7% of the *RSS*-, and 2–3% of the *SR*/*SR*/*S*- with a clear trend of a higher proportion of the *RRR*- with increasing lactation time on the expense of the other 2*R*-isomers. The stereoisomer composition of α-tocopherol in the plasma of sows fed the synthetic vitamin E and their piglets followed the same pattern as for the milk composition, except for a higher contribution (~17%) of the *SR*/*SR*/*S*- in plasma of sows at day 108 of gestation on expense of all four the 2*R*-forms. Thus, these results confirmed the data obtained by Lauridsen *et al.* (2002a,b) that sows discriminated between the stereoisomer forms of α-tocopherols, which resulted in higher milk and plasma α-concentrations arising from the *RRR*-form.

**2. At Weaning**

The sterosiomer composition of α-tocopherol in plasma and tissues of suckling piglets nicely reflects the stereoisomer profile of the milk, and the reported results above do not seem to indicate a further biodiscrimination by the suckling piglet than carried out by the lactating sow. However, when considering the piglets after weaning from the sow, one may speculate when the piglets are getting mature to be able to discriminate between stereoisomer form α-tocopherols. The role of α-TTP during the biodiscrimination is described below, and the expression of the protein by piglets is therefore reserved for this paragraph. In a recent study, we investigated the stereoisomer profile of α-tocopherol in plasma of piglets throughout the postweaning period, and the piglets were weaned from sows fed 70-IU all-*rac*-α-tocopheryl acetate on day 28 of age. Piglets were throughout the experiment (day 28–49 of age) given 70 IU of vitamin E either in the form all-*rac*-α-tocopherol or in the form of all-*rac*-α-tocopheryl acetate. Regarding the stereoisomer composition of α-tocopherol in plasma of the piglets, *RRR*-α-tocopherol contributed with the highest proportion (32%) in plasma, followed by *RRS*- (27%), *RSR*- (22%), *RSS*- (17%), while the four 2*S*-forms together only provided 1.7% of the α-tocopherol (Lauridsen and Jensen, 2006b).

The age of the piglets (day 35, 42, and 49) had no influence on the stereoisomer composition of α-tocopherol. By using the quantitative method in this study, we were not able to distinguish the "old pool" of vitamin E (provided with the sow milk) from the "newly absorbed pool" of vitamin E (provided with the weaning feed), and as such it is not possible to conclude regarding the eventual discrimination capability of pigs during this age period.

Besides the stereoisomer form of vitamin E, another important issue of the molecule when addressing pigs after weaning is the alcohol versus the acetate form. The vitamin E in milk of animals or humans is provided in the form of alcohol, that is free vitamin E. However, supplementation of vitamin E to animal feed is normally with the acetate-bound form, α-tocopheryl acetate, which may not be well absorbed by young infants having an immature digestive tract. In the study referred to above (Lauridsen and Jensen, 2006b), the α-tocopherol concentration in plasma and tissues of piglets fed the alcohol form of vitamin E (all-*rac*-α-tocopherol) was higher than the acetate (although no statistical differences were obtained).

Although several lines of evidence illustrate that plasma enrichment with *RRR*-α-tocopherol depends on the hepatic α-TTP, and that no discrimination of stereoisomers takes place during the absorption process of the small intestine (Kiyose *et al.*, 1995), it may be speculated that the steroisomer configuration of the dietary α-tocopherol plays a role for the hydrolysis process. The principal enzyme-hydrolysing tocopheryl acetate (Müller *et al.*, 1976) is carboxyl ester hydrolase (CEH), and the presence of bile salts is a prerequisite both as emulsifier and as activators of this enzyme (Lauridsen *et al.*, 2001a).

A study (Knarreborg *et al.*, 2004) provided evidence *in vivo* that the bioavailability of α-tocopheryl acetate is highly dependent on an adequate amount of bile salts to generate enzymatic hydrolysis of α-tocopheryl acetate and subsequent absorption of α-tocopherol to the blood plasma. Besides their role as detergents and CEH-activators, bile acids furthermore modulate CEH's chiral selectivity (Moore *et al.*, 1994). In addition to the chiral properties of the tocopherol molecule, the rate of hydrolysis of the α-tocopheryl esters will depend on the affinity of the CEH toward the ester, at this may depend on the affinity of the CEH toward the ester, and this may depend on the presence of other dietary molecules, for example retinol (Lauridsen *et al.*, 2001a) or tocopherol derivates.

Pigs may have an insufficient secretion of pancreatic enzymes after weaning, and it cannot be excluded that a discrimination of the esters (the *RRR*- versus the all-*rac*-α-tocopheryl acetate) could be caused by the enzyme's specific substrate recognition.

### 3. During Reproduction

Vitamin E deficiency has been shown to affect reproduction in several animal species, resulting in fetal death and resorption (Nielsen *et al.*, 1979). Consequently, the vitamin E standards for reproducing swine have increased over the past 25 years from 10- to 44-IU/kg diet (Mahan *et al.*, 2000; NRC, 1998). Given the differences in the biological activities of the stereoisomer forms of α-tocopherol, dietary sources of natural (D-α-tocopheryl acetate) or synthetic (DL-α-tocopheryl acetate) vitamin E have called for attention in reproducing sows for several parities (Mahan *et al.*, 2000). No effect was

TABLE II. Concentration of α-Tocopherol in Feed (mg/kg), Plasma (mg/liter) and Fetuses (μg/Fetus), and Composition of Stereoisomers (%) in Gilts at Day 28 of Gestation (Mean and SD in Parentheses)

| | | α-Tocopherol stereoisomers | | | | |
|---|---|---|---|---|---|---|
| | Total | *RRR*- | *RRS*- | *RSS*- | *RSR*- | *SR/SR/S*- |
| Feed | 57.6 | 28.9 | 11.7 | 9.8 | 8.4 | 41.2 |
| Plasma | 1.49 (0.35) | 43.2 (2.9) | 20.9 (2.5) | 16.9 (4.1) | 16.4 (1.6) | 2.2 (1.1) |
| Fetus | 1.07 (0.82) | 42.3 (3.1) | 20.3 (1.7) | 16.5 (2.6) | 16.5 (1.4) | 2.1 (0.52) |

obtained for vitamin E source or level (30 versus 60 IU/kg) on the various sow reproductive measurements, litter size, or incidences of mastitis–metritis–agalactia (MMA) or fluid discharges from the vagina, but serum and liver α-tocopherol contents in 21-day-old-nursing pigs were higher when the sow had been fed the natural compared with the synthetic source or when the 60-IU level had been fed (Mahan *et al.*, 2000).

In a preliminary study, the stereoisomer composition of plasma and fetus obtained from gilts at day 28 of gestation was investigated (personal communication Charlotte Lauridsen). Homogenates of fetus obtained from each gilt ($n = 24$) were prepared and analyzed for the content of α-tocopherol. The gilts were provided a feed supplemented with 32 mg/kg of vitamin E in the form of all-*rac*-α-tocopheryl acetate. Table II shows the stereoisomer distribution of α-tocopherol in the feed, plasma, and fetus of the gilts.

The data confirm other animal assays providing evidence that the chiral center at the position 2 is the major and possibly sole determinant of the biological differences between α-tocopherol stereoisomers. Obviously, the stereoisomer composition of α-tocopherol in the plasma and fetus of the gilts showed the same proportions, thus indicating that no further biodiscrimination by the fetus or the placenta at this stage of reproduction. Interestingly, when compared with plasma of lactating sows (Lauridsen and Jensen, 2006a), the *RRR*-form contributed with a higher proportion on expense of the other stereosiomers in plasma of the gilts at day 28 of gestation. This result might indicate a trend toward a higher biopotency of the *RRR*-form during the critical phase of reproduction, which should be investigated further in the future.

### C. HUMANS

With regard to the α-tocopherol stereoisomers in humans, relatively little information is available on the bioavailability and the secretion into milk, and the studies performed on pigs as described above may therefore provide

important information applicable to human nutrition. Investigations on the comparison of natural and synthetic vitamin E in humans have mainly been performed using the deuterium-labeled vitamin E, and these results have indicated overall that the natural vitamin E (*RRR*-α-tocopheryl acetate) has roughly twice the availability of synthetic vitamin (all-*rac*-α-tocopheryl acetate) (Burton *et al.*, 1998).

However, the focus has been given specifically to the ratio of *RRR*:*rac*, and very little information is available on the distribution of the single stereoisomer forms of α-tocopherol in humans after consumption of a dose of all-*rac*-α-tocopheryl acetate. In 1993, Ueda *et al.* developed a new chiral HPLC method to separate the eight stereosisomers of α-tocopherol into four peaks—the first peak was composed of the four 2*R*-isomers, the second peak of the *SSS*- and *SSR*-α-tocopherol, the third peak of *SRR*-α-tocopherol, and the four peak of *SRS*-α-tocopherol (Ueda *et al.*, 1993).

Kiyose *et al.* (1997) studied the single 2*S*-stereoisomer forms and the sum of the 2*R*-forms in women, who had received oral administration of natural and synthetic α-tocopheryl acetate. After oral administration, the concentration of 2*S*-isomers increased gradually but was significantly lower than that of 2*R*-isomers. The 2*S*-isomers contributed with around 4% of all-*rac*-α-tocopherol in LDL on the first day of oral administration, and on day 28 the 2*S*-isomer proportion had increased to ~20%. In HDL, the concentration of 2*S*-isomers was lower than that of HDL on days 1, 7, and 14 after administration. The concentrations of 2*S*-isomers were in the order (*SSS* = *SSR*) > *SRS* > *SRR* in HDL and LDL. This study by Kiyose *et al.* (1997) confirmed that the bioavailability of *RRR*-α-tocopherol was greater than that of all-*rac*-α-tocopheryl acetate because the 2*R*-isomers were preferentially incorporated in the serum lipoproteins of humans administered all-*rac*-α-tocopherol.

In addition, several studies suggest that the relative bioavailability of all-*rac*- and *RRR*-α-tocopherols varies with dosage in humans ingesting multiple doses sufficient to achieve steady-state conditions (Blatt *et al.*, 2004), and it would be interesting to know to which extent the actual proportion of the 2*R*-isomers varies when different dosages of the synthetic vitamin E form when administered to humans.

Our data (using our recent developed chiral HPLC method) showing that the 2*S*-forms of α-tocopherol contributed with <3% in human milk (Lauridsen and Jensen, 2006a), and that the ranking of the 2*R*-forms seemed to be similar to the data obtained on sow milk, lead to some suggestions for the future research: that focus should be given on the biological significance of the 2*R*-forms of α-tocopherol, rather than just differentiating between the *RRR*-form versus the other seven forms. Furthermore, that the relative bioavailability of the stereoisomer forms of α-tocopherol varies between tissues and with dosage, and even between animal species.

### D. MINK

Mink (*Mustella vision*) is a carnivore belonging to the marten family (*mustelidae*) and is domesticated because of its excellent fur. A major part of mink feed is different fish by-products, as well as slaughter offal. Due to this high intake of by-products with a high proportion of polyunsaturated fatty acids, mink has been shown to have a high vitamin E requirement (Børsting *et al.*, 1998).

In this connection, we have been looking at the biodiscrimination of stereoisomers of α-tocopherol in lactating mink and suckling mink kits, where the dams were fed a diet containing 100-mg all-*rac*-α-tocopheryl acetate per 10 MJ. In Table III, the relative distribution of stereoisomers of α-tocopherol in feed, plasma, and milk of the lactating mink at day 28 and plasma from mink kit also at day 28 are shown. These data clearly demonstrate the efficient exclusion of the 2*S*-stereoisomers in plasma, resulting in a complete absence in tissues from mink kits. It is interesting to notice that in contrast to rats and pigs, mink kit liver does not contain 2*S*-stereoisomers. Mink heart contain 59% *RRR*-α-tocopherol.

### E. POULTRY

Although quite a few experiments where the bioactivity of various vitamin E compounds has been tested in poultry, only one experiment dealing with the distribution of stereoisomers of α-tocopherol has been performed (Cortinas *et al.*, 2004). In contrast to the other animal species investigated, chickens fed 100-, 200-, or 400-mg/kg diet all-*rac*-α-tocopheryl acetate the

TABLE III. Total Content and Relative Distribution of Stereoisomers of α-Tocopherol in Feed, Plasma, and Milk of Lactating Mink at Day 28 and Plasma, Liver, Heart, and White Adipose Tissue of Mink Kit at Day 28

| | | α-Tocopherol stereoisomer | | | | |
|---|---|---|---|---|---|---|
| | α-Tocopherol (mg/kg) | *RRR-* | *RRS-* | *RSS-* | *RSR-* | *SR/SR/S-* |
| Feed | 56 | 16.2 | 11.0 | 9.5 | 13.0 | 50.4 |
| Lactating mink plasma | 16.8 | 39.0 | 21.2 | 16.3 | 16.4 | 7.2 |
| Milk | 7.1 | 37.5 | 18.3 | 14.7 | 16.7 | 12.8 |
| Mink kit plasma | 23.6 | 46.2 | 25.3 | 13.5 | 13.6 | 1.5 |
| Liver | 78 | 37.8 | 24.8 | 15.3 | 22.2 | 0 |
| Heart | 15 | 59.0 | 14.2 | 12.0 | 14.8 | 0 |
| White adipose tissue | 12 | 44.4 | 27.0 | 14.2 | 14.4 | 0 |

distribution of α-tocopherol stereoisomers in liver and thigh was very similar. In general, both tissues predominantly accumulated the 2*R*-stereoisomers (52% in the diet, 74% in liver, and 70% in thigh).

Further, this experiment showed that increased oxidation pressure due to a higher inclusion of polyunsaturated fatty acids in the diet increased the proportion of 2*R*-stereoisomers on the expense of the 2*S*-stereoisomers in both liver and thigh muscle. Within the 2*R*-stereoisomers, the difference between liver and thigh muscle was negligible. Thus, the average distribution of α-tocopherol stereoisomers in liver and thigh of chickens fed synthetic vitamin E was of 20% *RRR*-, 21% RRS-, 17% RSS, 15% RSR-, and 28% of the four 2S-α-tocopherols.

## F. RUMINANTS

Studies with cattle and sheep have indicated that the natural form of vitamin E resulted in higher serum α-tocopherol concentrations than did the synthetic form Hidiroglou *et al.* (1992). However, until the work of Jensen *et al.* (2005) and Meglia *et al.* (2006) was published, no information existed about the distribution of the stereoisomers in ruminants. Meglia *et al.* (2006) fed cows daily with 917-mg all-*rac*-α-tocopheryl acetate in addition to the 300–525-mg *RRR*-α-tocopherol occurring naturally in the basal ration for 5 weeks around parturition and analyzed the distribution of stereoisomers in plasma and milk.

Likewise, Jensen *et al.* (2005) fed cows a daily dose of 3000-mg all-*rac*-α-tocopheryl acetate in addition to the 450-mg *RRR*-α-tocopherol occurring naturally in the basal ration for 16 days and analyzed the composition of the stereoisomers in plasma. These results are listed in Table IV together with

TABLE IV. Relative Distribution of α-Tocopherol Stereoisomers in Plasma and Milk from Ruminants Fed all-*rac*-α-Tocopheryl Acetate

| | | α-Tocopherol stereoisomer | | | | |
|---|---|---|---|---|---|---|
| | | *RRR-* | *RRS-* | *RSS-* | *RSR-* | *SR/SR/S-* |
| Cow plasma | Meglia *et al.*, 2006 | 88.1 | 4.7 | 4.3 | 2.3 | 0.6 |
| Cow milk | | 87.4 | 2.8 | 2.9 | 5.1 | 1.8 |
| Cow plasma | Jensen *et al.*, 2005 | 84.4 | 5.5 | 5.0 | 3.7 | 1.4 |
| Calf plasma[a] | Milk replacer, 250 mg/day | 34.1 | 22.5 | 20.0 | 17.1 | 6.3 |
| Lamb plasma[a] | 200 mg/day | 42.3 | 20.7 | 15.9 | 14.5 | 6.7 |

[a]Unpublished data.

unpublished results from calves fed milk replacer with 250-mg all-*rac*-α-tocopheryl acetate per kilogram DM and lambs fed a daily dose of 200-mg all-*rac*-α-tocopheryl acetate per day for 3 months.

From Table IV it is clear that cows even though they are fed a higher proportion of the natural stereoisomer has a preferential accumulation of *RRR*-α-tocopherol in plasma as well as secretion into milk. It is important to notice that the 2*S*-forms are almost absent from plasma and milk and that the three synthetic 2*R*-stereoisomers are present in a considerable lower concentration than *RRR*-α-tocopherol.

Calves fed solely on milk replacer with synthetic vitamin E, which means they have been provided with 12.5% of each of the eight stereoisomers shows also a clear biodiscrimination in favor of *RRR*-α-tocopherol as it constitutes 34% of total α-tocopherol in plasma. The proportion of 2*S*-stereoisomers in both calves and lambs were very low. The three synthetic 2*R*-stereoisomers in both calves and lambs were represented in the order *RRS* > *RSS* > *RSR*. Generally, the stereoisomer patterns in these young animals were very similar to the distribution reported in pigs.

## V. α-TOCOPHEROL-BINDING PROTEIN (α-TTP)

In contrast to the relatively well-investigated binding proteins for vitamins A and D, proteins that bind and transport vitamin E have only been identified in the past decade and many of their specific biological roles remain elusive. The term "tocopherol-associated proteins" has been used to distinguish a molecularly defined family of proteins that are capable of binding α-tocopherol (Zimmer *et al.*, 2000) with a higher affinity than other tocopherols (Yamauchi *et al.*, 2001) and are also capable of binding phospholipids.

The α-tocopherol transfer protein (α-TTP) is of 32-kilodalton (kDa) that acts primarily in the liver to specifically select α-tocopherol with the side chain linked to the chromanol ring in the *R*-configuration at position 2 for incorporation into nascent very low-density lipoprotein (VLDL) (Hosomi *et al.*, 1997). Thus, α-TTP preferentially binds α-tocopherol over the γ-homologue (or others) as it can discriminate between the number and position of methyl groups on the chromanol ring. The predominant homologue *in vivo* is therefore α-tocopherol because most of the γ-tocopherol is excreted into the bile while the α-tocopherol is preferentially retained.

The overall role of α-TTP is the maintenance of normal plasma tocopherol concentrations, which is a conclusion that has been supported by the existence of two human diseases, "FIVE" (familial-isolated vitamin E deficiency) and "AVED" (ataxia with vitamin E deficiency) apparently caused by a genetic defect of α-TTP (Ben Hamida *et al.*, 1993; Traber *et al.*, 1990). In patients, the genetic defect causes impaired incorporation of α-tocopherol

into nascent VLDL, leading to very low levels of plasma vitamin E. Absorption and transport of vitamin E in chylomicrons, however, is normal in these diseases.

α-TTP is predominantly expressed in liver but low concentrations have also been detected in brain, spleen, lung, and kidney (Hosomi *et al.*, 1998). The ability of hepatic α-TTP to discriminate between tocopherols based on the chemistry of the chromanol ring and the position C2 appears to be the main reason for the predominance of *RRR*-α-tocopherol *in vivo*. As well as discriminating between tocopherol homologues, α-TTP can also differentiate between different tocopherol stereoisomers.

This indication has mainly been attributed during the study of Burton *et al.* (1998). In that study, the bioavailability of synthetic α-tocopherol in humans was determined to be roughly the half of that of natural *RRR*-α-tocopherol, and the 2*R*-stereoisomers were preferentially retained and 2*S*-stereoisomers being eliminated. At present, it is therefore generally accepted in the literature that the tocopherol-binding and transport proteins discovered hitherto selectively select *RRR*-α-tocopherol, leaving *β*-, *γ*-, and *δ*-tocopherols and the synthetic stereoisomers of racemic α-tocopherol to degradation.

Our results on the stereoisomer composition of fluids in different animal species show, however, that the other 2*R*-forms also contribute with a considerable proportion of the α-tocopherol, and it would be interesting to know to which extent the biodiscrimination between these stereoisomer forms is dependent on the expression of α-TTP. As shown for rats, the *RRS*-α-tocopherol constituted the highest proportion of the four 2*R*-stereoisomers in plasma and all measured tissues with exception of the liver (Jensen *et al.*, 2006).

Interestingly, cows have a much stronger preference for the *RRR*-form than other animal species or even calves, and to which extent the above mentioned results between animals species is exclusively related to the discrimination by α-TTP remains to be investigated. How many other α-tocopherol-binding proteins exist apart from α-TTP is still unclear (Brigelius-Flohé *et al.*, 2002).

Dietary vitamin E influences hepatic α-TTP concentrations (Fechner *et al.*, 1998), however, when vitamin E-depleted rats were fed a diet containing α- or *δ*-tocopherol or both, α-TTP messenger RNA increased (Fechner *et al.*, 1998). This was not expected, because the biological activity of *δ*-tocopherol, which does not bind α-TTP (Hosomi *et al.*, 1997), differs strongly from that of α-tocopherol. This shows that other tocopherols may have similar effects on gene expression that still await detection. Other authors (Kim *et al.*, 1998) have found that vitamin E depletion of rats strongly increased α-TTP messenger RNA concentrations, which then decreased after refeeding of α-tocopherol to below the concentrations in the control rats. Thus, the biodiscrimination appears to depend on dietary tocopherols, and as for example shown for rats (Jensen *et al.*, 2006), the proportion of *RRR*-α-tocopherol decreased in plasma and tissues with increasing dietary content of all-*rac*-α-tocopheryl

acetate, whereas the three synthetic 2*R*-isomers remained fairly constant within these tissues, and only small changes were observed.

Obviously, α-TTP may reach a saturation limit with regard to the preference toward the *RRR*-form, or the general biodiscrimination among the 2*R*-forms are actually not notable. Unpublished results on piglets fed increasing levels of all-*rac*-α-tocopheryl acetate during the period after weaning showed no influence on the expression of hepatic α-TTP (C. Lauridsen, personal communication). Interestingly, we observed a higher concentration *RRR*-α-tocopherol on the expense of the other 2*R*-forms in immune cells of pigs supplemented with vitamin C at a level of 500 mg/kg during the period after weaning (Lauridsen and Jensen, 2005), which may reflect a regeneration of the *RRR*-form during absorption and transportation by this antioxidant supplementation. However, a direct influence of the antioxidant supplementation on the regulation and concentration of binding proteins may also be speculated as seen for vitamin A- and D-binding proteins (Baker *et al*., 1984; Ong, 1985).

## VI. CONCLUSIONS

The discussion on the bioavailability of *RRR*- and all-*rac*-α-tocopheryl acetate has primarily been based on human and animal studies using deuterium-labeled forms, whereby a higher biopotency of 2:1 (of *RRR*: all-*rac*) has been demonstrated, differing from the accepted biopotency ratio of 1.36:1. However, the quantitative separation of the individual stereoisomers of the 2*R*-forms allow us to get a more detailed picture of the bioavailability of natural and synthetic vitamin E forms.

In agreement with previous studies, the 2*S*-forms exert very little importance for the vitamin E activity due to their limited bioavailability. We find notable differences between animal species with regard to the biodiscrimination between the 2*R*-forms, and with regard to cows, the preference of *RRR*-form is by far the most predominant one.

The distribution of the stereoisomer forms varies from tissue to tissue, and in some cases, higher levels of the other 2*R*-forms than the *RRR*-form are obtained, for example, for rats. However, the biodiscrimination of the stereoisomers forms is influenced by other factors such as age, dietary levels, and time after dosage. More focus should be given on the bioactivity of the individual 2*R*-forms rather than just the comparison between *RRR*- and all-*rac*-α-tocopheryl acetate.

## REFERENCES

Ames, S. R., Ludwig, M. I., Nelam, D. R., and Robeson, C. D. (1963). Biological activity of an 1-Epimer of d-α-tocopheryl acetate. *Biochem.* **2,** 188–190.

Baker, L. R. I., Clark, M. L., Fairclough, P. D., and Goble, H. (1984). Identification of two intestinal vitamin D-dependent calcium-binding proteins in the X-linked hypophosphataemic mouse. *Biochem. Biophys. Res. Commun.* **119,** 850–853.

Ben Hamida, C., Doerflinger, N., Belal, S., Linder, C., Reutenauer, L., Dib, C., Gypay, G., Vignal, A., Le Pasilier, D., Cohen, D., Pandolfo, M., Mokini, V., *et al.* (1993). Localization of Friedreich ataxia phenotype with selective vitamin E deficiency to chromosome 8q by homozygosity mapping. *Nat. Genet.* **5,** 195–200.

Blatt, D. H., Pryor, W. A., Mata, J. E., and Rodriguez-Proteau, R. (2004). Re-evaluation of the relative potency of synthetic and natural α-tocopherol: Experimental and clinical observations. *J. Nutr. Biochem.* **15,** 380–395.

Bramley, P. M., Elmadfa, I., Kafatos, A., Kelly, F. J., Manios, Y., Roxborough, H. E., Schuch, W., Sheehy, P. J. A., and Wagner, K.-H. (2000). Review. Vitamin E. *J. Sci. Food Agric.* **80,** 913–938.

Brigelius-Flohé, R., Kelly, F. J., Salonen, J. T., Neuzil, J., Zingg, J.-M., and Azzi, A. (2002). The European perspective on vitamin E: Current knowledge and future research. *Am. J. Clin. Nutr.* **76,** 703–716.

Burton, G. W., and Traber, M. G. (1990). Vitamin E: Antioxidant activity, biokinetics, and bioavailability. *In* "Annual Review of Nutrition" (R. E. Olson, D. M. Brier, and D. B. McCormick, Eds.). Annual Reviews Inc., Palo Alto, CA.

Burton, G. W., Traber, M. G., Acuff, R. V., Walters, D. N., Kayden, H., Hughes, L., and Ingold, K. U. (1998). Human plasma and tissue alpha-tocopherol concentrations in respons to supplementation with deuterated natural and synthetic vitamin E. *Am. J. Clin. Nutr.* **67,** 669–684.

Børsting, C. F., Engberg, R. M., Jensen, S. K., and Damgaard, B. M. (1998). Effects of high amounts of dietary fish oil of different oxidative quality on performance and health of growing-furring male mink (*Mustela vision*) and nursing female mink. *J. Anim. Physiol. Anim. Nutr.* **79,** 210–223.

Cohen, N., Scott, C. G., Neukom, C., Lopresti, R. J., Weber, G., and Saucy, G. (1981). Total synthesis of all eight stereoisomers of α-tocopheryl acetate. Determination of their diastereoisomeric and enantiomeric purity by gas chromatography. *Helv. Chim. Acta* **64,** 1158–1173.

Cortinas, L., Baroeta, A. C., Galobart, J., and Jensen, S. K. (2004). Distribution of α-tocopherol stereoisomers in liver and thigh of chickens. *Br. J. Nutr.* **92,** 295–301.

DellaPenna, D. (2005). A decade of progress in understanding vitamin E synthesis in plants. *J. Plant Physiol.* **162,** 729–737.

Derendorf, H., Lesko, L. J., Chaikin, P., Colburn, W. A., Lee, P., Miller, R., Powell, R., Rhodes, G., Stanski, D., and Venitz (2000). Pharmacokinetic/pharmacodynamic modeling in drug research and development. *J. Clin. Pharmacol.* **40,** 1399–1418.

Drotleff, A. M., and Ternes, W. (2001). Determination of RS, E/Z-tocotrienols by HPLC. *J. Chromatogr. A* **909,** 215–223.

Evans, H. M., and Bishop, K. S. (1923). Existence of a hitherto unknown dietary factor essential for reproduction. *J. Am. Med. Assoc.* **81,** 889–892.

Fechner, H., Schlame, M., Guthmann, F., Stevens, P. A., and Rüstow, B. (1998). α- and δ-tocopherol induce expression of hepatic α-tocopherol-tranfer protein mRNA. *Biochem. J.* **331,** 577–581.

Harris, P. L., and Ludwig, M. I. (1949). Relative vitamin E potency of natural and of synthetic alpha-tocopherol. *J. Biol. Chem.* **179,** 1111–1115.

Hidiroglou, N., McDowell, L. R., Papas, A. M., Antapli, M., and Wilkinson, N. S. (1992). Bioavailability of vitamin E compounds in lambs. *J. Anim. Sci.* **70,** 2556–2561.

Hosomi, A., Arita, M., Sato, Y., Kiyose, C., Ueda, T., Igarashi, O., Arai, H., and Inoue, K. (1997). Affinity for α-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. *FEBS Lett.* **409,** 105–108.

Hosomi, A., Goto, K., Kondo, H., Iwatsubo, T., Yokota, T., Ogawa, M., Arita, M., Aoki, J., Arai, H., and Inoue, K. (1998). Localization of α-tocopherol transfer protein in rat brain. *Neurosci. Lett.* **256,** 159–162.

Ingold, K. U., Burton, G. W., Foster, D. O., Hughes, L., Lindsay, D. A., and Webb, A. (1987). Biokinetics of and discrimination between dietary RRR- and SRR-alpha-tocopherols in the male rat. *Lipids* **22,** 163–172.

IUPAC-IUB Joint Commission on Biochemical Nomenclature (1981). "Nomenclature of Tocopherols and Related Compounds" (G.P. Moss, Ed.). http://www.chem.qmul.ac.uk./iupac/misc/toc.html

Jensen, S. K., Engberg, R. M., and Hedemann, M. S. (1999). *All-rac*-alpha-tocopherol acetate is a better vitamin E source than all-*rac*-alpha-tocopherol succinate for broilers. *J. Nutr.* **129,** 1355–1360.

Jensen, S. K., Kristensen, N. B., Lauridsen, C., and Sejersen, K. (2005). Enrichment of cows' milk with natural or synthetic vitamin E. *In* "Proceedings of the 10th Symposium on Vitamins and Additives in Nutrition of Man and Animal." 28–29 September, pp. 78–83. Jena/Thuringia, Germany.

Jensen, S. K., Nørgaard, J. V., and Lauridsen, C. (2006). Bioavailability of α-tocopherol stereoisomers in rats depends on dietary doses of *all-rac*-a-tocopheryl acetate or RRR-a-tocopheryl acetate. *Br. J. Nutr.* **95,** 477–487.

Kim, H.-S., Arai, H., Arita, M., Sato, Y., Ogihara, T., Inoue, K., Mino, M., and Tamai, H. (1998). Effect of α-tocopherol status on α-tocopherol transfer protein expression and its messenger RNA level in rat liver. *Free Radic. Res.* **28,** 87–92.

Kiyose, C., Muramatsu, R., Ueda, T., and Igarashi, O. (1995). Rats after intravenous administration. *Biosci. Biotech. Biochem.* **59,** 791–795.

Kiyose, C., Muramatsu, R., Kameyama, Y., Ueda, T., and Igarashi, O. (1997). Biodiscriminaion of α-tocopherol stereoisomers in humans after oral administration. *Am. J. Clin. Nutr.* **65,** 785–789.

Knarreborg, A., Lauridsen, C., Engberg, R. M., and Jensen, S. K. (2004). Dietary antibiotic growth promoters enhance the bioavailability of α-tocopheryl acetate in broilers through mediations of the lipid absorption processes. *J. Nutr.* **134,** 1487–1492.

Lauridsen, C., and Jensen, S. K. (2005). Influence of supplementation of *all-rac*-α-tocopheryl acetate preweaning and vitamin C postweaning on α-tocopherol and immune responses of piglets. *J. Anim. Sci.* **83,** 1274–1286.

Lauridsen, C., and Jensen, S. K. (2006a). Transfer of vitamin E in milk to the newborn. *In* "The Encyclopedia of Vitamin E" (V. R. Preedy and R. R. Watson, Eds.), Chapter 47, pp. 508–516. CABI International, Wallingford, Oxfordshire, UK.

Lauridsen, C., and Jensen, S. K. (2006b). Forms of vitamin E and α-tocopherol status of weaned piglets. *In* "Proceedings of the 19th International Pig Veterinary Society Congress," 16–19 July, p. 293. Copenhagen, Denmark.

Lauridsen, C., Hedemann, M. S., and Jensen, S. K. (2001a). Hydrolysis of tocopheryl and retinyl esters by porcine caboxyl ester hydrolase is affected by their carboxylate moiety and bile acids. *J. Nutr. Biochem.* **12,** 219–224.

Lauridsen, C., Leonard, S. W., Griffin, D. A., Liebler, D. C., McClure, T. D., and Traber, M. G. (2001b). Quantitative analysis by liquied chromatography-tandem mass spectrometry of deuterium-labeled and unlabeled vitamin E in biological samples. *Anal. Biochem.* **289,** 89–95.

Lauridsen, C., Engel, H., Morrie Craig, A., and Traber, M. G. (2002a). Relative bioactivity of dietary RRR- over *all-rac*-α-tocopheryl acetatetes in swine assessed with deuterium-labeled vitamin E. *J. Anim. Sci.* **80,** 702–707.

Lauridsen, C., Engel, H., Jensen, S. K., Morrie Craig, A., and Traber, M. G. (2002b). Lactating sows and suckling piglets preferentially incorporate RRR- over *all-rac*-α-tocopherol into milk, plasma and tissues. *J. Nutr.* **132,** 1258–1264.

Lodge, J. K. (2005). Vitamin E bioavailability in humans. *J. Plant Physiol.* **162,** 790–796.

Mahan, D. C., Kim, Y. Y., and Stuart, R. L. (2000). Effect of vitamin E sources (RRR- or *all-rac*-α-tocopheryl acetate) and levels on sow reproductive performance, serum, tissue, and milk α-tocopherol contents over a five-parity period, and the effects on the progeny. *J. Anim. Sci.* **78,** 110–119.

Meglia, G. E., Jensen, S. K., Lauridsen, C., and Waller, K. P. (2006). α-Tocopherol concentration and stereoisomer composition in plasma and milk from dairy cows fed natural or synthetic vitamin E around calving. *J. Dairy Res.* **73,** 227–234.

Moore, A., Burton, G. W., and Ingold, K. U. (1994). Modulation of cholesterol esterase's diastereoselectivity by chiral auxiliaries. The very large effect of a dihydroxy bile acid on the hydrolysis of α-tocoperyl acetates. *J. Am. Chem. Soc.* **116,** 6945–6946.

Müller, D. P. R., Manning, J. A., Mathias, P. M., and Harries, J. T. (1976). Studies of the intestinal hydrolysis of tocopheryl esters. *Int. J. Vitam. Nutr. Res.* **46,** 207–210.

Nelis, H. J., D'Haese, E., and Vermis, K. (2000). Vitamin E. *In* "Modern Chromatographic Analysis of Vitamins" (A. P. Leenheer, W. E. Lambert, and J. F. van Bocxlaer, Eds.), 3rd ed., pp. 143–228. Marcel Dekker, Inc., New York.

Nielsen, H. E., Danielsen, V., Simesen, M. G., Gissel-Nielssen, G., Hjarde, W., Leth, T., and Basse, A. (1979). Selenium and vitamin E deficiency in pigs. 1. Influence on growth and reproduction. *Acta Vet. Scand.* **20,** 276–288.

NRC (1998). "Nutrient Requirements of Swine," 10th ed. National Academy Press, Washington, DC.

Ong, D. E. (1985). Vitamin A binding proteins. *Nutr. Rev.* **43,** 225–232.

Packer, L. (1994). Vitamin E is nature's master antioxidant. *Sci. Am. Sci. Med.* **1,** 54–63.

Scherf, H., Machlin, L. J., Frye, T. M., Krautmann, B. A., and Williams, S. N. (1996). Vitamin E biopotency: Comparison of various 'natural-derived' and chemically synthesized alpha-tocopherols. *Anim. Feed Sci. Tech.* **59,** 115–126.

Traber, M. G. (2000). The bioavailability bugaboo. *Am. J. Clin. Nutr.* **71,** 1029–1030.

Traber, M. G., Sokol, R. J., Burton, G. W., Ingold, K. U., Papas, A. M., Huffaker, J. E., and Kayden, H. J. (1990). Impaired ability of patients with familiar isolated vitamin E deficiency to incorporate α-tocopherol into lipoproteins secreted by the liver. *J. Clin. Investi.* **85,** 397–407.

Ueda, T., Ichikawa, H., and Igarashi, O. (1993). Determination of α-tocopherol-tocopherol stereoisomers in biological speciments using chiral phase igh-performance liquid chromatography. *J. Nutr. Vitaminol.* **39,** 207–219.

Vecchi, M., Walther, W., Glinz, E., Netscher, T., Schmid, R., Lalonde, M., and Wetter, W. (1990). Chromatographische Trennung und quantitative Bestimmung aller acht Stereoisomeren von α-Tocopherol (Separation and quantitation of all eight stereoisomers of α-tocopherol by chromatography). *Helv. Chim. Acta* **73,** 782–789.

VERIS Research summary (1999). A comparison of natural and synthetic vitamin E. http://www.veris-online.org

Weiser, H., and Vecchi, M. (1981). Stereoisomers of α-tocopheryl acetate. II. Characterization of samples by physico-chemical methods and determination of biological acitivities in the rat resorption-gestation tests. *Int. J. Vitam. Nutr. Res.* **51,** 100–113.

Weiser, H., and Vecchi, M. (1982). Stereoisomers of α-tocopheryl acetate. II. Biopotencies of all eight stereoisomers, induvidually or in mixtures, as dertermined by rat resorption-gestation tests. *Int. J. Vitam. Nutr. Res.* **52,** 351–370.

Weiser, H., Riss, G., and Kormann, A. W. (1996). Biodiscrimination of the eight alpha-tocopherol stereosismers result in preferential accumulation of the four 2R forms in tissues and plasma of rats. *J. Nutr.* **126,** 2539–2549.

Yamauchi, J., Iwamoto, T., Kida, S., Massushige, S., Yamada, K., and Esashi, T. (2001). Tocopherol-associated protein is a ligand-dependent transcriptional activator. *Biochem. Biophys. Res. Commun.* **285,** 295–299.

Zimmer, S., Stocker, A., Sarbolouki, M. N., Spycher, S. E., Sassoon, J., and Azzi, A. (2000). A novel human tocopherol-associated protein: Cloning, *in vitro* expression, and characterization. *J. Biol. Chem.* **275,** 25672–25680.