"Vitamin CE," a Novel Prodrug Form of Vitamin E

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Reaction of 5a-bromo-α-tocopherol with ascorbic acid produces 5a-tocopheryl ascorbate which is designated "vitamin CE." This novel tocopherol derivative represents an interesting prodrug form of α-tocopherol (vitamin E) that is stable under acidic conditions, but regenerates finely dispersed vitamin E in basic media. The reaction mechanism of the base-induced decomposition of vitamin CE involves elimination of ascorbate and production of an *ortho*-quinone methide intermediate that oxidizes ascorbate, and is reduced to vitamin E. Kinetic experiments showed the reaction to proceed in the pH range of 8 to 11 under physiological conditions. Tissue culture measurements demonstrated that vitamin E generated from the novel derivative is absorbed at much higher rates than conventional preparations and can even be absorbed under simulated conditions of malabsorption where there is no uptake of conventional vitamin E medications.

5a-Bromo-α-tocopherol (**1**) is obtained in quantitative yield by bromination of α-tocopherol with elemental bromine.[1] The compound is a versatile starting material for the preparation of 5a-substituted tocopherols and can be used as an auxiliary in synthesis,[2] a starting material for new tocopherol compounds,[3] and as a lipophilic drug carrier.[4] In the latter case, it renders the attached drug more lipophilic and, thus, more easily transportable into lipidic tissues, such as membranes.

The reaction of **1** with ascorbic acid (vitamin C, **2**) produces 5a-tocopheryl ascorbate (**3**) which is conveniently named "vitamin CE" (Fig. 1). The preparation of **3** must be carried out under non-basic conditions. Basic media lead to elimination of HBr from the starting material, producing the intermediate *ortho*-quinone methide (**4**) that undergoes subsequent reactions, either a hetero-Diels–Alder reaction to form the spiro-dimer of α-tocopherol (**5**)[5] or a rearrangement to *para*-tocopheryl quinone (**6**).[6] Consequently, basic auxiliaries as well as the application of pure sodium ascorbate as the co-reactant have to be avoided.

In the optimized experimental procedure, a mixture of sodium ascorbate with a tenfold excess of ascorbic acid was used to ensure the absence of basic conditions while maintaining a high reactivity. The reaction proceeds with moderate yields in any polar aprotic organic solvent. However, only dimethyl sulfoxide (DMSO) allows a virtually quantitative conversion of the reactants to the product. This behavior can be attributed to the fact that all starting compounds are soluble in DMSO causing a ready Williamson-type reaction, whereas in the case of other polar aprotic solvents at least the ascorbate remains insoluble causing a much slower two-phase reaction with a higher rate of the formation of the above-mentioned byproducts. The product **3**, a yellowish wax like α-tocopherol itself, is stable under acidic conditions and remains unaffected even by extended refluxing with 1 N mineral acids. In contrast, basic treatment of **3** leads to immediate deprotonation at the phenolic hydroxyl group followed by elimination of the ascorbate anion and formation of the *ortho*-quinone methide of α-tocopherol (**4**). This reaction path is typical of 5a-substituted tocopherols.[3] The intermediacy of **4** has been demonstrated by trapping reactions analogous to those previously reported.[1] In the case of 5a-tocopheryl ascorbate (**3**), however, the separated moieties of ascorbate and *ortho*-quinone methide join in a redox process in which ascorbate is oxidized to dehydroascorbate while the *ortho*-quinone methide (**4**) is reduced to α-tocopherol (see Fig. 2), or to the α-tocopherolate anion at a higher pH value. The yield of this reaction, usually above 85%, can be increased by additional ascorbic acid as would be expected from the reaction mechanism. One additional equivalent of ascorbic acid, for instance, allows a 96% reconversion of α-tocopherol.

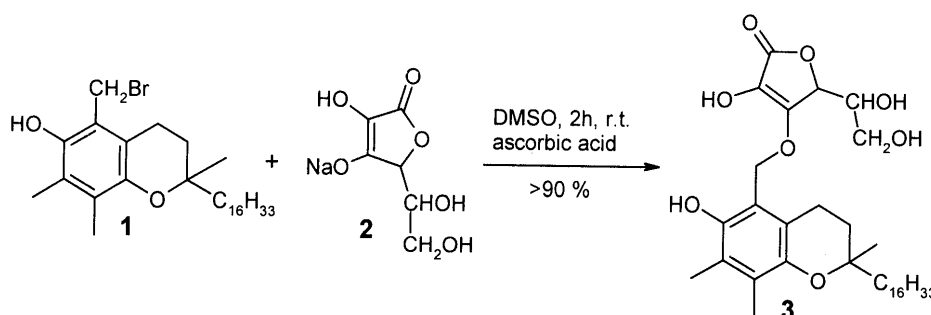The regenerated α-tocopherol is produced in a finely

Fig. 1.　Preparation of 5a-Tocopheryl Ascorbate (**3**)
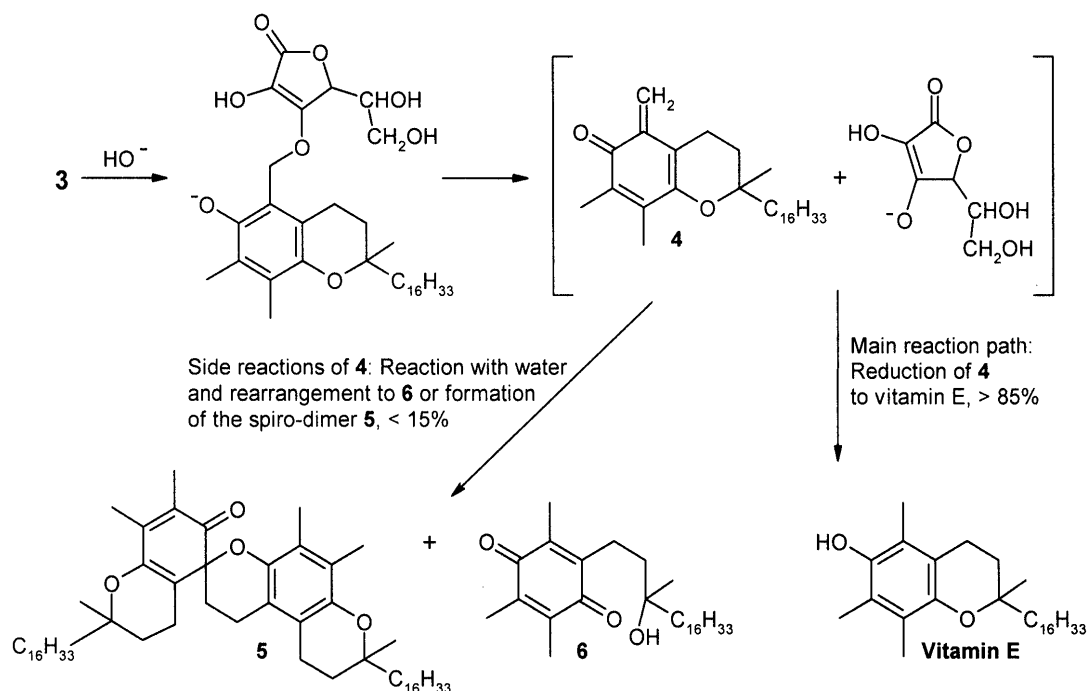
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Fig. 2. Regeneration of Vitamin E by Base-Induced Decomposition of 5a-Tocopheryl Ascorbate (3)

dispersed form, which can be observed either phenomeno-logically or by absorption experiments as described below. The byproducts of the regeneration of vitamin E, mainly para-tocopheryl quinone (6) and trace amounts of the spiro-dimer of α-tocopherol (5), are both natural metab-olites of vitamin E and fully biologically compatible. Before testing the compound in cell culture experiments, the kinetics of the base-induced decomposition of vitamin CE to α-tocopherol, i.e., the drug release from the prodrug form, was investigated. The dependence of the turnover on the pH value at different reaction times is illustrated in Fig. 3. The pH value required for the reaction to proceed corresponds with the basicity in the intestine, a prerequisite for the use of vitamin CE in cell culture or in in vivo experiments. Vitamin CE (3) becomes an illustrative example of the concept of pH-dependent drug release.

Many of the factors and processes necessary for the absorption of dietary lipids are also required for the absorption of tocopherol. Tocopherol must be emulsified and solubilized before its absorption across the brush-border membrane of the intestinal cells.[7] Emulsification begins in the stomach by mechanical forces that break up large particles into smaller ones. Within the small intestine, chyme is mixed with pancreatic and biliary secretions, which are necessary for the efficient absorption of vitamin E. Pancreatic lipase is required for the hydrolysis of triglycerides to monoglycerides and fatty acids. These lipolytic products—together with bile salts that are pro-duced by the liver and secreted into the small intestine by the gallbladder—form molecular aggregates, so-called mixed micelles. These micelles with a radius of 15—50 Å are able in turn to solubilize hydrophobic molecules, such as tocopherol, and are absorbed through the cell mem-branes by passive diffusion. Consequently, tocopherol can only be absorbed if both the biliary and the pancreatic factors are present in sufficient amounts.[7,8]
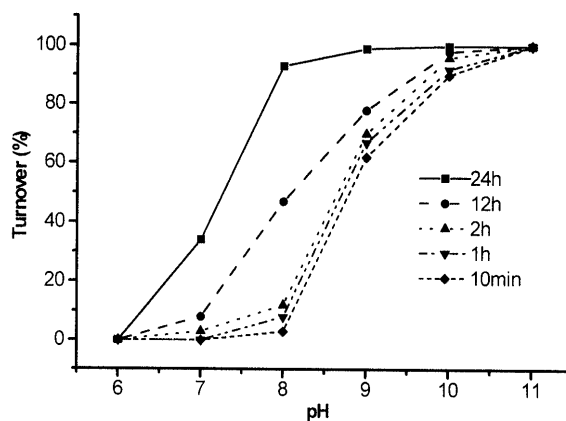

Fig. 3. Decomposition of Vitamin CE. Turnover versus pH Value at Different Reaction Times

The turnover expresses the degree of decomposition of compound 3 and is calculated as follows: turnover (%) = ([ascorbic acid] + [dehydroascorbic acid]) × [starting material $(3)]_0^{-1}$ × 100. The amount of unreacted ascorbate is equal to the amount of para-tocopheryl quinone (6) formed, and is a measure of the degree of side-reactions. As a proof of the high reconversion of vitamin E from 5a-tocopheryl ascorbate (3), the amounts of ascorbate and dehydroascorbate (in parentheses) are listed for the physiologically relevant pH values 8 and 9, both in per cent of the starting material 3: pH 8: 10 min: <0.3 (3), 1 h: 0.7 (8), 2 h: 1 (11), 12 h: 4 (43), 24 h: 8 (85); pH 9: 10 min: 5 (57), 1 h: 5.5 (62), 2 h: 6 (64), 12 h: 6.5 (72), 24 h: 8 (91). The ratio of dehydroascorbate to ascorbate can easily be calculated from these values and is 11 ± 0.4 for both pH 8 and 9. The ratio of α-tocopherol to para-tocopheryl quinone at the respective times is 11 ± 0.3 for pH 8 and 11 ± 0.2 for pH 9. The ratios at the other pH values tested are almost identical with those listed here. The ratio of α-tocopherol to para-tocopheryl quinone or dehydro-ascorbate to ascorbate, which is the ratio of the main reaction to side-reactions, is consequently largely independent of the pH.

α-Tocopherol derived from vitamin CE is produced exclusively extracellularly. Once the vitamin E has been generated, it is absorbed in the above-described manner exactly as genuine α-tocopherol. The extracellular de-composition of 3 to vitamin E and ascorbate is proved by the fact that the plasma tocopherol content increases through absorption, whereas the plasma ascorbate/dehy-
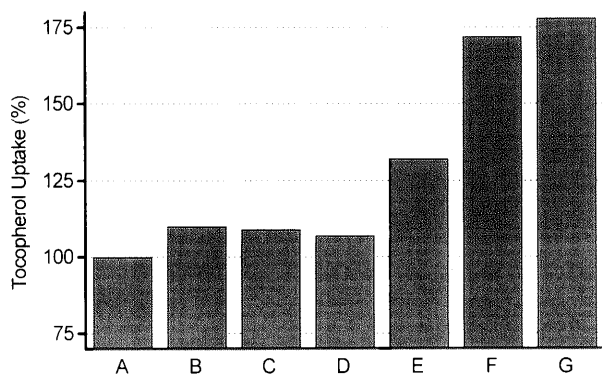
Fig. 4. Absorption of Vitamin E from Different Sources in Small Intestinal Cells after an Incubation Time of 2 h (for Details See Experimental)

A, normal content before absorption, (100%); B, α-tocopherol; C, α-tocopherol acetate; D, α-tocopherol + 1 eq sodium ascorbate; E, tocopheryl polyethylene glycol–1000 succinate (TPGS); F, vitamin CE; G, vitamin CE + 1 eq sodium ascorbate.
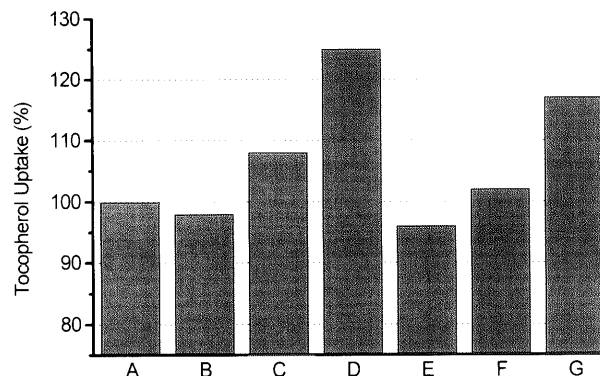
Fig. 5. Absorption of Vitamin E from Different Sources in Small Intestinal Cells under Simulated Conditions of Malabsorption after an Incubation time of 2 h (see Experimental)

A, normal content before absorption, (100%); B, α-tocopherol, absence of bile salts; C, TPGS, absence of bile salts; D, vitamin CE, absence of bile salts; E, α-tocopherol, absence of pancreatic juice; F, TPGS, absence of pancreatic juice; G, vitamin CE, absence of pancreatic juice.

droascorbate level remains unchanged. In contrast to natural α-tocopherol which must be thoroughly emulsified before forming mixed micelles and being absorbed, vitamin E generated from the new prodrug form **3** is produced by a chemical reaction as a fine dispersion. Thus, formation of micelles with subsequent absorption is greatly facilitated. It is even conceivable that this thoroughly dispersed form of α-tocopherol can be absorbed directly without prior formation of mixed micelles, as suggested by the results shown in Fig. 5 (see below). This, however, requires conformation. The facile absorption of α-tocopherol regenerated from 5a-tocopheryl ascorbate (**3**) could be demonstrated in uptake experiments with a small intestinal cell line: the new prodrug form of vitamin E was compared with traditional vitamin E preparations (Fig. 4).

With the average tocopherol concentration of healthy, normolipodemic intestinal cells set at 100%, uptake of α-tocopherol or α-tocopherol acetate under standard conditions for absorption experiments[9] caused an increase of about 10%, in agreement with results previously reported.[9,10] The effect in the case of a blank run—with α-tocopherol and ascorbic acid added as two separate compounds in an 1 : 1 ratio—is of similar magnitude. With vitamin CE (**3**) as the novel prodrug form of vitamin E, there is a 70% increase in the tocopherol content of the cell, or even more in case of one equivalent of ascorbate added. The latter effect is due to a better reconversion of the intermediate ortho-quinone methide (**4**) to α-tocopherol by the equivalent of ascorbate as an additional reductant, and results in higher tocopherol concentrations. 5a-Tocopheryl ascorbate (**3**) even outperforms tocopheryl polyethylene glycol–1000 succinate (TPGS), the only oral preparation of vitamin E known so far that appears to be absorbed by patients in early stages of certain diseases.[11]

In patients with liver diseases or biliary obstruction, luminal bile salt concentrations are less than the critical micellar concentration. Therefore, tocopherol cannot be solubilized in the small intestine.[12] Also, patients with pancreatic insufficiency, i.e., patients secreting a reduced amount of pancreatic enzymes, show drastically impaired absorption of vitamin E, presumably caused by a failure

to hydrolyze triglycerides and thereby to form mixed micelles.[13] Both groups of patients exhibit very low serum concentrations of α-tocopherol, but are unfortunately unable to absorb conventional oral preparations of vitamin E.[12] In these cases, the new prodrug form, vitamin CE (**3**), promises a solution: as already indicated, the compound is able to regenerate α-tocopherol in a fast chemical reaction as a finely dispersed form, almost on a molecular level. The resulting facilitated absorption in small intestinal cells has been demonstrated in another experiment, as illustrated in Fig. 5.

Here, conditions of vitamin E malabsorption were simulated by depleting the incubation medium of factors necessary for the formation of mixed tocopherol micelles. These substances are mainly bile salts, besides fatty acids and monoglycerides that are formed by the action of lipolytic pancreatic enzymes. Surprisingly, in the absence of biliary or pancreatic factors, when normal vitamin E preparations and even TPGS cannot be absorbed, there is still uptake of α-tocopherol generated from vitamin CE (see Fig. 5). This opens the way for new, convenient vitamin E medications that can replace conventional treatments, such as extremely large oral vitamin doses, replacement therapies, or non-oral administration.

In summary, we have described the synthesis of a novel prodrug form of vitamin E, 5a-tocopheryl ascorbate (**3**). The compound does not contain α-tocopherol in the traditional form of an ester or ether, but as a 5a-substituted derivative that generates finely dispersed α-tocopherol under basic conditions. The kinetics of this drug release has been investigated. Absorption experiments demonstrated that the new vitamin E preparation was superior to conventional vitamin E preparations.

Experimental

in tocopherol derivatives follows IUPAC recommendations.[14] Chemicals as well as ingredients for culture medium, stock solutions and tocopherol preparations were obtained from Sigma–Aldrich Chemie GmbH, unless otherwise indicated.

Preparation of 3-Hydroxy-5-(1,2-dihydroxyethyl)-4-[6-hydroxy-2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)-chroman-5-ylmethoxy]-furan-2(5H)-one (5a-Tocopheryl Ascorbate, 3) In a 250 ml flask equipped with an efficient magnetic stirrer, a mixture of dry and finely powdered sodium ascorbate (10 mmol, 1.981 g), powdered ascorbic acid (50 mmol, 8.806 g), and DMSO (50 ml) was stirred at 60 °C for 2 h and then cooled to room temperature. Under stirring, a solution of 5a-bromo-α-tocopherol (1) (10 mmol, 5.096 g) in tetrahydrofuran (THF) (10 ml) and DMSO (10 ml) was added within 2 h in an inert atmosphere. The mixture was stirred at 50 °C for 3 h and cooled to room temperature. Then 100 ml of n-hexane and 50 ml of water were added and the phases were separated. The aqueous layer containing the major portion of the DMSO was extracted twice with n-hexane, and the combined organic phases were washed once with 20 ml of 1 N HCl, and then with water until they were free of DMSO, normally 8 to 10 times. The obtained solution was carefully dried over sodium sulfate and absorbed on acidic aluminum oxide (60 g). The yellowish aluminum oxide was washed with diethyl ether until the filtrate was colorless (appr. 150 ml). Finally, the desired product was eluted with methanol. The obtained solution contained pure vitamin CE. Removal of the solvent under reduced pressure at room temperature produced pure 3 as a yellow wax in 63% yield. ^{1}H-NMR (DMSO-d_6/CDCl$_3$) δ: 2.11 (s, 3H, C$\underline{H}_3$-Ar), 2.13 (s, 3H, C$\underline{H}_3$-Ar), 2.63 (t, 2H, Ar-C$\underline{H}_2$-CH$_2$), 3.6 (d, 2H, CH-CH(OH)-C$\underline{H}_2$OH), 3.7 (m, 1H, CH-C$\underline{H}$(OH)-CH$_2$OH), 4.63 (s, 2H, Ar-C$\underline{H}_2$-O), 4.77 (d, 1H, C$\underline{H}$-CH(OH)-CH$_2$OH). The strong resonances of the isoprenoid side chain below 2.0 ppm are not listed. ^{13}C-NMR (DMSO-d_6/CDCl$_3$) δ: 11.8 (C-8b), 12.1 (C-7a), 19.5 (C-4a′), 19.6 (C-8a′), 20.8 (C-4), 21.0 (C-2′), 22.5 (C-13′), 22.6 (C-12a′), 23.6 (C-2a), 24.5 (C-6′), 24.8 (C-10′), 27.9 (C-12′), 31.6 (C-3), 32.6 (C-8′), 32.7 (C-4′), 37.3 (C-7′), 37.4 (C-5′), 37.5 (C-9′), 37.6 (C-3′), 39.4 (C-11′), 39.8 (C-1′), 61.9 (–CH$_2$OH), 64.8 (C-5a), 71.0 (C$\underline{H}$(OH)-CH$_2$OH), 74.3 (C-2), 77.9 (C$\underline{H}$-CH(OH)-CH$_2$OH), 115.1 (C^{Ar}), 116.1 (C^{Ar}), 119.5 (=$\underline{C}$-O-CH$_2$), 123.0 (C^{Ar}), 125.5 (C^{Ar}), 144.6 (C^{Ar}), 147.3 (C^{Ar}), 156.8 (=C-OH), 173.8 (CO). MALDI-TOF-MS m/z: 606 (M+H$^+$). Anal. Calcd for C$_{35}$H$_{56}$O$_8$ (604.84): C, 69.51; H, 9.33. Found: C, 69.68; H 9.51.

Kinetic Measurements of the Base-Induced Decomposition of 3 A 100 mg sample of 3 in 5 ml of aqueous methanol (v/v = 1 : 1) was added to 10 ml of buffer solution and 2 g of coarse-grained SiO$_2$. The pH was set to integral values between 6 and 14 with citrate/dihydrogenphosphate buffer according to McIlvaine for pH 6—7, glycine/borate buffer according to Sörensen for pH 8—12, and NaOH in brine for pH 13—14. The mixture was vigorously shaken over a specific period of time. Afterwards, the reaction was stopped by addition of 5 ml of 1 N HCl. The solids were filtered off and washed with acetone (2 ml), and the washing was added to the filtrate. The resulting aqueous mixture was extracted twice with 5 ml of n-hexane and the phases were carefully separated. The organic phase was washed with 5 ml of water, dried over NaSO$_4$, and analyzed by GC. The ratio of α-tocopherol and para-tocopheryl quinone was determined as the average value of two GC runs. Unreacted 3 that was also present in the organic phase cannot be detected by GC, and did not interfere with the measurement. The aqueous phase was divided into two equal volumes to allow verification by a second run. Ascorbic acid and dehydroascorbic acid were quantified according to literature procedures[15] by redox titration with 2,6-dichlorophenol-indophenol. The total of ascorbic acid and dehydro-ascorbic acid is equal to the turnover, i.e., the amount of 3 that reacted. The ratio of α-tocopherol and para-tocopheryl quinone obtained from GC experiments, and the ratio of dehydroascorbic acid and ascorbic acid determined by titration must be equal according to the underlying reaction mechanism. In all cases, the two corresponding values obtained differed from each other by less than 3%, thereby proving the accuracy of the procedure applied (cf. Fig. 3).

Absorption Experiments Cell Culture, Absorption Media and Tocopherol Preparations: A human intestinal cell line (Caco-2 cells) in tissue culture was used in absorption experiments according to the method of Traber et al.[9] The cells were cultured in medium consisting of Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum (FCS), 20 mM HEPES pH 7.3, 200 mg/l streptomycin, 200000 U/l penicillin and 1 mM α-tocopherol. Seven days before the absorption experiments the cells were dissociated and replated. For the absorption measurements 2 ml of medium (approximately 2×10^6 cells) was used on 60-mm dishes at 37 °C. Four stock solutions containing the following components were prepared. Solution A: 0.2 M Tris, pH 8.2, 0.15 M NaCl, 1% bovine serum albumin (BSA), 10 mM sodium taurocholate and 10 mM sodium cholate; solution B: 0.2 M Tris, pH 8.2, with 20% gum arabic; solution C: 10 mM glyceryl trioleate, 10 mM glyceryl oleate and 10 mM oleic acid in n-hexane; solution D: 0.1 M Tris, pH 7.5, 0.15 M NaCl. α-Tocopherol and α-tocopherol acetate were commercial products (Aldrich) and were used without further purification. Vitamin CE was prepared according to the above procedure. Tocopheryl polyethylene glycol–1000 succinate (TPGS, Eastman Kodak), obtained as a 10% solution in distilled water, was sterilized by ultrafiltration and stored at −5 °C. Pancreatic juice standard was frozen at −70 °C until used in the experiments. Stock solutions of all vitamin E derivatives in n-hexane (distilled water in the case of TPGS) containing exactly 0.1 mM of the respective vitamin E compounds were prepared.

Incubation Protocol: All samples were protected from light during the incubation and analyses. Solution C (3 ml) and the vitamin-derivative solution (3 ml) were mixed and dried under nitrogen. (In the case of TPGS, only an aliquot of solution C was taken to dryness, and an aliquot of TPGS solution was added directly to the mixture to be sonicated.) The residue was sonicated for 1 min at 0 °C with 5 ml of solution B. Then 15 ml of solution A was added, and the mixture was again sonicated for 3 min. The resulting emulsion was used directly for the absorption experiments. Cells were washed with DMEM, then 2 ml of DMEM, 2 ml of the respective vitamin E-containing emulsion, and 100 μl of pancreatic enzyme solution were added. The dishes were incubated for 2 h (see Figs. 4 and 5), and the cells were analyzed to determine tocopherol absorption. To study the absorption in the case of the absence of certain components, we followed the above given procedure, but without the addition of either pancreatic juice, or bile salts (see Fig. 5).

Analyses: The cells were washed with stock solution C, incubated with 5 ml of this solution for 10 min, and again washed twice with solution C. Then 1 ml of 1% sodium bisulfite in distilled water was added to the cell suspension which was then made up to a total volume of 3 ml with ethanol. The free tocopherol content was determined by sonicating the mixture for 15 min in a water-bath sonifier. Distilled water (2 ml) was added. By shaking for 10 min, the tocopherol was extracted into 5 ml of n-hexane. A 2 ml aliquot of the n-hexane was dried under nitrogen, the residue was dissolved in 0.2 ml methanol, and tocopherol was determined by HPLC (C$_{18}$ column, reversed phase) with electrochemical detection. The α-tocopherol content in the case of no addition of vitamin E preparation to the incubation medium corresponds to the α-tocopherol content of a normal intestinal cell. After determination, the latter value obtained was arbitrarily set at 100% to allow the application of a relative scale. All values obtained are means of triplicate samples with duplicate HPLC determination. Differences were considered statistically significant at the 95% confidence level (F-test).

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