Transport of Alpha-Tocopherol and Its Derivatives Through Erythrocyte Membranes

Francesco Bonina,[1] Maria Lanza,[2] Lucia Montenegro,[1] Loredana Salerno,[1] Patrizia Smeriglio,[2] Domenico Trombetta,[2] and Antonella Saija[2,3]

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Purpose. To investigate the transport of α-tocopherol (T), tocopherol succinate (TS) and tocopherol succinate-3-glucose (a newly synthetized, less hydrophobic T ester; TSG) through bovine erythrocyte membranes.

Methods. Our experiments were carried out on erythrocytes (obtained from heparinized fresh bovine blood), because they represent a suitable model for investigations of membrane transport.

Results. T was shown to reside almost completely in the suspension medium, while the greater part of TS disappeared from the suspension medium and was mainly incorporated into erythrocyte membranes. In comparison with T, a larger amount of TSG was incorporated into erythrocyte membranes and taken up by cells; however the TSG intracellular accumulation was significantly lower than that observed with TS. Furthermore, the transport of TS and TSG was partially inhibited by *p*-chloromercuribenzenesulfonate (which inhibits monocarboxylate uptake; PCMBS) and by maltose (a competitive inhibitor of glucose transport) respectively, with a concomitant increase in drug membrane incorporation. No significant change in drug transport was observed in the presence of 4,4′-diisothiocyanostilbene-2,2′-disulfonate, a selective and irreversible blocker of band 3 protein (DIDS).

Conclusions. Our results show 1) the existence of large differences in membrane incorporation of T, TS and TSG (very likely caused by differing abilities to fill spaces in the lipid bilayer) and 2) a specific contribution of the monocarboxylate transport protein and of the glucose transport protein in the cellular uptake of TS and TSG, respectively. A tempting suggestion is that the unique cytoprotective properties of TS may be related to the differences in the transmembrane mobility observed between T and its succinate ester. Furthermore, T conjugation to a monocarboxylate or glycoside moiety could provide suitable substrates for active membrane transport, thus appearing as a promising pharmaceutical strategy for the improved delivery of tocopherol derivatives.

KEY WORDS: tocopherol; tocopherol esters; erythrocytes; biomembranes.

INTRODUCTION

Lipid peroxidation is a universal mechanism of modification and damage of biomembranes and is related to significant pathological events. α-Tocopherol (T), a widespread naturally occurring compound, functions as the major lipophilic antioxidant in biological systems because of its ability to terminate the peroxidative process (1). The antioxidant activity of T is determined by the aromatic fragment of the molecule, while the hydrocarbon chain is needed for its proper orientation in the membrane (2,3). T is practically insoluble in water and is readily oxidized by atmospheric oxygen. Ester derivatives of T, such as tocopherol succinate (TS), are commonly used for both experimental and therapeutic purposes because of their high stability to oxidation; they provide a stable dosage form of T that can be broken down by cellular esterases to supplement endogenous T (4). However, besides causing problems with regard to parenteral use, the water insolubility of T and its esters is very likely the predominant factor responsible for their much diminished absorption following oral administration.

Penetration of a drug into cells is often the first step necessary for its biological activity. The degree of incorporation and the uniform distribution into lipid bilayers, and the rate of transport into cells, are particular factors influencing the efficiency of antioxidant compounds (5). However, so far, there are few data concerning the transport of T and its esters through biological membranes.

In the present study, the transport of T and TS through bovine erythrocyte membranes has been investigated. In fact, erythrocytes are not adhesive, but freely suspended cells, contain no membrane other than the plasma membrane and their external surface is entirely exposed to the outer medium; so they are a suitable model for investigation of membrane transport (6,7). Furthermore, in the mammalian erythrocyte membrane there are many transport proteins, such as band 3 protein (7,8), GLUT1 glucose transport protein (9) and monocarboxylate transport protein (10). Thus, this experimental model allows us to obtain preliminary data concerning the mechanisms of membrane permeation of drugs (lipid passive diffusion, channel- or carrier-mediated transport).

Furthermore, by means of the same experimental model, we have also investigated the erythrocyte uptake of tocopherol succinate-3-glucose (TSG), a newly synthetized, less hydrophobic T ester. In fact, derivatization with a glycoside moiety has been recently demonstrated to be a successful tool for development of novel prodrugs able to permeate through biomembranes by means of active transport carriers (11–15).

MATERIALS AND METHODS

Preparation of Erythrocytes

The method reported by Matsumoto and Ohsako (6) and by Matsumoto et al. (7) was used. Briefly, heparinized fresh bovine blood, obtained from a local slaughter house, was centrifuged at 1630 × g for 15 min at 4°C and the supernatant plasma was separated. After removing the buffy coat, the erythrocytes were washed 3 times with 140 mM NaCl containing 10 mM phosphate buffer (pH 7.4; isotonic phosphate buffered saline, PBS) and resuspended in PBS.

Determination of Drug Incorporation into Erythrocytes

T, TS and TSG were dissolved in 10 mM phosphate buffer, at a concentration of 0.75 mM, using the minimum amount of EtOH and Tween 40 (100 μl and 130 μl, respectively, in 100 ml of PBS); NaCl was then added to yield a final osmotic

[1] Deparment of Pharmaceutical Sciences, School Pharmacy, University of Catania, Italy.
[2] Department Farmaco-Biologico, School Pharmacy, University of Messina, Italy.
[3] To whom correspondence should be addressed.

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pressure of 285 mOsM (drug-containing PBS). The medium added to control tubes was PBS containing the same volume of ethanol and Tween 40 used in drug-containing PBS.

The washed erythrocytes were suspended in sufficient drug-containing PBS to give an hematocrit value of 45% (v/v). The suspension was incubated with mild shaking at 37°C for 1 h. No hemolysis occurred during incubation or handling, both in control and drug-treated samples. A duration of 1 hour was chosen on the basis of results obtained in a previous series of experiments, showing that for all drugs tested, equilibrium was attained within 50–60 min.

The erythrocytes were then separated by centrifugation at 2900 × g for 15 min at 4°C and the quantity of drug in the supernatant was determined. The erythrocytes (precipitate) were homogenized with distilled water of volume equal to the sediment volume, and the resultant hemolysate was centrifuged at 725 × g and 16260 × g for 15 min to give hemolysate supernatant (cytosol component) and precipitate (the membrane) respectively. Before analysis an aliquot of the hemolysate supernatant was deproteinized with acetonitrile. The samples were stored at −70°C until analysis. No evidence of drug hydrolysis or oxidation, due to experimental procedures, was observed during chromatographic runs.

Effect of Three Uptake Inhibitors on Drug Transport Through Erythrocyte Membranes

The effect of 4,4′-diisothiocyanostilbene-2,2′-disulfonate (a selective and irreversible blocker of band 3 protein; DIDS) (7,8), *p*-chloromercuribenzenesulfonate (an organomercurial which specifically inhibits lactate and monocarboxylate transport; PCMBS) (10) and maltose (a competitive inhibitor of D-glucose uptake) (16) on drug transport through erythrocyte membranes was studied.

A suspension of the washed erythrocytes, having an hematocrit value of 45%, was treated with DIDS (100 μM) or PCMBS (0.6 mM) in the dark for 30 min at 37°C. Drug-containing PBS was then incubated with the treated cells or with intact cells (which served as a control) as described above. Maltose (75 mM) was dissolved, together with the drug to be tested, in 10 mM phosphate buffer and the final osmotic pressure was adjusted to 285 mOsM by adding the appropriate amount of NaCl; the experiment was then carried out as previously described.

Drug Determination

Drug analysis of each fraction was performed using an HPLC apparatus (Varian 5000 system, Varian, Walnut Creek, CA, U.S.A.) equipped with a 20 μl loop, a Polychrom 3060 UV/VIS detector (Varian), a Pecosphere HS-5 HC ODS column (particle size: 10 μ; 15 cm × 4.6 mm I.D.; Perkin-Elmer, Norwalk, CT, U.S.A.) and a 4290 integrator (Varian). Samples were filtered prior to injection using a Millex HV13 filter (0.45 μ; Waters-Millipore Corporation, Milford, MA, U.S.A.) and an aliquot (20 μl) was injected into the HPLC apparatus. The mobile phase was methanol-water (93.5:6.5) containing 2.8 mM 1-octanesulfonic acid sodium salt. Detection was carried out at 282 nm. The flow-rate was set at 1.6 ml/min.

Recovery from hemolysate showed satisfactory results. A known amount of T, TS or TSG was added to 0.5 ml of the hemolysate (obtained as described before); then the hemolysate was deproteinized with acetonitrile and analyzed for drugs. The following recovery percentages (mean ± S.D. of 5 determinations) were calculated: T 99.2 ± 0.57%; TS 98.6 ± 0.75%; TSG 99.5 ± 0.67%.

The results, expressed as mean ± S.D. of at least 3 experiments, were analyzed statistically by the Wilcoxon test for unpaired data; statistical significance was accepted when $P < 0.05$.

Log Capacity Factor (log K′)

Reverse-phase chromatographic retention times can be used to estimate oil/water partition coefficients; a good correlation is found between log octanol/water partition coefficients and log K′ using octadecyl silica columns (5). Log K′ values for T, TS and TSG were determined by HPLC with UV/visible detection, as previously described.

Each drug was dissolved in PBS to give a final concentration of 10 μg/ml and an aliquot (20 μl) of the solution was injected into the HPLC apparatus. Log K′ values were calculated from the following relationship:

$$\log K' = \log \frac{T_r - T_0}{T_0}$$

where T_r is the retention time of the drug peak and T_0 is the retention time of the non-retained solvent peak.

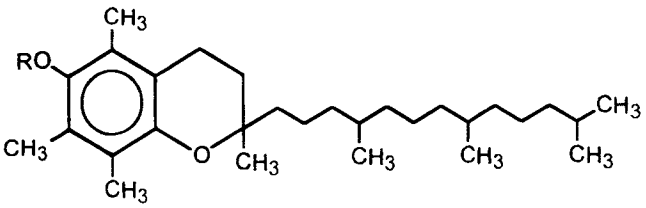
Synthesis of TSG

TSG, or [3-O-succinyltocopherole-(α,β)-D-glucopyranose] (compound **3,** fig. 1), was prepared by esterification of 1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose with (+)α-tocopherol acid succinate (compound **2,** fig. 1) in the presence of 1,3-dicyclohexylcarbodiimide (DCC) followed by successive acid hydrolysis of the protecting isopropylidene groups (17,18). Briefly, an ether solution of **2** (19.21 mmol), DCC (19.21 mmol), 1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose (19.21 mmol) and 4-pyrrolidino-pyridine (3 mmol) was stirred at room temperature overnight and then filtered to remove 1,3-dicyclohexyl urea. The ether phase, washed with 5% acetic acid solution (50 ml × 2) and then with water (50 ml × 2), was dried on anhydrous sodium sulphate; after which, the solvent was removed by vacuum. The residue obtained was warmed at 80–90°C for 5 min with a mixture (15 ml) of trifluoroacetic acid and water (9:1, v/v). After cooling, this solution was neutralized with drops of a saturated $NaHCO_3$ solution, then extracted with ethyl acetate and dried on anhydrous sodium sulphate. A white solid was obtained by reducing the solvent to a small volume; it was then filtered and crystalized from ethyl acetate to give a mixture of α and β anomers of pure **3**.

Elemental analysis was performed on a Carlo Erba Elemental analyzer model 1106; the data of C, H, and N were within ± 0.4% of the calculated values (calculated values for $C_{39}H_{64}O_{10}$: C 67.90; H 9.31. On analysis: C 67.39; H 9.37). ^{13}C-NMR was performed on a Bruker AC 200 MHz spectrometer at 80°C and was expressed as *delta* units referred to TMS as internal standard.

^{13}C-NMR for Benzopyranic Nucleus

10.86 (CH_3-7); 11.04 (CH_3-8); 11.71 (CH_3-5); 22.59 (CH_3-3); 28.31 (CH_2-3); 39.85 (CH_2-4); 73.93 (C-2); 116.41



(1) R - H tocopherol

(2) R = $HOOCCH_2CH_2CO-$ tocopherol succinate

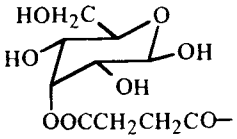
(3) R =  tocopherol succinate-3-glucose

Fig. 1. Structural formulae of tocopherol and its derivatives.

(C-8); 120.95 (C-4a); 124.03 (C-5); 125.56 (C-7); 139.75 (C-6); 147.92 (C-8a)

^{13}C-NMR for Aliphatic Chain

18.57 (CH_3-4); 18.64 (CH_3-8); 21.48, 21.48 (2 CH_3-12); 22.94 (CH_2-10); 23.26 (CH_2-6); 26.49 (CH-12); 27.86 (CH_2-2); 28.16 (CH_2-3); 30.12 (CH_2-1); 31.26 (CH-4); 31.31 (CH-8); 35.90 (CH_2-5); 36.01 (CH_2-9); 36.16 (CH_2-7); 39.43 (CH_2-11)

^{13}C-NMR for Succinic Moiety

19.14, 19.58 (2 CH_2); 159.56, 170.69 (2 COOR)

^{13}C-NMR for α-pyranose

60.41 (CH_2-6); 67.72 (CH-4); 69.71 (CH-5); 71.04 (CH-2); 72.19 (CH-3); 91.46 (CH-1)

^{13}C-NMR for β-pyranose

60.41 (CH_2-6); 67.72 (CH-4); 75.60 (CH-2); 75.84 (CH-5); 78.18 (CH-3); 96.08 (CH-1)

Drugs Used

(±)-α-tocopherol, (+)α-tocopherol acid succinate, DIDS, PCMBS, maltose, 1-octane-sulfonic acid sodium salt, 1,-2:5,6-Di-O-isopropylidene-α-D-glucofuranose, 4-pyrrolidinopyridine and DCC were purchased from Sigma Chemical Co. (St. Louis, MO, USA); KH_2PO_4, K_2HPO_4, $NaHCO_3$, NaCl, acetonitrile, ether, acetic acid, ethanol, Tween 40, trifluoroacetic acid, ethyl acetate, methanol and anhydrous sodium sulphate were obtained from Carlo Erba Reagenti (Milano, Italy). All chemicals used were of the purest analytical grade.

RESULTS AND DISCUSSION

In Table I the data on intracellular uptake and membrane incorporation of T, TS, and TSG are presented. T resides almost completely in the suspension medium, resulting in only 6.91% intracellular uptake and 9.87% membrane incorporation. Conversely, the greater part of TS disappears from the suspending medium (85.64%), with 60.61% located in erythrocyte membranes. In comparison with T, a larger amount of TSG was incorporated into erythrocyte membranes (18.37%) and taken up by cells (14.64%); however TSG intracellular accumulation was significantly lower than that observed with TS.

Table I. Distribution of T, TS, and TSG in the Suspension Medium, Erythrocyte Cytosol and Erythrocyte Membrane. Data Represent the Mean ± S.D. of (n) Experiments. See Text for Details

		Percentage of drug detected in each fraction		
Drug	(n)	Suspension medium	Cytosol components	Membrane
T	(5)	83.22 ± 2.66	6.91 ± 1.88	9.87 ± 0.79
TS	(3)	14.36 ± 1.15	25.03 ± 5.61	60.61 ± 4.45
TSG	(4)	66.99 ± 3.03	14.64 ± 2.64	18.37 ± 0.40

In the same way as the long-chain homologues, alpha-tocopherol molecules have been shown to be significantly associated in clusters and fully localized in the hydrophobic zone of the lipid bilayer (2); such localization is due to hydrogen bonding between the OH group of the chromanol nucleus and the carbonyl oxygen atom of membrane glycerophosphatides as well as the hydrophobic interaction of the phytol fragment of the T molecule with the fatty acid residues of phospholipids. Thus the T molecules appear to be firmly fixed in the bilayer, which sharply restricts their transbilayer mobility.

In TS, the OH group on the tocopherol aromatic head is esterified (see Fig. 1), so that drug mobility through the erythrocyte membrane should be limited only by the hydrophobic interactions between the tocopherol hydrocarbon tail and the membrane fatty acid residues. Furthermore, the large differences observed in membrane incorporation between T and TS might be due to different abilities to fill spaces in the lipid bilayer.

A similar hypothesis may be suggested to explain the results obtained with TSG. The lower intracellular transport and membrane incorporation of TSG, when compared to TS, is very likely due both to the higher TSG hydrophility (in fact

the log K′ values calculated for TS and TSG were respectively 0.448 and 0.253) and to the steric hindrance of the succinate-glucose residue.

Generally drug transport is fundamentally related to P (the partition coefficient), because lipophilicity is believed to be the most important character affecting drug transport from the circulating blood to tissues and organs. However, the higher incorporation of TS into erythrocytes cannot be explained in terms of drug lipophility; in fact, TS (log K′ = 0.448) exhibits a lower liposolubility than T (log K′ = 0.620). Moreover, TS water solubility is so low as to justify the sequestration of the drug in the lipid phase.

Recently, TS has shown unique cytoprotective properties in hepatocytes exposed to hyperoxic conditions; such cytoprotection elicited by TS appears to correlate with cellular accumulation of the intact TS molecule and not with T accumulation (4,19). Taking our data into account, one can speculate that the cytoprotective activity of TS may be related to the differences in the transmembrane mobility observed between T and its succinate ester. However, the mechanism by which TS functions at intracellular level (release and accumulation of T at critical cellular sites, release and utilization of succinate or a protective effect of the TS molecule itself) remains unclear.

Since drugs may permeate through phospholipid bilayer membranes or through pores or may be involved in carrier-mediated transport, we performed a second series of experiments to clarify the transport route of TS and TSG. Tables II and III show the effect of maltose, DIDS and PCMBS on erythrocyte uptake and membrane incorporation of the drugs tested. The transport of TS and TSG was partially inhibited by PCMBS (which inhibits monocarboxylate uptake) and by maltose (a competitive inhibitor of glucose transport) respectively, with a concomitant increase in drug membrane incorporation. No significant change was observed in the presence of DIDS (a blocker of band 3-mediated transport).

These results show a specific contribution of the monocarboxylate transport protein and of the glucose transport protein in the cellular uptake of TS and TSG respectively. In other words, the succinate residue and the succinate-glucose residue appear to make T transport not only more efficient, but also more selective.

The development of T prodrugs has been generally aimed at obtaining an improvement in water solubility and oxidation stability. Interestingly, our results seem to suggest that T conjugation to a monocarboxylate or glycoside moiety, by providing suitable substrates for active membrane transport, might appear as a promising pharmaceutical strategy for an improved delivery of tocopherol derivatives. The preferential delivery of drugs or their derivatives to target cells or tissues is known to be facilitated by a specific mechanism involving recognition and uptake by cell surface receptors. Particularly, conjugation with tyrosine or glucose has been recently shown to be a successful means of selective drug delivery; for example, phosphonoformate-L-tyrosine conjugate is actively transported, by means of active amino acid carriers, through monolayers of porcine brain microvessel endothelial cells (13) and a glycosyl phosphotriester prodrug of AZT shows a good delivery to the central nervous system (11). In addition, conjugating glucose or galactose to poorly absorbable drugs can improve their intestinal absorption by means of glucose transport carriers in the small intestine (14,15).

Finally, one has to point out that the glycosidic residue conjugated to T could be tailored to meet more properly specific requirements of the receptors of targeted cells (12); moreover, the kinetics of reconversion of the esters to the parent drug must be carefully evaluated. Thus further intensive efforts are still needed for the development of novel, more efficacious T prodrugs.

Table II. Effect of Maltose on Distribution of T, TS and TSG in the Suspension Medium, Erythrocyte Cytosol and Erythrocyte Membrane. Data Represent the Mean ± S.D. of (n) Experiments. See Text for Details

			Percentage of drug detected in each fraction		
Drug	Treatment	(n)	Suspension medium	Cytosol components	Membrane
T	—	(4)	82.88 ± 2.73	7.13 ± 1.94	9.99 ± 0.80
T	*Maltose*	(3)	84.74 ± 3.30	6.99 ± 0.96	8.27 ± 2.35
TS	—	(3)	14.55 ± 1.23	24.87 ± 5.83	60.58 ± 4.56
TS	*Maltose*	(3)	13.37 ± 3.18	23.17 ± 5.21	63.46 ± 2.05
TSG	—	(4)	67.44 ± 3.51	15.28 ± 2.32	17.28 ± 1.19
TSG	*Maltose*	(4)	62.71 ± 3.29	9.09 ± 2.48[a]	28.20 ± 0.76

[a] $P < 0.05$ versus the respective control.

Table III. Effect of DIDS or PCMBS on Distribution of T, TS, or TSG in the Suspension Medium, Erythrocyte Cytosol, and Erythrocyte Membrane. Data Represent the Mean ± S.D. of (n) Experiments. See Text for Details

			Percentage of drug detected in each fraction		
Drug	Treatment	(n)	Suspension medium	Cytosol components	Membrane
T	—	(3)	83.12 ± 2.55	7.26 ± 2.64	9.57 ± 1.12
T	*DIDS*	(4)	82.35 ± 3.46	7.03 ± 1.21	8.66 ± 1.88
T	*PCMBS*	(4)	83.65 ± 2.92	6.84 ± 1.13	8.58 ± 2.47
TS	—	(4)	10.67 ± 1.44	25.68 ± 1.52	63.65 ± 0.11
TS	*DIDS*	(3)	11.20 ± 2.25	20.88 ± 4.85	68.72 ± 2.65
TS	*PCMBS*	(3)	12.72 ± 1.86	19.41 ± 3.61[a]	67.37 ± 1.73
TSG	—	(4)	66.81 ± 1.96	15.26 ± 2.13	17.93 ± 0.20
TSG	*DIDS*	(5)	64.76 ± 1.74	15.21 ± 1.45	20.03 ± 0.32
TSG	*PCMBS*	(3)	66.49 ± 3.08	16.85 ± 2.10	16.66 ± 0.95

[a] $P < 0.05$ versus the respective control.

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