Biochimica et Biophysica Acta, 684 (1982) 200–206
Elsevier Biomedical Press

BBA 71029

SPECIFIC BINDING SITES FOR D-α-TOCOPHEROL ON HUMAN ERYTHROCYTES *

ABBAS E. KITABCHI ** and JAY WIMALASENA

Departments of Medicine and Biochemistry, and Clinical Research Center, University of Tennessee Center for the Health Sciences, Memphis, TN 38163 (U.S.A.)

(Received July 29th, 1981)

Key words: Vitamin E; Erythrocyte membrane; Tocopherol binding; (Human blood)

Since vitamin E deficiency is associated with increased susceptibility of erythrocytes to hemolysis, we investigated the presence of tocopherol binding sites in human red blood cells. Erythrocytes were found to have specific binding sites for D-α-[^{3}H]tocopherol with properties of receptors. Kinetic studies of binding demonstrated two binding sites: one with high affinity (equilibrium association constant $K_a = 2.6 \cdot 10^7$ M^{-1}), low capacity (7600 sites/cell) and the second with low affinity ($K_a = 1.24 \cdot 10^6$ M^{-1}), high capacity (150000 sites/cell). These sites are at least partly protein in nature.

Introduction

Vitamin E (D-α-tocopherol), besides its well-known effect as biological antioxidant, is proposed to have a role in a wide variety of biological processes [1–3], including ACTH-activated, membrane-bound adenylate cyclase [4] and ACTH-induced steroidogenesis [5,6] in the rat as well as decreasing erythrocyte fragility as evidenced by increased hemolysis under conditions of oxidative stress and decreased red cell survival time [7,8] in vitamin E deficiency. The above changes in blood corpuscle functions and adrenal cells may be related to changes in membrane constitution.

We have recently demonstrated that rat adrenocortical cell membranes have specific binding sites for D-α-tocopherol [9]. In this communication we demonstrate that human erythrocytes have specific binding sites for D-α-tocopherol.

Materials and Methods

D-α-[^{3}H]Tocopherol (specific activity 13 Ci/mmol, purity 98%) was purchased from Amersham-Searle Radiochemicals. D-α-Tocopherol and other vitamin E analogs were obtained from Eastman Kodak and Hoffman-LaRoche. Trypsin and lima bean trypsin inhibitor were obtained from Millipore Laboratories. The sources of other chemicals were as stated earlier [9]. D-α-[^{3}H]Tocopherol stock solution was aliquoted and kept at −60°C under nitrogen to prevent oxidation. Specific binding of D-α-[^{3}H]tocopherol did not change during storage under these conditions for 2–3 months.

Blood from normal volunteers was used to obtain washed erythrocytes as described previously [10].

Binding assay

In the routine binding assay an aliquot of fresh red cell suspension ($(4–5) \cdot 10^9$ cells/ml) was in-

* Presented as an abstract at Federation of American Societies for Experimental Biology; Atlanta, GA; April 12–17, 1981.

** To whom correspondence should be addressed: 951 Court Avenue, Rm. 327B, Memphis, TN 38163, U.S.A.

Abbreviations: K_a, equilibrium association constant; Trolox, *rac*-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; k_1 and k_2, rate constants for forward and reverse reactions, respectively.

cubated for 4 h at 37°C in a total volume of 1.0 ml of Tris-Ringer (0.025 M Tris, 0.120 M NaCl, 0.0012 M $MgSO_4$, 0.0025 M KCl, 0.01 M glucose, 0.001 M EDTA) bovine serum albumin (1%) pH 7.1 in 12 × 75 mm plastic tubes. D-α-Tocopherol or other vitamin E analogs were added in ethanol; the final concentration of the latter in all assay tubes was 0.5%. Routinely 220000–280000 cpm of D-α-[^{3}H]tocopherol were used/assay. At the end of incubation, 1 ml of ice cold Tris-Ringer buffer and 1 ml of dibutyl phthalate were added/tube and the tubes were centrifuged at 800 × g in a Beckman TJ-R centrifuge for 15 min. The red cell pellets were solubilized in 15 ml of Scinti-Verse (Fisher-Scientific) and counted in a Nuclear Chicago scintillation spectrometer at an efficiency of 40%. Quenching of ^{3}H by hemoglobin was corrected by decolorizing the hemoglobin with 5% H_2O_2 or by counting D-α-[^{3}H]tocopherol standard after addition of the same quantity of red cell pellets to counting vials as in the test samples. These red blood cells were incubated under the same conditions as the assay tubes except for the exclusion of D-α-[^{3}H]tocopherol during the preincubation. Specific binding for D-α-tocopherol was defined as total binding of D-α-[^{3}H]tocopherol minus nonspecific binding. The latter was determined by measuring cpm of D-α-[^{3}H]tocopherol in the presence of 110 μM of unlabeled D-α-tocopherol. Nonspecific binding did not exceed 10–20% of the total count. Binding was measured in triplicate and the individual values were within 10% of the mean. Cells were counted in a Hi Cell counter. Each experiment reported herein was repeated and the number of experiments (n) is indicated in Results.

Results

Specific binding of D-α-[^{3}H]tocopherol was time dependent and reached saturation at 4 h of incubation at 37°C (Fig. 1). The initial rate of binding and the amount bound at equilibrium increased with increasing quantities of D-α-[^{3}H]tocopherol. Non-specific binding (data not shown) did not increase with time and was 18% of total binding at 4 h. The initial rate of specific binding of 37°C > 30°C > 21°C (data not shown) ($n = 2$).

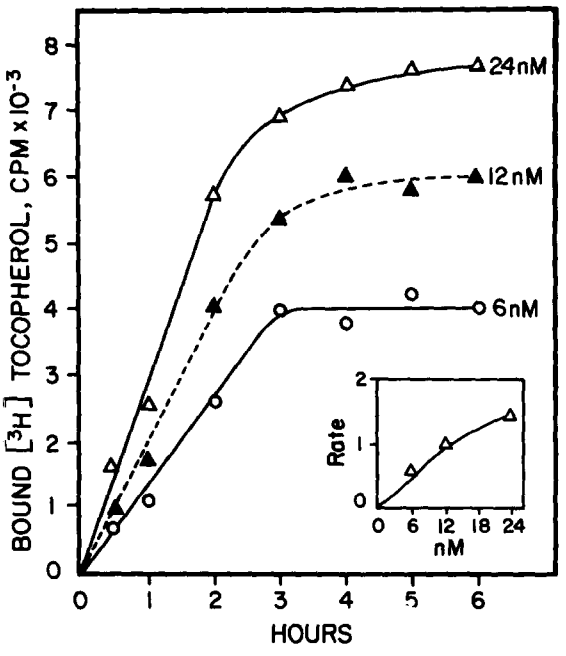

Fig. 1. Time course of binding. The binding of D-α-[^{3}H]tocopherol to red blood cells at the concentrations shown in the figure was measured at several time intervals. The slopes of the linear parts of these curves are plotted against the concentration of D-α-tocopherol in the inset. Binding was measured as in Methods.

The quantity of specific binding increased with number of cells linearly up to 80 · 10^7 cells/assay (data not shown). Routine assays were performed with (50–80) · 10^7 cells/reaction ($n = 2$).

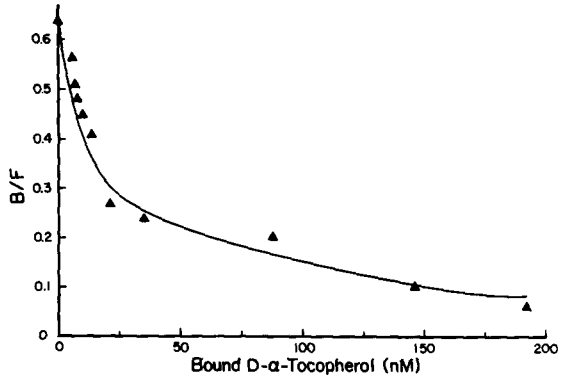

Fig. 2. Comparative analysis of binding. The quantity of D-α-[^{3}H]tocopherol bound at equilibrium to red blood cells in the presence of several concentrations of unlabeled D-α-tocopherol was measured. These data are presented as a Scatchard plot. Binding was measured as in Methods.

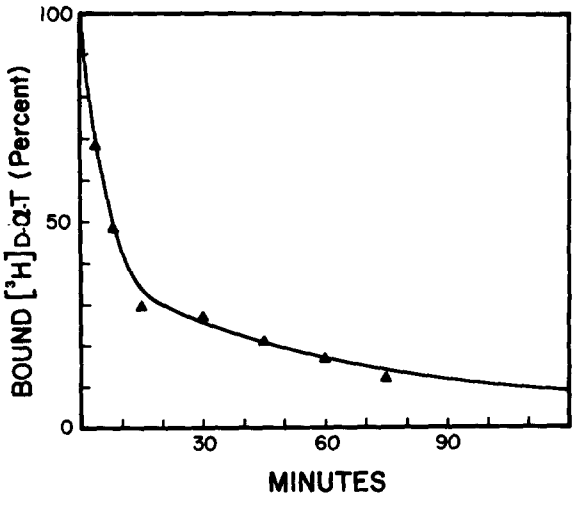

Fig. 3. Time course of the dissociation reaction of bound D-α-[^{3}H]tocopherol. See text for details.

The Scatchard plot [11] generated by measuring binding of D-α-[^{3}H]tocopherol at several concentrations of unlabeled D-α-tocopherol is shown in Fig. 3. From these data the equilibrium association constants (K_a) and binding capacities (R) were calculated according to the method of Thakur et al. [12] for a two independent site model. For the high affinity site $K_{a1} = (2.64 \pm 0.4$ (S.E.)) $\cdot 10^7$ M^{-1}, $R_1 = 7000$–8000 sites/cell; for the low affinity site, $K_{a2} = (1.24 \pm 0.3) \cdot 10^6$ M^{-1}, $R_2 = 140000$ –160000 sites/cell. Three out of three experiments demonstrated the presence of two binding sites.

Dissociation of specifically bound D-α-[^{3}H]tocopherol was studied as follows. After a 4-h incubation of $0.8 \cdot 10^9$ cells with D-α-[^{3}H]tocopherol, the cells were separated from the incubation medium and resuspended at a 100-fold dilution with buffer. To one incubation mixture

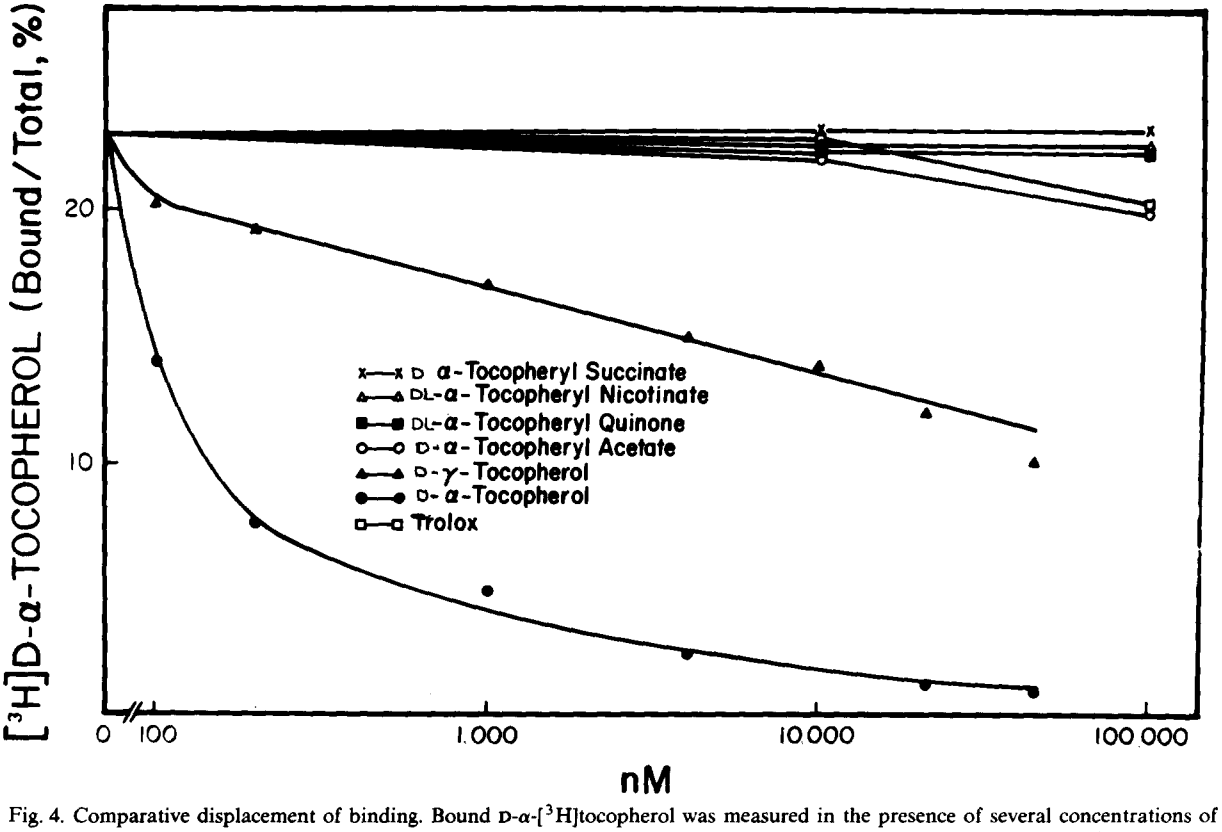

Fig. 4. Comparative displacement of binding. Bound D-α-[^{3}H]tocopherol was measured in the presence of several concentrations of D-γ-tocopherol, D-α-tocopherol and other tocopherol analogs as described in Methods. These results are expressed as the percent of added D-α-[^{3}H]tocopherol which was specifically bound to $1 \cdot 10^9$ red blood cells.

unlabeled D-α-tocopherol was added at a concentration of 110 μM, while an equivalent volume of absolute alcohol was added to a second incubation mixture. Samples were removed at several time points during the dissociation reaction at 37°C and specific binding was measured (Fig. 3). While the specific binding was readily reversible, addition of unlabeled D-α-tocopherol did not produce a significant effect over dilution alone ($n = 2$). The K_a values computed from the rate constants derived from the data in Figs. 1 and 3 confirmed the presence of two binding sites; the values for K_{a1} ($4.7 \cdot 10^7$ M^{-1}), K_{a2} ($3.4 \cdot 10^6$ M^{-1}) were in reasonable agreement with those from Fig. 2.

The pH optimum for the binding reaction was 7–7.2 (data not shown). The specific binding of D-α-[^{3}H]tocopherol was not decreased by D-α-tocopheryl acetate, DL-α-tocopheryl nicotinate, D-α-tocopheryl quinone or the biologically inactive vitamin E analog *rac*-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Fig. 4) ($n = 3$). D-α-[^{3}H]tocopherol binding was inhibited, however, by D-γ-tocopherol, with a potency of ≤ 1% at concentrations up to 10 μM (Fig. 4). D-γ-Tocopherol is known to be a biologically less active analog of D-α-tocopherol [13].

We tested a number of agents which act as prooxidant (ascorbate), antioxidant (selenium) or interact with erythrocyte membranes (H_2O_2). In addition, we assessed binding of D-α-tocopherol to red blood cells with protease inhibitor and sulfhydryl agents. The results of these studies are presented in Table I. H_2O_2 almost totally inhibited binding at 1%. Selenium dioxide was partially effective in blocking the inhibition by H_2O_2, although by itself selenium dioxide had no effect.

Binding of D-α-[^{3}H]tocopherol was markedly inhibited by preincubation of red blood cells at 65°C for one hour. Preincubation with trypsin at 1.25 mg/ml drastically decreased binding (assayed after neutralization with trypsin inhibitor, 4 mg/ml, centrifugation, wash and resuspension) while addition of trypsin inhibitor before trypsin blocked the effects of trypsin. However, when red blood cells with previously bound D-α-[^{3}H]tocopherol were exposed to trypsin, there was only a 30% decrease in bound D-α-[^{3}H]tocopherol after one hour of incubation.

Preincubation of red blood cells with *N*-ethylmaleimide for 1 h at 37°C decreased binding by 50%, while dithiothreitol by itself had no effect on binding. Dithiothreitol completely blocked the inhibition due to *N*-ethylmaleimide. These results taken together strongly suggest that specific binding of D-α-tocopherol is at least partly due to a thermolabile protein which may have sensitive disulfide bonds.

Studies in seven normal volunteers revealed that 18–24% of added D-α-[^{3}H]tocopherol was bound (per 10^9 cells/4 h), with a mean of 20.4 ± 0.9% (S.E.). The day-to-day variability of binding was less than 10% between subjects and between groups.

Although the physiological significance of the D-α-tocopherol binding sites is presently unknown, the data illustrated in Fig. 5 demonstrate that upon preincubation of red blood cells with 1.1 μM D-α-tocopherol subsequent hemolysis by H_2O_2 was significantly reduced. The effect of D-γ-tocopherol at the same concentration was approx. 50% that of D-α-tocopherol. However at a concentration of 11

TABLE I

EFFECTS OF VARIOUS AGENTS ON D-α-[^{3}H]TOCOPHEROL ERYTHROCYTE BINDING

Red blood cells were preincubated for 1 h as indicated, centrifuged, resuspended, and assayed for binding as described under Methods. The control which is taken as 100% consists of value of 20% binding of D-α-[^{3}H]tocopherol per $1 \cdot 10^9$ cells. Results are the mean of two experiments with three measurements per experiment.

Additions	Binding as percent of control
Control	100
Sodium ascorbate (1 mM)	94
Calcium chloride (1 mM)	98
Hydrogen peroxide (1%)	14
+ Selenium dioxide (1 mM)	34
Selenium dioxide (1 mM)	97
Trypsin (45 min at 37°C)	27
+ Trypsin inhibitor	82
Trypsin (1 h at 37°C) subsequent to binding [a]	66
Dithiothreitol (DTT) (10 mM)	100
N-Ethylmaleimide (NEM) (10 mM)	46
Dithiothreitol + *N*-ethylmaleimide	126

[a] Cells were incubated with D-α-[^{3}H]tocopherol for 3 h and treated with trypsin (1.25 mg/ml) before measurement of bound D-α-[^{3}H]tocopherol.

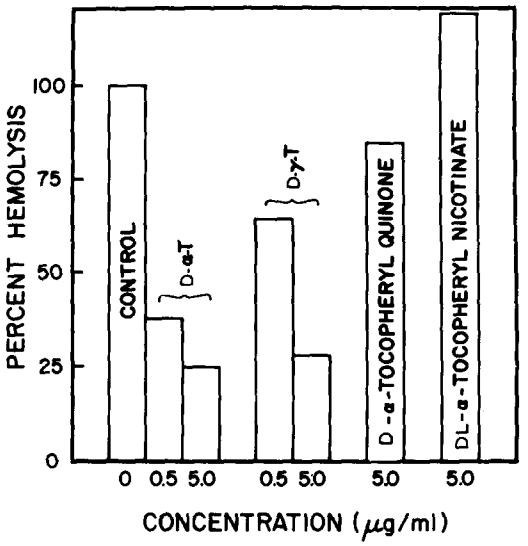

Fig. 5. Effects of tocopherols on hemolysis. $0.4 \cdot 10^9$ red blood cells were preincubated (37°C) with the tocopherols at the concentrations depicted in the figure for 3 h. They were centrifuged, washed (4°C) and incubated with 5% H_2O_2 for 45 min at 37°C and hemolysis was measured [28]. To the control tubes only C_2H_5OH (carrier for vitamin E) was added. The hemolysis in control is taken as 100% and represents 14–16% of total hemoglobin released when the red blood cells were hemolysed with H_2O. The results depicted are the means of two experiments.

μM, D-γ-tocopherol had an antihemolytic effect of the same magnitude. D-α-Tocopheryl quinone and DL-α-tocopheryl nicotinate were inactive. In fact, the nicotinate increased hemolysis by 20% when compared to the control incubation ($n = 2$). These results are compatible with the binding displacement data (Fig. 4) where it is seen that only D-γ-tocopherol was able to compete with D-α-[^{3}H]tocopherol for the binding sites and that this competition increased with increasing concentrations of D-γ-tocopherol. In comparison, the binding competition by D-α-tocopherol increased by only a small amount between 1 and 10 μM concentrations. The other tocopherol analog, δ-tocopherol, exhibited no competitive binding with D-α-[^{3}H]tocopherol (data not shown).

Although we have not examined the possibility that D-α-[^{3}H]tocopherol was metabolized during the incubation, the following observations suggest that there is minimal metabolism of tocopherol conjugates in erythrocytes and erythrocyte membranes. (1) When red blood cells were preincubated with various tocopherol analogs, only D-α-tocopherol was able to inhibit subsequent binding of D-α-[^{3}H]tocopherol. (2) Whereas a number of esterified tocopherols are biologically active in vivo, due to well defined mechanisms of metabolism [14], only D-γ-tocopherol is able to compete for binding in both red blood cells and membranes. The latter is a D-α-tocopherol partial agonist. Other tissues which were studied for tocopherol binding sites included polymorphonuclear cells and platelets, neither of which showed any specificity for D-α-[^{3}H]tocopherol binding.

Since the specific binding of D-α-[^{3}H]tocopherol to red blood cells could also be due to uptake into intact cells, erythrocytes were hemolysed according to the method of Fairbanks et al. [15] and the ghost pellet was homogenized in a glass/glass homogenizer. The suspension was washed with Tris buffer four times by centrifugation (40000 × g) and resuspension. Binding of D-α-[^{3}H]tocopherol to these membrane suspensions demonstrated that 80–90% of the specific binding of D-α-[^{3}H]tocopherol was recoverable in the membrane derived from an equivalent number of cells ($0.8 \cdot 10^9$ cells bound 20% of added D-α-

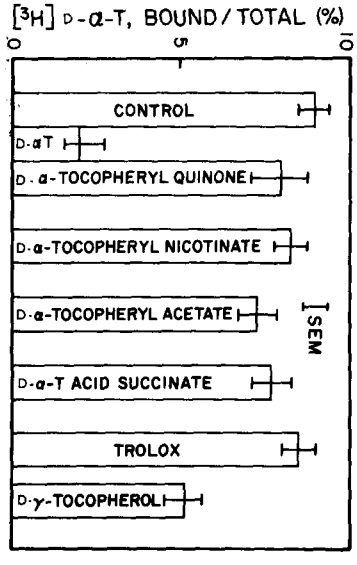

Fig. 6. Specificity of binding of D-α-[^{3}H]tocopherol to membranes from $0.8 \cdot 10^9$ red blood cells. Binding was measured for 3 h at 37°C using the same buffer as for red blood cels, with 200000 cpm of D-α-[^{3}H]tocopherol. Tocopherol concentration 5 μg/ml.

[^{3}H]tocopherol; membranes from $0.8 \cdot 10^9$ cells bound 17.6% of added D-α-[^{3}H]tocopherol) [16]. Binding to membranes had the same specificity as with the intact red blood cells (Fig. 6) and was temperature and time dependent [16].

Discussion

The results presented in this communication suggest that human erythrocytes have specific, saturable binding sites for D-α-tocopherol which demonstrate properties expected of a biologically significant receptor. These binding sites are at least partly protein in nature. The kinetic parameters for these binding sites are similar to those observed with those of the rat adrenocortical membranes [9]. Although we have not investigated tocopherol binding sites for other human tissue cells, such as liver, muscle, etc., our preliminary results on polymorphonuclear cells and platelets suggest that these cells are devoid of a specific binding site for D-α-[^{3}H]tocopherol. Catignani [17] and Catignani and Bieri [18] have observed a specific binding site for D-α-tocopherol in the rat liver cytosol but these authors did not report equilibrium binding data. Nair et al. [19] proposed that the rat liver nuclei may have a binding site for D-α-tocopherol. The concentration of D-α-tocopherol in rat red blood cells is 10–12 μM [13] and in the human some of the D-α-tocopherol binding sites could be endogenously occupied. Since we were able to measure saturable specific binding of 6 nM D-α-[^{3}H]tocopherol, either exogenously added D-α-tocopherol must bind to unoccupied sites or the endogenously bound tocopherol must have been removed during the washing procedure.

D-α-Tocopherol is a well-known antioxidant in vitro [20] and has been demonstrated to decrease the hemolytic damage to red blood cells by oxidative stress [21,22]. Since the disruption of red blood cell membrane is a likely locus of action of oxidative stress and D-α-tocopherol has been proposed to be important in the maintenance of membrane structure and function [1,13,23–27], it is possible that D-α-tocopherol binding proteins or receptors are present on the red blood cell surface. The demonstration that D-α-tocopherol and, to a lesser extent, D-γ-tocopherol can inhibit H_2O_2 hemolysis suggests that these specific binding sites may be of physiological significance in protecting red blood cells from hemolytic damage.

Acknowledgments

The authors wish to acknowledge Dr. W.E. Scott of Hoffman-LaRoche for the generous gifts of various tocopherol analogs, Linda Balentine for editorial assistance, and Richard Fleming for technical assistance.

This work was supported in part by U.S. Public Health Service Training Grant AM07088, National Institutes of Health Grant AM19717, and General Clinical Research Center Grant RR00211.

References

1 Nelson, J.S. (1980) in Vitamin E (Machlin, L.J., ed.), pp. 397–428, Marcel Dekker, New York
2 Slater, T.F. and Sawyer, B.C. (1971) Biochem. J. 123, 823–828
3 Kitabchi, A.E. (1980) in Vitamin E (Machlin, L.J., ed.), pp. 348–371, Marcel Dekker, New York
4 Nathans, A.H. and Kitabchi, A.E. (1975) Biochim. Biophys. Acta 399, 244–253
5 Kitabchi, A.E., Nathans, A.H. and Kitchell, L.C. (1973) J. Biol. Chem. 248, 835–840
6 Civen, M., Leeb, J.E., Wishnain, R.M. and Morin, R.J. (1980) Int. J. Vit. Nutr. Res. 50, 70–78
7 Corash, L., Spielberg, S., Bartsocas, C., Boxer, L., Steinherz, R., Sheetz, M., Egan, M., Schlessleman, J. and Schulman, J.D. (1980) N. Engl. J. Med. 303, 416–420
8 N.I.H. Conference (1980) (Schulman, J.D., moderator) Ann. Int. Med. 93, 330–346
9 Kitabchi, A.E., Wimalasena, J. and Barker, J. (1980) Biochem. Biophys. Res. Commun. 96, 1739–1746
10 Yasuda, K. and Kitabchi, A.E. (1980) Diabetes 29, 811–814
11 Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–670
12 Thakur, A.K., Jaffe, M. and Rodbard, D. (1980) Anal. Biochem. 107, 279–290
13 Bieri, J.G. and Farrell, P.M. (1976) Vitam. Horm. 31, 31–75
14 Gallo-Torres, H.E. (1980) in Vitamin E (Machlin, L.J., ed.), pp. 193–268, Marcel Dekker, New York
15 Fairbanks, G., Steck, T.L. and Wallach, F.H. (1971) Biochemistry 10, 2606–2617
16 Wimalasena, J., Davis, M. and Kitabchi, A.E. (1981) Fed. Proc. 40, 874
17 Catignani, G.L. (1975) Biochem. Biophys. Res. Commun. 67, 66–72
18 Catignani, G.L. and Bieri, J.G. (1977) Biochim. Biophys. Acta 497, 349–357
19 Nair, P.P., Patnaik, R.N. and Hauswirth, J.W. (1978) in Tocopherol, Oxygen and Biomembranes (De Duve, C. and Hayaishi, O., eds.), pp. 121–130, Elsevier, Amsterdam

20 Tappel, A.L. (1972) Ann. NY Acad. Sci. 203, 12–28
21 McCay, P.B. and King, M. (1980) in Vitamin E (Machlin, L.J., ed.), pp. 289–317, Marcel Dekker, New York
22 Weiss, S.J. (1980) J. Biol. Chem. 255, 9912–9917
23 Molennar, I., Hulstaert, C.E. and Hardonk, M.J. (1980) in Vitamin E (Machlin, L.J., ed.), pp. 372–390, Marcel Dekker, New York
24 Kitabchi, A.E., Nathans, A., Barker, J., Kitchell, L. and Watson, B. (1978) in Tocopherol, Oxygen and Biomembranes (De Duve, C. and Hayaishi, O., eds.), pp. 201–219, Elsevier, Amsterdam
25 Green, J. (1972) Ann. NY Acad. Sci. 203, 29–44
26 Lucy, J.A. (1972) Ann. NY Acad. Sci. 203, 4–11
27 Diplock, A.T., Baum, H. and Lucy, J.A. (1971) Biochem. J. 123, 721–729
28 Farrell, P.M., Bieri, J.G., Fratantoni, J.F., Wood, R.F. and DiSant'Agnese, P.A. (1977) J. Clin. Invest. 60, 233–241