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CHARACTERIZATION AND SOLUBILIZATION OF THE SPECIFIC BINDING SITES FOR *d*-α-TOCOPHEROL FROM HUMAN ERYTHROCYTE MEMBRANES

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Abstract—Previous work from our laboratory has demonstrated the presence of specific binding sites for *d*-α-tocopherol (vitamin E) in intact human erythrocytes [A. E. Kitabchi and J. Wimalasena, *Biochim. biophys. Acta* **684**, 300 (1982)]. The binding was time, temperature and cell concentration dependent. To localize the binding sites, red blood cells were further fractionated; greater than 90% of the tocopherol binding sites were localized on membranes. The washed membrane fraction from normal human erythrocytes has specific binding sites for *d*-α-tocopherol with properties suggestive of protein receptors. Two binding sites with K_a values of $3.31 \times 10^7\ M^{-1}$ and $1.51 \times 10^6\ M^{-1}$ were demonstrated, and solubilized *d*-α-tocopherol binding site complexes were resolved to a major component with an M_r of 65,000 and a minor component with an M_r of 125,000.

For a number of years, vitamin E (*d*-α-tocopherol, dαT) has been known to function as an antioxidant [1, 2] and to influence membrane-bound enzyme systems [3–5]. The ability of dαT to protect erythrocytes (RBC) and leucocytes against oxidative damage as observed during hemolysis and decreased phagocytosis may be due to the ability of membrane-bound dαT to scavenge H_2O_2, free radicals and the superoxide anion [6–12]. We previously reported that rat adrenocortical cell membrane fractions have two specific binding sites for dαT [13], and more recent investigations revealed the presence of two similar binding sites for dαT in intact normal human RBC [14]. Therefore, it was of interest to investigate whether the major organelle of RBC (viz. plasma membranes) has specific binding sites for dαT and to attempt to solubilize these binding sites by detergent extraction.

MATERIALS AND METHODS

[^{3}H]*d*-α-Tocopherol (purity 98%) was purchased from Amersham–Searle Radiochemicals (Arlington Heights, IL). *d*-α-Tocopherol (dαT) and vitamin E analogs were obtained from the Eastman–Kodak Co. (Rochester, NY) or were gifts from Hoffmann-LaRoche (Nutley, NJ). Trypsin and lima bean trypsin inhibitor were obtained from Millipore Laboratories (Elkhart, IN). The sources of other chemicals were as stated earlier [13, 14]. [^{3}H]*d*αT stock solution was aliquoted and kept at −60° under nitrogen. Specific binding of [^{3}H]*d*αT 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 [14].

Human RBC ghosts were prepared from fresh normal blood according to the method of Fairbanks *et al.* [15]. The washed RBC ghost pellet was homogenized in 5 mM phosphate buffer (pH 8) by hand in a glass homogenizer with a glass pestle. This suspension was centrifuged at 39,000 *g* for 20 min in a Beckman J 2-21 centrifuge using the JA 20 rotor at 4°. The pellet was washed three to four times by resuspension and centrifugation. The washed membranes were finally resuspended in a volume of Tris–Ringer buffer (described below) equal to that of the starting blood volume. Aliquots of this suspension were used for the *d*αT binding assay. Binding assays were routinely performed in 12 × 75 mm polypropylene tubes in a final volume of 1 ml containing Tris–Ringer buffer (pH 7.1) (Tris, 0.025 M; NaCl, 0.12 M; $MgSO_4$, 0.0012 M; KCl, 0.0025 M; glucose, 0.01 M; EDTA, 0.001 M) and 0.2 ml membrane suspension. When dαT was used, it was dissolved in absolute ethanol and added at a final concentration of 5 μg/ml to determine nonspecific binding. The concentration of alcohol in all reactions was 0.5%. Total binding and nonspecific binding were measured by the addition of [^{3}H]dαT, 220,000–260,000 cpm (sp. act. 12 Ci/mmole) to initiate the reaction. After 2 hr of incubation at 37°, 2 ml of ice-cold Tris–Ringer was added to each reaction tube; 1.5 ml of this mixture was transferred to another tube containing 1.5 ml of 20% ice-cold sucrose. After centrifugation at 39,000 *g* for 15 min, the supernatant fraction was aspirated and the bottom of the tube containing the membrane pellet was cut and collected in a scintillation vial. Bound [^{3}H]dαT was counted in a Nuclear

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Chicago counter in 15 ml of Scintiverse (Fisher) at an efficiency of 40%. Each measurement was in triplicate. Protein was measured by the method of Lowry *et al.* [16] using bovine serum albumin as standard.

In solubilization studies, membranes previously incubated with [^{3}H]dαT for 2 hr at 37° were pelleted and washed with 2 ml of ice-cold Tris–Ringer buffer. The washed pellet was homogenized in a glass homogenizer in 1.0% Triton, Tris–Ringer at 4° for 30 min. At the end of the incubation, the suspension was rehomogenized and centrifuged at 40,000 *g* for 20 min in a Beckman J-21 centrifuge at 4°. In a few experiments, the Triton extract was centrifuged at 100,000 *g* for 1 hr in a Beckman L2-65 ultracentrifuge at 4° in an SW 40 rotor. Ultracentrifugation yielded results similar to the 40,000 *g* centrifugation. The supernatant fractions from the centrifugations were dialyzed for 18 hr at 4° against a 200-fold excess of Tris–Ringer ethanol (1%) at 4°. Dialysates (0.5 ml), centrifuged at 10^5 *g* for 1 hr, were chromatographed on Sepharose 6B or Sephadex G-100 columns (0.9 × 45 cm) previously equilibrated with Tris–Ringer ethanol at 4°. One-ml fractions were collected, and aliquots from each fraction were used for [^{3}H]dαT counting and O.D.$_{280}$ measurements. The Triton extraction solubilized 60–70% of the protein in the membrane pellet.

In rechromatography studies, three fractions of each peak with the highest bound [^{3}H]dαT were pooled and concentrated by vacuum dialysis; 0.5-ml fractions were rechromatographed as described in the appropriate figure legends.

Solubilization of unlabeled membranes was performed as described above for labeled membranes. The 100,000 *g* supernatant fraction was used for binding studies as discussed in Results. All experiments reported herein were repeated; the number of experiments (N) is included in the figure legend.

The Sepharose 6B column was calibrated with soybean trypsin inhibitor, ovalbumin, bovine serum albumin, concanavalin A and human IgG.

RESULTS

Washed RBC membranes were used for binding studies as described in Materials and Methods. Binding of [^{3}H]dαT was linear with added protein concentrations up to 500 μg/ml. The quantity of [^{3}H[dαT bound by 500 μg of membrane protein accounted for 80–90% of the binding of [^{3}H]dαT by 0.8 × 10^9 erythrocytes, and 500 μg/ml of membrane protein was prepared from 0.8 × 10^9 RBC. Thus, most of the binding sites for [^{3}H]dαT were localized in membranes, and routine assays were performed with 400–500 μg of membrane protein.

Binding of [^{3}H]dαT to RBC membranes was time and temperature dependent (Fig. 1A) confirming our previous work [14]. Both the initial rate of binding and the quantity of bound [^{3}H]dαT at equilibrium were greater at 37° than at 30° (> 22°). At 37° the nonspecific binding was 10–20% of total binding (Fig. 1B), and specific binding reached equilibrium by 2 hr. This time period was used for all other binding reactions. The data from time–course experiments are plotted according to the second-order rate

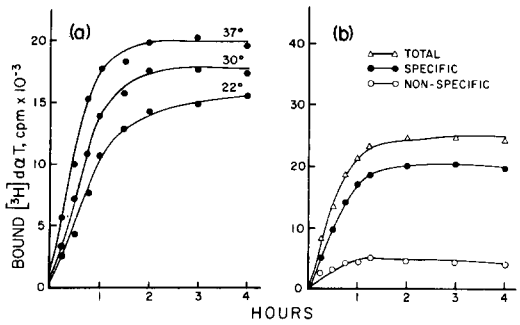


Fig. 1. (A) Time course of the binding reaction and effect of temperature. Four hundred micrograms of RBC membrane was incubated with [^{3}H]dαT at the temperatures depicted in the panel. Reactions were terminated after several time intervals, and the specific binding of [^{3}H]dαT was measured as described in Materials and Methods. Results are the means of two experiments. (B) Specific binding of [^{3}H]dαT as a function of time. In an experiment identical to that of panel A, total and nonspecific binding at 37° was determined after several time intervals as described in Materials and Methods. Specific binding was determined by subtracting nonspecific binding from total binding at each time point. Results are the means of two experiments.

equation [17] in Fig. 2A, and the forward reaction rate constants (k_1) for the two binding sites were calculated using the binding capacities of the two binding sites (Table 1 and Fig. 3).

Dissociation of bound [^{3}H]dαT was measured by resuspending labeled membrane pellets in 100-fold excess of Tris–Ringer and incubating at 37° (Fig.

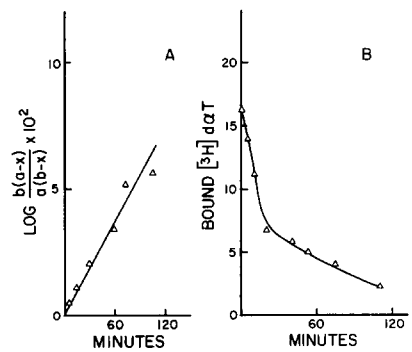


Fig. 2. Kinetics of association and dissociation. (A) Data from a time–course experiment analogous to that in Fig. 1B are plotted according to the second-order rate equation using the number of high affinity binding sites determined from the Scatchard plot. Results are the mean of two experiments. Ordinate: a = molarity of [^{3}H]dαT, b = molarity of high affinity binding sites, and x = fraction bound. (B) A standard binding reaction was performed with 400 μg of RBC membrane protein and [^{3}H]dαT. At the end of 2 hr, membranes were collected by centrifugation as described in Materials and Methods, resuspended by homogenization in Tris–Ringer buffer at 4°, and diluted in the same buffer to 100 times the original assay volume. This suspension was incubated at 37° and aliquots were withdrawn at increasing time intervals to measure the quantity of bound [^{3}H]dαT. A similar experiment in the presence of 5 μg/ml of unlabeled dαT was simultaneously performed to measure nonspecific binding. Results depicted are for specific binding and are the means of two experiments.

Table 1. Kinetic parameters of dαT binding proteins

	K_a* (M^{-1})	Binding capacity (pmoles/mg protein)	Rate constant data
Site 1	3.3 ± 0.2† $\times 10^7$	4.1	2.7×10^6‡
Site 2	1.5 ± 0.4† $\times 10^6$	66	4.7×10^4‡
Fast			0.06§
Slow			0.0075§

* From Scatchard analysis.
† Standard error.
‡ $k_1 \times M^{-1} \times min^{-1}$.
§ $k_2 \times min^{-1}$.

2B). When the data from this experiment were plotted according to the first-order dissociation equation, two well-defined slopes were observed, suggestive of dissociation from two binding sites or two rates of dissociation from the same binding site. The two dissociation rate constants (k_2) and association rate constants (k_1) yielded four equilibrium association constants: 3.62×10^8, 4.54×10^7, 6.30×10^6, 7.88×10^5 M^{-1}. Addition of an excess of unlabeled dαT did not change the rate of dissociation (data not shown).

Measurement of equilibrium binding at several unlabeled dαT concentrations was used to construct a Scatchard plot (Fig. 3). The data were analyzed by the two-site model of Thakur *et al.* [19] and yielded the two equilibrium association constants (K_a) as indicated in Table 1. The K_a values for the high-affinity fast dissociation and the low-affinity fast dissociation systems, 4.54×10^7 and 7.88×10^5 M^{-1}, are in reasonable agreement with the K_a from equilibrium data obtained from the Scatchard plot (3.31×10^7 M^{-1} and 1.51×10^6 M^{-1}).

Specificity of tocopherol binding. Binding of [^{3}H]dαT to RBC membranes was highly specific (Fig. 4). Out of a series of tocopherols only *d*-γ-tocopherol was able to compete with binding of [^{3}H]dαT. However, the potency of *d*-γ-tocopherol, a partial agonist of dαT *in vitro* [12] was less than 5% when compared to unlabeled dαT in binding competition (Fig. 4). Therefore, the binding of [^{3}H]dαT to RBC membranes is a highly specific reaction.

Studies on biochemical characteristics of binding sites. To investigate the nature of the binding sites, we pretreated membranes with trypsin in the presence and absence of trypsin inhibitor. Pretreatment of membranes with trypsin produced a time-dependent decrease in the ability of membranes to bind [^{3}H]dαT (Fig. 5C). This action of trypsin was inhibited by soybean trypsin inhibitor. Therefore, the specific binding sites for [^{3}H]dαT are at least partly protein in nature.

Thermolability of the tocopherol binding sites was investigated by incubating membranes at 65°. Heating decreased subsequent binding of [^{3}H]dαT (Fig. 5A), and the half-life of the binding sites at 65° was 75 min (Fig. 5B). Incubation of the membrane at 90° for 1 hr reduced by 75% (Fig. 5A).

Pretreatment of membranes with 10 mM *N*-ethylmaleimide at 37° for 30 min decreased binding by 60% (data not shown). The pH optimum for binding was between pH 7 and 7.5 (data not shown).

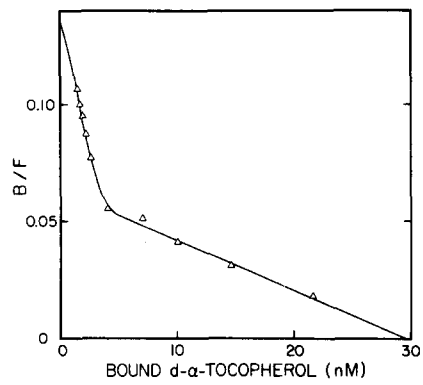


Fig. 3. Quantitative analysis of the specific binding of [^{3}H]dαT to RBC membranes. Binding of [^{3}H]dαT to 500 μg of RBC membranes was measured as described in Materials and Methods in the presence of increasing concentrations of unlabeled dαT. These data are plotted according to the Scatchard equation [18], and the equilibrium association constants and number of binding sites were calculated by the method of Thakur *et al.* [19]. We have assumed that 1 mole of [^{3}H]dαT binds to 1 mole of binding sites. Results are representative of four experiments.

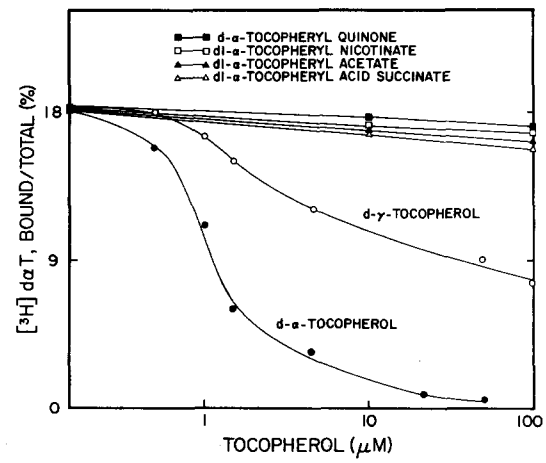


Fig. 4. Specificity of dαT binding to RBC membranes. The specific binding of [^{3}H]dαT to 450 μg of RBC membrane protein was measured as described in Materials and Methods. Binding was measured in the absence of unlabeled tocopherol and in the presence of increasing concentrations of unlabeled tocopherols indicated in the figure. Results are the means of two experiments.

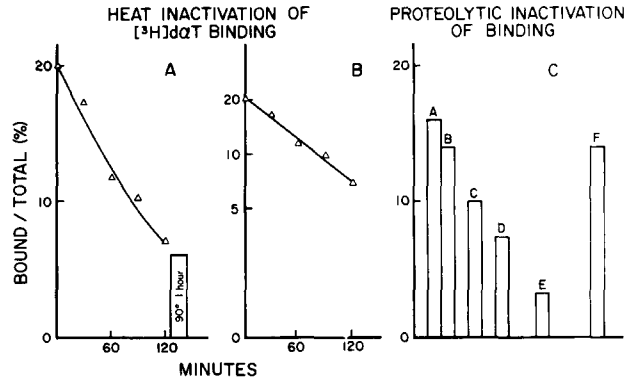


Fig. 5. Thermal and trypsin inactivation of RBC membrane [^{3}H]dαT binding. (A) RBC membranes (450 μg) were incubated in the binding reaction mixture (minus [^{3}H]dαt) for increasing time intervals at 65°. The membranes were collected by centrifugation, resuspended, and assayed for binding as described in Materials and Methods. A separate aliquot of membranes was heated at 90° for 1 hr before binding was measured. Results are the means of two experiments. (B) The data from the heat-inactivation experiment are plotted on a semilogarithmic scale to determine the half-life of binding sites at 65°. (C) RBC membranes (500 μg) were not preincubated at 37° (column A), were preincubated without trypsin at 37° in the standard reaction mixture for 30 min (B), and were preincubated with trypsin (1.25 mg/ml) for 15 min (C), 30 min (D), and 1 hr (E). In (F), membranes were preincubated with trypsin and 4 mg/ml of soybean trypsin inhibitor for 11 hr at 37°. After preincubation, membranes were collected by centrifugation, washed once, resuspended, and assayed for [^{3}H]dαT binding as described in Materials and Methods. Results are the means of two experiments.

Solubilization studies. The above evidence suggested that RBC membranes have specific binding sites for [^{3}H]dαT. Therefore, it was of interest to solubilize and characterize these membrane-bound specific binding sites as the first step in studies leading to purification of the specific binding sites. RBC membranes were detergent extracted as described in Materials and Methods, and binding of [^{3}H]dαT to free binding sites was studied by incubation of 200 μg of solubilized protein with [^{3}H]dαT at 37° for 3 hr and at 4° for 18 hr in Tris–Ringer buffer. We attempted to separate bound [^{3}H]dαT from free [^{3}H]dαT by dextran charcoal treatment, polyethylene glycol precipitation, hydroxyapatite precipitation, Millipore ultrafiltration (0.45 μm), Whatman glass fiber filtration, and methylene chloride extraction [20]. None of these methods yielded satisfactory results. Dextran charcoal and polyethylene glycol separated bound fractions had $>40\%$ of added [^{3}H]dαT, and there was no specific binding. These results indicated that free [^{3}H]dαT was contaminating the bound fraction. Except for hydroxyapatite precipitation, none of the other methods demonstrated any significant specific binding. Hydroxyapatite precipitation, performed as described for steroid receptors [20], demonstrated 1–2% specific binding which increased with increasing quantities of soluble membrane protein added but was never greater than 5% specific binding (data not shown). We have not studied separation of bound and free [^{3}H[dαT by diethylaminoethylcellulose paper [21] extensively, as preliminary studies were not promising. An alternative possibility which we have not used may be heparin-Sepharose to separate bound and free [^{3}H]dαT.

The above studies may have been inconclusive due to a number of reasons. First, the solubilized binding protein was unstable in the presence of Triton X-100. Second, the lipid soluble [^{3}H]dαT may have formed a macromolecular complex with Triton X-100. Third, bound [^{3}H]dαT may have dissociated during separation of bound and free [^{3}H]dαT. The studies described below suggest that the second alternative may explain the results discussed before.

Four hundred micrograms of RBC membrane protein was incubated with [^{3}H]dαT in the presence of 0.1, 0.5 and 1.0% Triton X-100 in the usual binding reaction mixture. At the end of a 1-hr incubation at 37°, insoluble material was pelleted by centrifugation at 40,000 g for 20 min. The supernatant fractions were dialyzed overnight against Tris–Ringer buffer (Materials and Methods), and the pellet and dialysates were counted. As shown in Fig. 6, there was a progressive increase in bound [^{3}H]dαT in the soluble fraction with increasing concentrations of Triton while that bound to the membrane fraction decreased. At 1.0% Triton, approximately 90% of the cpm that bound to an equivalent membrane fraction was found bound to the soluble fraction. Thus, the presence of Triton X-100 in the binding reaction mixture did not inhibit the binding reaction. Rather, Triton X-100 either solubilized membrane binding sites which subsequently bound [^{3}H]dαT or [^{3}H]dαT that was bound to membranes was solubilized during the incubation with Triton X-100. Mixtures of [^{3}H]dαT in Triton X-100 did not yield retained [^{3}H]dαT on dialysis, demonstrating that dialysis separated bound from free [^{3}H]dαT in the presence of Triton X-100 (legend, Fig. 6). Chromatography of the binding reaction mixture containing 1% Triton extract of membranes on Sephadex G-100 in the presence of 1% Triton yielded one peak, but a free [^{3}H]dαT peak was not observed at the V_t (Fig. 7A). Chromatography of a [^{3}H]dαT, Triton (1%) mixture produced a very similar peak. Again, only a small quantity of free [^{3}H]dαT was observed. These results strongly suggest that [^{3}H]dαT formed a macromolecular complex with Triton which could

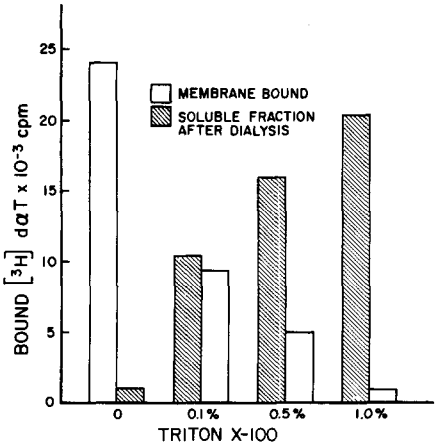
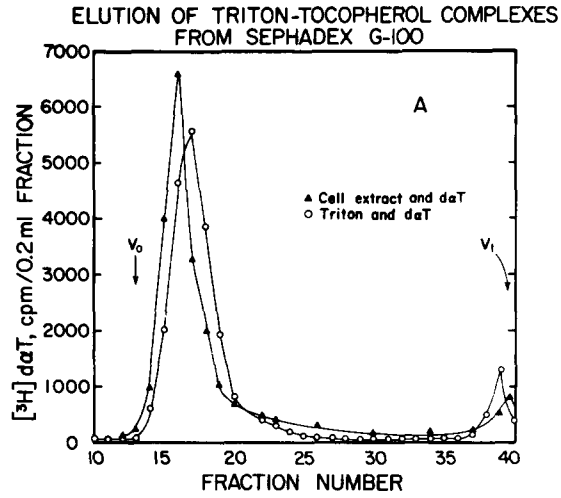
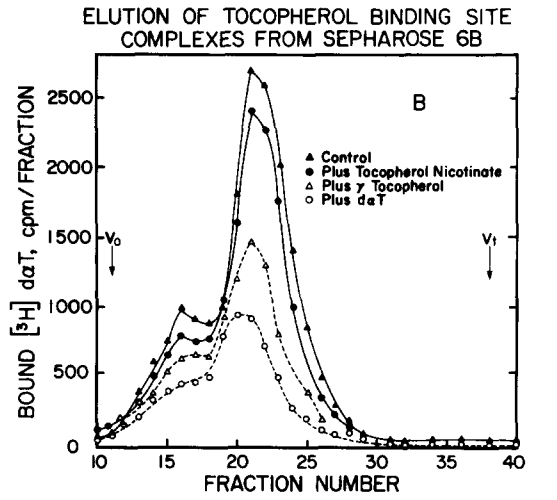


Fig. 6. Solubilization of dαT binding sites by Triton X-100. RBC membranes (475 μg protein) were incubated for 1 hr in the standard reaction mixture as described in Materials and Methods in the presence of increasing concentrations of Triton X-100 (v/v) depicted in the figure. At the end of the incubation, contents of three assay tubes were pooled and centrifuged as described in Materials and Methods. Supernatant fractions were collected and dialyzed overnight, and bound [^{3}H]dαT in the dialysates as well as the membranes was counted. Results depicted are normalized for one reaction mixture and are the means of two experiments. When 200,000 cpm of [^{3}H]dαT and 1% Triton were dialyzed, < 1% cpm was retained in the dialysate.





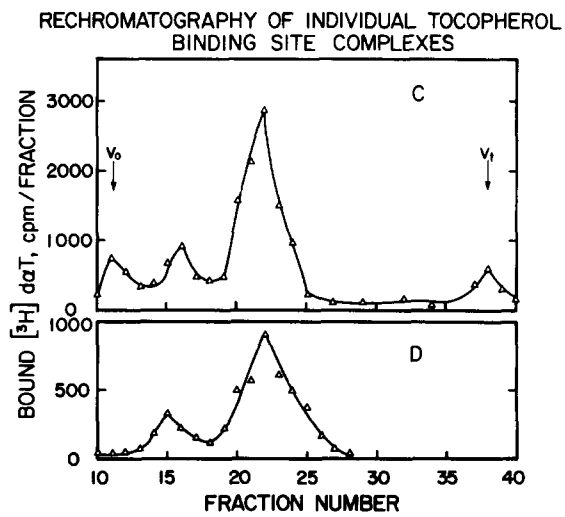


Fig. 7. Gel chromatography of [^{3}H]dαT binding site complexes. (A) A Triton X-100 (1%) extract of RBC membranes prepared as described in Materials and Methods was incubated in the reaction mixture with [^{3}H]dαT (cell extract). At the termination of the 37° incubation, an aliquot of the reaction mixture was chromatographed on a Sephadex G-100 column as described in the text. Aliquots of each eluted fraction were counted. (V_0 = void volume, measured with blue dextran; V_t = total volume, measured with [^{3}H]leucine.) In another experiment (Triton and dαT), all procedures were identical except for the absence of solubilized membrane extract in the incubation mixture. Results are from a single experiment. (B) Standard binding reactions were performed with 425 μg of RBC membrane protein as described in Materials and Methods. Membranes were collected by centrifugation, washed once in ice-cold Tris–Ringer buffer, and extracted with Triton X-100 as described in the text. The solubilized extracts of three reactions were pooled and dialyzed overnight, and an aliquot was chromatographed on Sepharose 6B; 0.5-ml aliquots of each fraction were counted for [^{3}H]dαT and 0.1 ml was used for O.D.$_{280}$ measurements. Control and plus dαT experiments were repeated six times and results depicted are representative of five experiments; plus *d*-γ-tocopherol and *dl*-α-tocopheryl nicotinate experiments were performed once. (Tocopherols were added at 5 μg/ml to prelabeling reaction mixtures as described in the text.) Recovery of applied radioactivity was 70–80%. (C) Fractions 21–23 from two experiments (control) were pooled, concentrated by vacuum dialysis, and rechromatographed on Sepharose 6B as described in Materials and Methods. Fractions eluted (1 ml) were counted *in toto*. (D) Same experiment as in panel C, except that fractions 15–17 from four (control) chromatographic experiments were pooled.

not be separated from bound [^{3}H]dαT unless dialysis was first performed (Fig. 6). Dialysis probably decreased the Triton concentration substantially so that the major fraction of free [^{3}H]dαT did not form macromolecular complexes.

We performed all subsequent solubilization studies with membrane pellets prelabeled with [^{3}H]dαT. When prelabeled membranes were extracted with 1% Triton as described in Materials and Methods, > 80% of bound [^{3}H]dαT was extracted into the soluble fraction (100,000 *g* supernatant) (Fig. 6). Chromatography of dialyzed soluble fractions on Sepharose 6B yielded two bound [^{3}H]dαT peaks (Fig. 7B). Although there was 10–20% variability in the actual values of bound cpm, two peaks were consistently observed (N = 5) and the peak with the larger K_{av} value was always predominant (Fig. 7B, control). When a membrane extract from incubation in the presence of 5 μg/ml dαT was chromatographed similarly, the quantity of bound [^{3}H]dαT was 25–38% of that bound in extracts incubated without excess dαT (Fig. 7B). However, when extracts of membranes incubated with 5 μg/ml *dl*-α-tocopheryl nicotinate were chromatographed, there was < 10% displacement of bound [^{3}H]dαT. The partial agonist *d*-γ-tocopherol did, however, displace bound [^{3}H]dαT by approximately 40% (Fig. 7B). These results suggest that [^{3}H]dαT binding to the solubilized dαT binding site complex has a similar specificity to that of RBC membranes (Fig. 4). As demonstrated in Fig. 1, the nonspecific binding of [^{3}H]dαT to RBC membranes was < 20% of total binding. Therefore, the higher bound [^{3}H]dαT, not displaced by excess unlabeled dαT in the solubilized complexes, was unexpected. It is probable that some of the specifically bound [^{3}H]dαT dissociated during washing of membranes and dialysis at 4°, whereas the nonspecifically bound [^{3}H]dαT may have dissociated to a lesser extent. In support of this suggestion, the recovery of bound [^{3}H]dαT after extraction and dialysis (without excess dαT) was 65–75% of that bound to membranes, whereas in samples treated with excess dαT recovery was over 80% (data not shown).

Rechromatography of individual peaks demonstrated that the major part of the larger molecular species dissociated to the smaller molecular species (Fig. 7D). The major part of the smaller molecular species had the same K_{av} as the original extract. Minor fractions, however, were eluted at V_o and the K_{av} of the larger molecular species (Fig. 7C). These results suggest that the larger molecular species may have dissociated into the smaller molecular species.

By calibrating the Sepharose 6B column with molecular weight standards [22], the molecular weights of the two [^{3}H]dαT binding site complexes were estimated. The M_r values of 125,000 and 65,000 and the observation that the M_r 125,000 species dissociated partly to the M_r 65,000 species (Fig. 7D) suggests that the larger species was a dimer of the smaller species.

DISCUSSION

The evidence presented here suggests that RBC membranes have specific binding sites for dαT. These binding sites have properties generally expected of receptor sites, viz. specificity, saturability, moderate affinity, time, protein and temperature dependence, and reversibility of binding. The equilibrium association constants derived from equilibrium and rate constant data are in reasonable agreement. Since the biological concentration of dαT in RBC is 4.8 μM [1], these binding sites may be of physiological importance in the human RBC, perhaps in the antihemolytic actions of dαT [6–12]. However, the equilibrium constants we have estimated are within the limitations imposed by the low specific activity of the dαT. The membrane binding data confirm our previous observations on the intact RBC [14] and the K_a values are similar to those observed previously with partially purified adrenal membranes [13].

Attempts at solubilization of a ligand free binding site by Triton X-100 extraction of RBC membranes were unsuccessful. While the exact reasons for the lack of demonstration of soluble unlabeled binding sites are unknown, evidence presented above suggests that formation of Triton [^{3}H]dαT complexes was a major reason. While we were able to separate bound and free [^{3}H]dαT by dialysis, this method could not be used for development of a rapid assay for soluble binding sites. Extraction of membranes with 2% cholate [23] solubilized > 70% of the binding sites. However, this method did not improve results of bound and free [^{3}H[dαT separation (results not shown). The recently developed zwitterionic solubilizing reagent 3-[3-cholamidopropyl)dimethylamonio] - 1 - propanesulfonate [24] failed to extract the major part of the [^{3}H]dαT binding site complexes.

The results presented above indicated that > 90% of membrane-bound [^{3}H]dαT binding site complexes could be solubilized in a stable form by Triton X-100 extraction. Gel chromatography data indicated that this complex consisted of two molecular species with M_r values of 65,000 and 125,000. The larger molecular weight species could be resolved into the smaller molecular weight species upon rechromatography; thus, these complexes may have a monomer–dimer relationship. Since these complexes may have bound detergent, molecular weight estimations may overestimate actual molecular weights [25], and it is also possible that the binding proteins are not globular. Previous studies of rat liver cytoplasmic [^{3}H]dαT binding site complexes [26] suggested an M_r of 30,000; thus, human RBC membrane-bound binding sites may be oligomers of a smaller subunit which also occurs in the cytoplasm. We have, however, no evidence for a cytoplasmic binding site in human RBC. The relationship, if any, of the two soluble [^{3}H]dαT binding site complexes to the two membrane-bound binding sites (demonstrated by kinetic data) is unknown at present. Clearly, further characterization of the two soluble [^{3}H]dαT binding site complexes would be of value in understanding the relationship of the two soluble molecular forms to the membrane-bound binding sites and in understanding the mechanism of action of dαT as an antihemolytic agent. Previous work by us suggested that preincubation of human RBC with dαT produced a dose-dependent inhibition of subsequent hemolysis by H_2O_2 [14].

Although binding proteins for dαT have been proposed to occur in both cytoplasm and nucleus [26–28], the receptor-like properties of these binding sites were not characterized in detail. There is considerable evidence that there are soluble cytoplasmic receptor proteins for 1,25-dihydroxy vitamin D_3 in several tissues [29–32]. However, solubilization of specific binding proteins for fat soluble vitamins from membranous structures has not been demonstrated before. The possibilities that RBC membrane-bound binding sites for dαT may play some role in endoperoxide formation [33, 34] and adrenocortical hormonal responsiveness [4, 5] are important questions for future investigations.

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