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Transmembrane distribution of sterol in the human erythrocyte

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The transbilayer cholesterol distribution of human erythrocytes was examined by two independent techniques, quenching of dehydroergosterol fluorescence and fluorescence photobleaching of NBD-cholesterol. Dehydroergosterol in conjunction with leaflet selective quenching showed that, at equilibrium, 75% of the sterol was localized to the inner leaflet of resealed erythrocyte ghosts. NBD-cholesterol and fluorescence photobleaching displayed two diffusion values in both resealed ghosts and intact erythrocytes. The fractional contribution of the fast and slow diffusion constants of NBD-labelled cholesterol represent its inner and outer leaflet distribution. At room temperature the plasma membrane inner leaflet of erythrocyte ghosts as well as intact erythrocytes cells contained 78% of the plasma membrane sterol. The erythrocyte membrane transbilayer distribution of sterol was independent of temperature. In conclusion, dehydroergosterol and NBD-cholesterol data are consistent with an enrichment of cholesterol in the inner leaflet of the human erythrocyte.

Introduction

It has been well-established that phospholipids are asymmetrically distributed in biological membranes (for reviews, see Refs. 1 and 2). In contrast, there is little agreement in the literature on the transbilayer distribution and migration of cholesterol in biological membranes [3–6]. The human erythrocyte membrane has been extensively examined and transbilayer migration rates of sterols ranging between seconds to hours and nearly opposite cholesterol transbilayer distributions have been reported (for review, see Ref. 3). A primary difficulty in determining cholesterol transbilayer migration rate and distribution has been the lack of adequate methods. For example, recently cholesterol oxidase and exchange were used to conclude that cholesterol is enriched in the inner (cytofacial) leaflet of the human erythrocyte membrane [7], a finding opposite to that of some others (for review, see Ref. 3). Whether the cholesterol oxidase method correctly established the transbilayer cholesterol distribution in erythrocytes is difficult to evaluate since cholesterol oxidase apparently can perturb the membrane and enhance sterol transbilayer migration rate [8,9]. Thus, it is still unclear whether cholesterol is enriched in the outer or inner leaflet of the erythrocyte.

Herein, two independent methods, fluorescence quenching by Forster energy transfer and fluorescence photobleaching, are used to determine transbilayer distribution of sterol in human erythrocyte membranes. The fluorescent sterols dehydroergosterol and 25-NBD-26-norcholesterol (NBD-cholesterol) are used to

Abbreviations: dehydroergosterol $\Delta^{5,7(11),22}$-ergostatetraene-3β-ol; diphenylhexatriene, 1,6-diphenyl-1,3,5-hexatriene; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; NBD-cholesterol, 25-NBD-26-norcholesterol; modulated fringe pattern photobleaching, fluorescence photobleaching; SUV, small unilamellar vesicle; TNBS, trinitrobenzenesulfonic acid.

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examine the transbilayer migration rate and the transbilayer distribution of sterol. The advantages of the use of these sterols as cholesterol analogues to determine transbilayer cholesterol distribution were recently reviewed [3]. These sterols differ in that the fluorophore of dehydroergosterol is an intrinsic part of the cyclopentanophenanthrene ring structure of the sterol nucleus and is sensitive to the sterol microenvironment closer to the polar/hydrocarbon interface as compared to NBD-cholesterol [10]. In contrast, NBD-cholesterol has an extrinsic fluorophore reporter group that is linked to the alkyl side chain of the sterol. This NBD is deeply buried in the interior of the membrane bilayer [11]. The NBD fluorophore attached to lipids or proteins can be photobleached and has been extensively used to examine lateral diffusion of molecules by fluorescence photobleaching techniques [12]. These two probe molecules in conjunction with fluorescence quenching (by Forster energy transfer) and fluorescence photobleaching allow independent examination of transbilayer distribution of sterol in erythrocyte membranes.

Materials and Methods

Materials

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) was purchased from Avanti Biochemical Inc. (Birmingham, AL). Dehydroergosterol was synthesised and purified by high performance liquid chromatography as described previously [13,14]. Purity was confirmed by high performance liquid chromatography, absorbance peak ratios, and comparison with dehydroergosterol standard. 1,6-Diphenyl-1,3,5-hexatriene and NBD-cholesterol were obtained from Molecular Probes Inc. (Eugene, OR). [1a,2a(n)-^{3}H]cholesterol (46 mCi/mmol) and cholesteryl [1-^{14}C]oleate (57 mCi/mmol) were obtained from Amersham, Arlington Heights, IL, and from New England Nuclear, Boston, MA, respectively. Purity of these lipids was confirmed by silica gel G (Analtech, Newark, DE) thin-layer chromatography developed with chloroform/methanol (98:2, v/v). Trinitrobenzenesulfonic acid and phospholipase A_2 (*Naja mocambique mocambique*) were from Sigma Chemical Co., St. Louis, MO.

Preparation of liposomes

Small unilamellar vesicles (SUV) were prepared as previously described [14–17]. The following lipids were dissolved in chloroform, dried with N_2, and sonicated to provide SUV in 4 ml Tris (50 mM, pH 7.3): POPC (2.8 mg), dehydroergosterol (0.159 mg), 5 μCi [^{3}H]cholesterol, and 1 μCi cholesteryl [^{14}C]oleate. The lipid composition of the resultant SUV was determined also as described previously [14–17].

Erythrocyte trinitrobenzenesulfonic acid labelling and resealed ghost isolation

Human erythrocytes from healthy volunteers were collected in tubes containing heparin anticoagulant. The erythrocytes were sedimented at $1000 \times g$ for 10 min and washed three times with 6 ml of isotonic Tris buffer (172 mM, pH 7.6). Aliquots (400 μl) of packed erythrocytes were resuspended in 10 ml buffer (40 mM NaCl, 120 mM $NaHCO_3$ (pH 8.5)) at 4 °C without or with 8 mM trinitrobenzenesulfonic acid. The erythrocytes were incubated for 45 min at 4 °C. The trinitrophenylation reaction was terminated by addition of 10 ml 1% bovine serum albumin in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2PO_4, 6.5 mM KH_2PO_4, (pH 7.3)). Erythrocytes were sedimented at 4 °C at $1000 \times g$ for 10 min and washed three times with buffer (40 ml NaCl, 120 mM $NaHCO_3$ (pH 8.5)). The washed erythrocytes were resuspended in 30 ml of hypotonic lysis buffer (5 mM Tris, 1 mM EDTA (pH 8.5)). The ghosts were sedimented at $40\,000 \times g$ for 30 min. The lysis process was repeated three or more times until the ghosts were clear of hemoglobin. The ghosts are resealed right-side-out as described earlier [18]. The resealed ghosts were resuspended in 1 ml of phosphate-buffered saline (pH 7.3).

Erythrocyte phosphatidylethanolamine transbilayer distribution

Trinitrobenzenesulfonic acid and phospholipase A_2. Human erythrocytes were treated with trinitrobenzenesulfonic acid as described above. The % of plasma membrane phosphatidylethanolamine trinitrophenylated was determined as described earlier [19–21]. The exposure of phosphatidylethanolamine in the outer leaflet of erythrocytes was also determined with phospholipase A_2 as follows: Packed erythrocytes (250 μl) were suspended in glycylglycine, buffer (100 mM KCl, 50 mM NaCl, 0.25 mM $MgCl_2$, 44 mM sucrose, 10 mM glycylglycine, and 0.25 mM $CaCl_2$ (pH 7.4)). Phospholipase A_2 (50 U) was added and the samples were incubated at 37 °C in a shaking water bath. At varying time points a sample was removed, the reaction stopped with 90 mM EDTA, and placed on ice. An aliquot of the supernatant was removed to determine extent of hemolysis. Erythrocytes were then washed two times with isotonic Tris (0.172 M, pH 7.6). The final pellet was lysed in distilled water. Lipids were extracted with isopropanol and chloroform [22]. Phospholipids and lysophospholipids were resolved using two-dimensional thin-layer chromatography on silica gel H and compared to standards (Analtech, Newark, NJ). The first direction consisted of chloroform/methanol/glacial acetic acid/water (50:25:8:4, v/v); second direction consisted of chloroform/methanol/water (5:10:1, v/v). Spots were visualized with iodine, scraped, and phosphorus assayed [23].

Incorporation of dehydroergosterol into trinitrophenylated and control resealed erythrocytes. A 0.5 ml aliquot of POPC/dehydroergosterol SUV (90:10 mol%) was mixed with 0.4 ml of resealed erythrocyte ghosts (40–45 μg protein) to provide a 1:10 ratio of donor:acceptor membrane lipid. The mixture was incubated in a shaking water bath at 37 °C. Samples (100 μl aliquots) were removed at 0, 5, 15, 30, 60, 90, and 120 min. SUV and erythrocyte ghosts were separated by sedimentation in a 200 μl polyethylene or polyallomer tube for 4 min at 63000 × g with a Beckman Airfuge (Beckman Instr., Fullerton, CA). The supernatant containing SUV was removed. The pellet containing erythrocyte ghosts was resuspended in Tris buffer (50 mM, pH 7.3), removed from the tube and diluted to 2 ml with Tris buffer. Fluorescence intensity was measured with a SLM 4800 fluorometer updated to 1–250 MHz multifrequency phase and modulation capability (155 Instr., Champaign, IL). Fluorescence intensity was corrected for light scatter, usually less than 5% of signal, when necessary. After fluorescence intensity measurement, each 2 ml sample was extracted with 1 ml hexane/diethyl ether (1:1, v/v). The organic (upper) phase was removed, placed into a glass scintillation vial, solvent was evaporated, and 10 ml ACS scintillation cocktail (Amersham, Arlington Heights, IL) was added. Radioactivity (^{3}H and ^{14}C) was measured by a Beckman 7000 (Fullerton, CA) beta counter.

Erythrocyte and erythrocyte ghost preparation for fluorescence photobleaching

Human blood was obtained from healthy volunteers or from a local blood bank (Centre National de Transfusion Sanguine). Blood collected on EDTA was stored at 4 °C and used within 5 days. Erythrocytes were obtained by four washes of blood (1000 × g for 5 min) with the following buffer: 20 mM Hepes (pH 7.4), 145 mM NaCl, 5 mM KCl, 0.1 mM EGTA, 1 mM $MgSO_4$, 10 mM inosine, 10 mM glucose. Pink resealed ghosts containing 3 mM ATP and a regenerating system (creatine phosphate and creatine kinase) were prepared as described earlier [24].

Erythrocyte and resealed erythrocyte ghost labelling with NBD-cholesterol for fluorescence photobleaching recovery

Red cell labelling was carried out according to Avignan [25] as modified by Jonas [26]. Briefly, the fluorescent NBD-cholesterol was fixed on Celite; Celite was then incubated with high density lipoprotein-3 (HDL_3). The lipoproteins labeled with NBD-cholesterol were afterwards incubated with intact erythrocytes or resealed ghosts [27].

Celite 545 (Frolabo, Paris) was washed with diluted HCl (1 M), with water, with methanol, and finally dried overnight under vacuum. NBD-cholesterol in chloroform was incubated with Celite (4% w/w). The mixture was then dried under vacuum.

HDL_3 was prepared by ultracentrifugal flotation [28]. Dialysis for 24 h against buffer (20 mM Hepes (pH 7.4), 0.1 mM EGTA) was utilized to eliminate potassium bromide. Lecithin:cholesterol acyl-transferase activity was denatured by 30 min incubation at 56 °C, followed by 30 min centrifugation at 13000 rpm.

Celite (50 mg) with NBD-cholesterol was incubated with 1 ml of HDL_3 (1 mg protein/ml) for 20 h at 37 °C. Celite was eliminated after the incubation period by centrifugation and filtration. Approximately 10% of the NBD-cholesterol initially on Celite was incorporated in the HDL_3. The erythrocytes or resealed ghosts (30 μl pelleted erythrocytes or ghosts) were incubated with 160 μl HDL_3-NBD-cholesterol (1 mg/ml of protein) and 160 μl NaCl (300 mM) in water for 20 min at 37 °C, followed by sedimentation to separate cells and lipoprotein.

Fluorescence measurements

Fluorescence lifetime measurements, nonlinear least-squares analysis, and Lorentzian continuous distributional analysis were performed as described earlier [10,16,17,29]. Forster energy transfer distances were measured as described earlier [29,30]. Fluorescence photobleaching experiments were carried out as described earlier by using the modulated fringe pattern photobleaching technique [12,31]. The very rapid bleaching of NBD was controlled as described therein and in more detail in Davoust [32]. The observed decay curves were fitted by multi-exponentials using the method of Pade-Laplace [33].

Results

Sidedness selectivity of trinitrophenylation in the erythrocyte

The degree of trinitrobenzenesulfonic acid penetration through the erythrocyte membrane (leakiness) was carefully monitored to assure that under nonpenetrating conditions the reagent is not labeling both sides of the erythrocyte plasma membrane. Several control procedures were used.

First, phosphatidylethanolamine is localized primarily in the inner leaflet of the cell membrane and phosphatidylserine is almost exclusively an inner leaflet phospholipid [1,2,34]. The degree of phosphatidylethanolamine and phosphatidylserine trinitrophenylation reflects the degree of cell membrane inner leaflet labelling by trinitrobenzenesulfonic acid. When intact cells were trinitrophenylated at 4 °C, 8.1 ± 1.2% and 0% of membrane phosphatidylethanolamine and phosphatidylserine were trinitrophenylated, respectively (Table I). When intact cells were incubated with trinitrobenzensulfonic acid at 37 °C, 31.8 ± 2.6% of phos-

phatidylethanolamine was trinitrophenylated as was 4.3% of phosphatidylserine. The possibility that trinitrophenylated phosphatidylethanolamine migrated across the erythrocyte membrane under the conditions used herein was ruled out since further exposure of phosphatidylethanolamine was not observed.

Second, the above observations with TNBS and transbilayer distribution of aminophospholipids were confirmed with phospholipase A_2. When the intact erythrocytes were exposed to phospholipase A_2, $10 \pm 1\%$ of phosphatidylethanolamine was accessible to phospholipase A_2 hydrolysis (Table I). None of the phosphatidylserine was hydrolyzed under these conditions (Table I).

Third, the fluorophore diphenylhexatriene partitions equally across membrane bilayers and between fluid and solid lipids. This property has, in conjunction with selective quenching by covalently linked trinitrophenyl groups, proven extremely useful in monitoring sidedness of the TNBS-membrane reaction [20,35]. Under conditions in which TNBS does not label the inner leaflet aminophospholipids, about 40% of diphenylhexatriene fluorescence is quenched by TNBS (Fig. 1). In contrast, under conditions where aminophospholipids in both leaflets are trinitrophenylated about 87% of diphenylhexatriene is quenched (Fig. 1).

Fourth, trinitrophenyl groups in close proximity to diphenylhexatriene quench fluorescence by Forster nonradiative enery transfer [20,35]. This energy transfer should reduce the fluorescence lifetime of diphenylhexatriene in trinitrophenylated erythrocyte membrane leaflets. The fluorescence lifetime of diphenylhexatriene in control red blood cell plasma membranes was examined both by nonlinear least squares and Lorentzian distributional analysis and determined to best fit (χ^2 of 1.9 to 2.2) for two components with the major one near 11.1 ns representing more than 0.98 fractional fluorescence (Fig. 2). A minor component near 3 ns represented less than 0.02 of the fluorescence and is an artifact [36] unaltered by trinitrophenylation. Fitting the data to a single component or to more components either increased the χ^2 to 17 or did not significantly improve χ^2, respectively. Treatment of erythrocytes with TNBS at 4°C resulted in two major lifetime components near 11.9 and 6.4 ns, fractional fluorescence of 0.71 and 0.29, respectively (Table II). When the fractional fluorescence values are converted to mole fractions, 57% and 43% of the diphenylhexatriene were localized in the outer and inner leaflets of the erythrocyte plasma membrane, respectively. When both leaflets were trinitrophenylated (37°C), only the lifetime component near 6 ns appeared. Quenching of as little as 10% of the fluorescent probe molecules present on the inner membrane leaflet by TNBS labelled molecules present on this same surface, would have resulted in a significant reduction of the fluorescence lifetime of diphenylhexatriene. The data show that this was not the case.

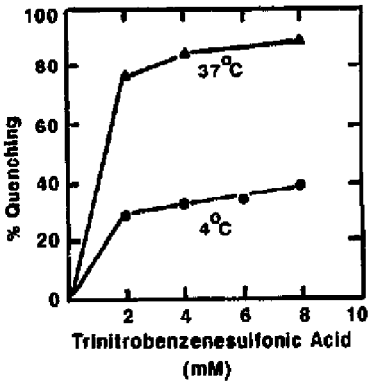

Fig. 1. Effect of trinitrophenylation on diphenylhexatriene fluorescence in resealed erythrocyte ghosts. Human erythrocytes were treated with buffer with or without TNBS under penetrating (37°C) or nonpenetrating (4°C) conditions as described in Materials and Methods. Non-trinitrophenylated and trinitrophenylated resealed erythrocyte ghosts were prepared, diphenylhexatriene was incorporated (1:1000 molar ratio to erythrocyte ghost lipid) and fluorescence intensity was determined also as described in Materials and Methods. Values represent the average of 1–3 determinations with a range less than 5% from the average.

TABLE I

Erythrocyte membrane leaflet selectivity of trinitrobenzenesulfonic acid (TNBS) and phospholipase A_2

Erythrocytes were treated with trinitrobenzenesulfonic acid or phospholipase A_2 at the indicated temperature as described in Methods. Values represent the means ± S.E. ($n = 3$). n.d., none detected.

Reagent	Temp. (°C)	% phosphatidylethanolamine trinitrophenylated or hydrolyzed	% phosphatidylserine trinitrophenylated or hydrolyzed
TNBS	4	8.1 ± 1.2	n.d.
	37	31.8 ± 2.6	4.3 ± 1.1
Phospholipase A_2	37	10.0 ± 1.0	n.d.

Energy transfer from dehydroergosterol to trinitrophenyl groups in erythrocyte plasma membranes

The trinitrophenyl quenching method was applied to examine the transbilayer sterol distribution in erythrocyte plasma membranes. This procedure is an extension of the same method applied to determine transbilayer sterol distribution in cultured cell [19,37–40] and brain synaptic plasma membranes [21]. Several conditions in addition to those described in the preceding sections must be met in order that the quenching by trinitrophenyl groups be maximal.

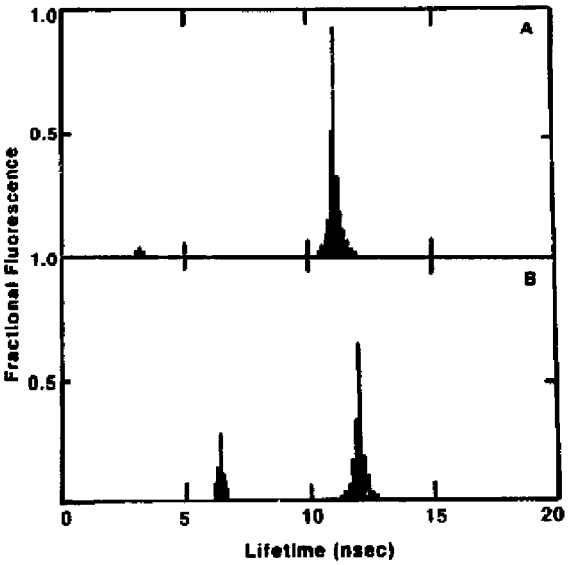

Fig. 2. Lorentzian distributional analysis of diphenylhexatriene lifetime. Fluorescence lifetime of diphenylhexatriene in nontrinitrophenylated and trinitrophenylated (4 °C) resealed erythrocyte ghosts was determined by phase and modulation (1–250 MHz) at 14 different different modulation frequencies and the results were fitted to one or two components both by nonexponential least-squares fit and continuous distributional fit to one or two Lorentzian distributions. In either case the χ^2 were significantly improved by two component fits. The centers of lifetime distribution shown in the figure were nearly identical to the two lifetime values obtained from two component nonlinear least-squares fit (Table II).

First, the surface density of trinitrophenyl acceptor groups must be sufficiently large for maximal energy transfer efficiency [41]. Trinitrobenzenesulfonic acid trinitrophenylates free amino groups on both proteins and aminophospholipids. When intact erythrocytes were labelled under nonpenetrating conditions, trinitrophenyl groups attached to plasma membrane proteins were measured by the difference in absorbance at 434 nm (wavelength of maximal absorbance of covalently linked trinitrophenyl groups) between the intact membranes and the extracted lipids. Approximately 10 times as many trinitrophenyl groups were attached to membrane proteins as compared to aminophospholipids. Both protein and aminophospholipid linked trinitrophenyl groups can act as fluorescence quenchers. The erythrocyte membrane surface density of trinitrophenylated phosphatidylethanolamine alone is 0.04. When the protein attached trinitrophenyl groups are taken into account, the trinitrophenyl acceptor group surface density is in in excess of 0.3. The acceptor density of trinitrophenylated phosphatidylethanolamine alone is sufficient to account for a greater than 90% transfer efficiency (when R_0, the distance for half maximal transfer efficiency is about 30 ångstrom) of the dehydroergosterol/trinitrophenyl donor/acceptor pair [42]. As shown below, the R_0 for this pair was 28 ångstrom.

Second, the relative contribution of trinitrophenyl groups attached to aminophospholipid and protein responsible for quenching fluorescence of dehydroergosterol incorporated into erythrocyte membranes was determined. Both protein and aminophospholipid linked trinitrophenyl groups can act as fluorescence quenchers. Under conditions in which both sides of membranes were exposed to trinitrophenyl groups: (a) in lipid vesicles containing dehydroergosterol and trinitrophenyl groups inside and outside the vesicle, trinitrophenyl groups maximally quench 81% of dehydroergosterol fluorescence and (b) in unsealed membrane ghosts (both aminophospholipids and proteins trinitrophenylated) the quenching was 96%. Thus, under both nonpenetrating and penetrating conditions an excess of trinitrophenyl acceptor groups is present in the trinitrophenylated erythrocyte membranes.

Third, quenching of dehydroergosterol in one leaflet by acceptor trinitrophenyl groups in the opposite leaflet must not occur. For the dehydroergosterol/trinitrophenyl donor/acceptor pair the Forster R_0, the distance at which the transfer efficiency is 50%, was determined to be 28 ångstrom. The distance between the dehydroergosterol and trinitrophenyl groups, R, was calculated from the R_0 and the transfer efficiency (96%) as described earlier [29,30,42] and found to be 16 ångstrom. The conjugated triene fluorophore of dehydroergosterol is localized in the B/C rings of the sterol cyclopentanophenanthrene ring structure, about 3 ångstrom from the aqueous interface of membrane bilayers with a width of approx. 40 ångstrom. Transbilayer distances between trinitrophenyl groups in one leaflet and dehydroegosterol fluorophores in the opposite leaflet would be in excess of 37 ångstrom. Energy transfer from dehydroergosterol in the inner leaflet to trinitrophenyl groups localized in the exofacial leaflet of the membrane will not be significant because the transfer efficiency varies as $1/R^6$.

TABLE II

Analysis of the fluorescence emission decay of diphenylhexatriene in resealed human erythrocyte ghosts

C, W, and F refer to the lifetime, width of distribution, and fractional contribution, respectively. The subscripts refer to component 1 or 2, respectively.

Reagent	Analysis (NLS or LD)					
	C_1 (ns)	W_1 (ns)	F_1	C_2 (ns)	W_2 (ns)	χ^2
Nonlinear least squares						
None	12.1	–	0.93	4.3	–	1.9
Lorentzian distribution						
None	11.1	0.05	0.98	3.1	0.05	2.2
TNBS, 4 °C	11.9	0.05	0.71	6.4	0.09	2.2

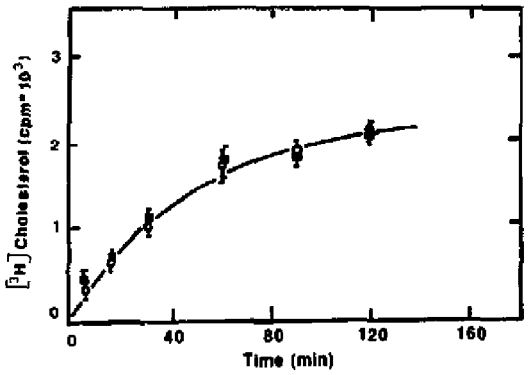

Fig. 3. Incorporation of [^{3}H]cholesterol into trinitrophenylated and non-trinitrophenylated resealed erythrocyte ghosts. Human erythrocytes were treated with buffer with or without trinitrobenzenesulfonic acid under non-penetrating conditions (4°C) and resealed erythrocyte ghosts were isolated as described in Methods. The resealed ghosts were incubated with POPC/dehydroergosterol SUV (1:10 molar ratio of SUV:ghost lipid) containing trace amounts of [^{3}H]cholesterol and cholesteryl [1-^{14}C]oleate as described in Methods. Exchange of [^{3}H]cholesterol into the resealed control (○) and trinitrophenylated (□) resealed ghosts was measured also as described in Methods. As determined by the presence of the non-exchangeable marker, cholesteryl [1-^{14}C]oleate, less than 5% of the radioactivity incorporated into the resealed erythrocyte ghosts was due to sticking or fusion with SUV.

Transbilayer distribution and transbilayer migration rate of fluorescent dehydroergosterol in erythrocyte membranes

The transbilayer distribution of sterol in erythrocyte membranes was examined by exchange of fluorescent dehydroergosterol into erythrocyte ghosts without or with covalently attached trinitrophenyl groups in the outer monolayer. It is essential that: (1) trinitrophenylation not affect sterol entry into erythrocyte membranes; (2) fluorescent dehydroergosterol mimic the exchange of cholesterol into the erythrocyte membranes; (3) self-quenching of dehydroergosterol not occur, and (4) that fusion of erythrocyte membranes with SUV not occur. These criteria are met as follows:

The exchange curves of [^{3}H]cholesterol from SUV to erythrocyte ghosts and to trinitrophenylated erythrocyte ghosts were nearly identical (Fig. 3). The [^{3}H]cholesterol exchanged into control and trinitrophenylated ghosts with half-times of 34.9 and 33.5 min, respectively.

The exchange of [^{3}H]cholesterol (Fig. 3) and dehydroergosterol (Fig. 4, top curve) from SUV to ghosts were very similar. The [^{3}H]cholesterol and dehydroergosterol exchanged into the ghosts with half-times of 34.9 and 34.0 min, respectively. Dehydroergosterol exchange mimics cholesterol exchange between SUV and erythrocyte ghosts.

Above 6 mol% self-quenching of dehydroergosterol occurs [10,15,16]. Under the conditions used herein, at equilibrium less than 2% of erythrocyte ghost acceptor lipid is dehydroergosterol.

A non-exchangeable marker, cholesteryl [1-^{14}C]oleate, was incorporated into the SUV to monitor SUV adherence to or fusion with acceptor ghosts. In all cases, less than 5% of the radioactivity of [^{3}H]cholesterol or fluorescence of dehydroergosterol could be accounted for by SUV adherence or fusion of SUV with ghosts.

Since the above controls validate the use of trinitrophenylated erythrocyte ghosts to determine the transbilayer distribution with dehydroergosterol, the equilibrium transbilayer distribution of dehydroergosterol and transbilayer migration rate of dehydroergosterol can be measured. At equilibrium, 25.6 ± 3.1% of the dehydroergosterol fluorescence intensity is quenched in the red cell ghosts with trinitrophenylated outer leaflet (Table III). When both leaflets are trinitrophenylated, quenching was 96% (Table III). The transbilayer migration rate of sterol in erythrocyte ghosts was also determined with dehydroergosterol. Appearance of dehydroergosterol in the outer monolayer of resealed erythrocyte ghosts and fluorescence signal are simultaneous. In contrast, in the trinitrophenylated ghosts the fluorescence of dehydroergosterol in the outer leaflet is quenched. Only after the dehydroergosterol has migrated from the outer to the inner monolayer of the trinitrophenylated ghost is the fluorescence detectable. Thus the fluorescence versus time curve in trinitrophenylated ghosts is shifted to longer time (Fig. 4). The half-time of this shift, the transbilayer migration rate was 6 ± 2 min (Table III).

Fluorescence photobleaching experiments of NBD-cholesterol in human erythrocytes and erythrocyte ghosts

In the modulated fringe pattern photobleaching technique, the contrast between the fluorescence in-

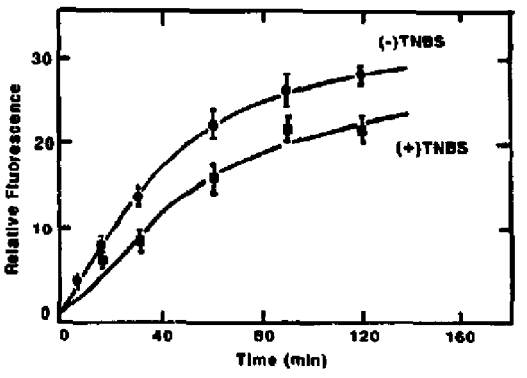

Fig. 4. Incorporation of dehydroergosterol into trinitrophenylated and non-trinitrophenylated resealed erythrocyte ghosts. All conditions were as described in Methods. Dehydroergosterol fluorescence intensity was determined in the acceptor resealed red cell ghosts.

TABLE III

Erythrocyte ghost transbilayer sterol distribution and migration time: quenching of dehydroergosterol

Dehydroergosterol was incorporated into resealed erythrocyte ghosts from erythrocytes pretreated without and with trinitrobenzenesulfonic acid (TNBS) under nonpenetrating conditions (4°C). Dehydroergosterol was incorporated into the resealed ghosts by exchange from POPC/dehydroergosterol SUV. At equilibrium, the mol% of sterol represented by dehydroergosterol in the resealed ghosts was less than 2 mol%. The resealed ghost sterol/phospholipid ratio was unaltered by incorporation of dehydroergosterol. Values represent the means ± S.E. ($n = 3$).

Parameter	TNBS labelling temperature (°C)	% Fluorescence quenched	$t_{1/2}$ (min)
Transbilayer distribution	4	25.6 ± 3.1	-
	37	96.0 ± 3.0	-
Transbilayer migration	4	-	6 ± 2

tensity of bleached regions and unbleached regions determined by the fringe pattern, is analyzed as a function of time. The decay of the contrast follows a simple exponential law in the case of a single diffusion constant [31,33]. Thus, a semilogarithmic transformation of the contrast curve gives a straight line for a single diffusion. Fig. 5 shows the result of the accumulation of several fluorescence contrast curves obtained in different areas of the same microscope slide containing erythrocytes labelled with NBD-cholesterol. In spite of the sensitivity of the modulated fringe pattern technique, 10 to 20 accumulations are necessary to obtain a good signal to noise ratio because of the light absorption by hemoglobin. Very similar curves were obtained with pink resealed ghosts, the number of accumulations necessary for good signal to noise ratio being less in the latter case (from 1 to 4). Fig. 5A is the direct accumulated recording, while Fig. 5B is the semilogarithmic transformation. The curve in Fig. 5B shows clearly that the contrast curve cannot be accounted for by the diffusion of an homogeneous population of fluorescent molecules, that is, with a single diffusion constant. In fact all samples (erythrocytes and ghosts, at any temperature) corresponded to a multi-exponential decline, which after analysis revealed that the fast component always accounts for 75 ± 5% of the fluorescent molecules. Table IV shows the actual percentage of fast component obtained at different temperatures in the ghosts and at 22°C in intact erythrocytes. The values of the diffusion constants are also indicated. These diffusion values are remarkably similar to the two diffusion constants of NBD-labelled phospholipids in the same cells as determined by Morrot et al. [12]. In these previous experiments the fast diffusion was attributed to phospholipids in the inner monolayer, while

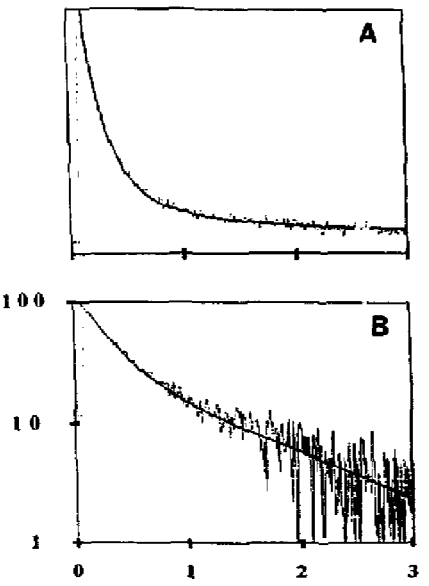

Fig. 5. Decay of the fluorescence contrast after photobleaching (second harmonic of the modulation) for NBD-cholesterol in human erythrocytes. Experiments were carried out at 22°C. Interfringe spacing was 1.67 μm; bleaching time was 50 ms. (A) Accumulation of 20 bleaches on the same microscope slide, direct recording; (B) Semi-logarithmic transformation showing bi-exponential behavior.

the slow diffusion was attributed to phospholipids in the outer monolayer [12,43]. Thus, it is likely that the two components of cholesterol diffusion represent also NBD-cholesterol in the inner and outer monolayers, respectively. The immobilized fraction of NBD-cholesterol varied from sample to sample but never exceeded 10% of the probes. The significance of this

TABLE IV

Erythrocyte membrane transbilayer sterol distribution: fluorescence photobleaching experiments of NBD-cholesterol

At each temperature several curves were accumulated and then analyzed. The uncertainty in the diffusion value is approximately 10%, due to errors in the measurement of the interfringe and in the simulation of the biexponential curves. The % fast component is determined with and accuracy of ±5% of the mobile fraction of NBD-cholesterol.

Temperature (°C)	Diffusion constant (10^{-9} cm²/s)		Fast component (%)
	D_1	D_2	
Ghosts resealed with ATP			
6	0.56	0.11	70
25	4.30	0.93	78
30	5.40	1.40	72
Red blood cells			
22	3.50	0.86	78

immobilized cholesterol fraction is not known, but its variability suggests an experimental artifact, for example a small fraction of trapped HDL_3 on the surface of the erythrocytes.

Discussion

Two independent techniques, quenching of dehydroergosterol fluorescence and fluorescence photobleaching of NBD-cholesterol, were used to determine the transbilayer distribution of cholesterol analogues in human erythrocytes. Both methods showed that erythrocyte sterol is localized primarily in the inner leaflet.

In the first method, dehydroergosterol was used as a cholesterol analogue in conjunction with selective leaflet quenching to determine resealed erythrocyte ghost transbilayer sterol distribution. At equilibrium, 75% of the fluorescent dehydroergosterol was localized in the inner leaflet of the erythrocyte plasma membrane. This distribution is independent of whether the TNBS-labelled ghost vesicles are completely resealed, since the intact cell was used to trinitrophenylate only the outer monolayer of the erythrocyte membrane. The validity of this approach depends on two factors: the selectivity of the TNBS as a non-penetrating probe and the adequacy of using dehydroergosterol as an analogue for cholesterol.

It should be noted that the term non-penetrating used in conjunction with TNBS may not be most appropriate. Certainly, in the present work the term refers to TNBS labelling conditions in which very little, if any, trinitrophenylation of the inner leaflet occurs. This may not, however, be completely synonymous with lack of penetration of TNBS in all circumstances. For example, there appears to be some uncertainty in the literature with regard to calling TNBS a non-penetrating probe for erythrocytes. Unarguably, under certain conditions, e.g. 37 °C, TNBS can permeate the erythrocyte membrane [44]. However, at 4 °C this penetration is either much less [44] or insignificant [45–47,19] in erythrocytes. Most important for the non-penetrating conditions, even if a small amount of TNBS penetrated the erythrocyte it preferentially reacts with hemoglobin amino groups [45,47–49] and glutathione sulfhydryl groups [44]. TNBS and phospholipases have both been used to determine the aminophospholipid asymmetry in erythrocyte [46,41,50,51], platelet [51,52], and sarcoplasmic reticulum [53] membranes. Although the transbilayer distribution of erythrocyte and sarcoplasmic reticulum membranes were nearly opposite, in either case the TNBS and phospholipase results indicated essentially similar asymmetry. Last, in cultured cells the results obtained with TNBS were confirmed with both penetrating and non-penetrating acetimidate derivatives [54]. Nevertheless, it is important for each system in which TNBS is used that appropriate control experiments be performed to assure that under non-penetrating conditions the reagent did not label in inner leaflet. The experiments performed herein with TNBS were designed on the basis of the earlier work on TNBS labelling of erythrocytes and conditions were chosen to minimize penetration of TNBS [45–47]. The trinitrophenylation reaction was carefully monitored to assure that the reagent did not label both sides of the membrane by using a series of control procedures. The same control procedures have been documented earlier for the use of TNBS to label cultured fibroblasts [5,20,55] and synaptosomes [35].

Dehydroergosterol is an excellent analogue of cholesterol for studies of sterol domains as indicated by a considerable body of model and biological membrane data from this and other laboratories (for reviews, see Refs. 3,4 and 56). Fluorescent sterols such as dehydroergosterol and cholestatrienol codistribute with cholesterol in membranes both laterally [10,15,57] and transbilayerly (for reviews, see Ref. 3). In addition, these sterols have similar desorption rates as cholesterol as determined by exchange studies [4,16,58,59,56]. Probes that are structurally dissimilar from cholesterol have significantly different desorption rates and are therefore not useful for examining sterol domains (for reviews, see Refs. 3 and 56). Moreover, dehydroergosterol is nonperturbing since it: (1) is a natural product comprising up to 20% of membrane sterols [33]; (2) is bioincorporated into cultured cells without adverse effect [19,30,37–40].

The transbilayer distribution of cholesterol determined by dehydroergosterol (as well as NBD-cholesterol) independently establishes that cholesterol is enriched in the inner leaflet of the erythrocyte. These data, taken together with cholesterol oxidase results [7], indicate that three different methods consistently show that the inner leaflet of the human erythrocyte is enriched in cholesterol. Moreover, the results presented herein also substantiate that cholesterol oxidase, when used appropriately [7] can be used to determine transbilayer cholesterol distribution despite the limitations of the latter method [8,9]. Last, the results with enrichment of erythrocyte cholesterol in the inner leaflet are also in line with the transbilayer distribution of dehydroergosterol and other sterols reported in eukaryotic cell surface membranes and in model membranes (for reviews, see Ref. 3).

The second technique, NBD-cholesterol and fluorescence photobleaching showed that the cholesterol analogue partitions in two different environments, characterized by a different diffusivity. The two diffusion values measured with NBD-cholesterol are remarkably similar to the two diffusion values obtained with fluorescent phospholipid in resealed ghosts or intact erythrocytes [12]. Since previous investigators have shown that cholesterol analogues diffuse like the

phospholipid in which they are embedded [60–62], we conclude that the NBD-cholesterol is distributed between the inner and outer monolayers of erythrocytes with 75 ± 5% in the *inner* monolayer which is the layer associated with the fast diffusion. Due to the approximately 10% of immobilized fluorescent cholesterol, the uncertainty in the asymmetry of the fluorescent cholesterol analogue may be slightly larger. However, it cannot be less than 65% in the inner monolayer. Thus, the inner monolayer which has a higher lipid diffusivity contains more cholesterol than the outer monolayer. This conclusion may appear paradoxical. However, in a recent study on model systems [43], we have shown that a difference in cholesterol population cannot explain the difference in diffusivity of the two erythrocyte layers. The asymmetric 'diffusivity' must be due to the difference in phospholipid composition between the inner and outer monolayers of erythrocyte membranes.

The cholesterol transbilayer distribution has been measured in plasma membranes from only a limited number of biological tissues and species. Results with human erythrocytes presented herein and with cholesterol oxidase [7] provide data consistent with the conclusion that the inner leaflet of human red blood cell membranes is enriched with cholesterol. Transbilayer cholesterol migration appears intimately involved in the mechanisms of cholesterol desorption from membranes and spontaneous exchange between membranes. Fluorescence techniques provided a relatively fast cholesterol transbilayer migration half-time near 6 min in human erythrocyte plasma membranes. It should be noted that the erythrocyte ghosts may be slightly leaky and the measured transbilayer migration half-time would represent a maximal value. However, the data in Fig. 4 show that the ghosts were not significantly leaky since a shift in the fluorescence curve was indeed observed.

The results presented herein with regard to transbilayer sterol distribution and migration rate may be affected by at least two parameters. (1) If the transbilayer migration rate of sterol is slow, then measurements of cholesterol transbilayer distribution and/or migration rates may not be correct (for reviews, see Ref. 56). Nevertheless, it now appears that the transbilayer migration rate of sterols is rapid (data presented herein and elsewhere, see reviews in Refs. 3 and 56). Thus, it is unlikely that dehydroergosterol, added into the red cell membrane by exchange, may not completely equilibrate across the bilayer. (2) The presence of multiple cholesterol domains in the lateral plane of the bilayer may complicate determination of cholesterol transbilayer rates. Multiple lateral cholesterol domains have been documented in phospholipid/cholesterol vesicles (see reviews in Ref. 56). These domains were similar in both leaflets of the model membrane vesicles. In the case of erythrocytes, two exchangeable pools representing 15% and 85% of cholesterol have been reported [63]. Most important, the same pools were observed in intact erythrocytes and inside-out plasma membrane vesicles derived therefrom. Similarly, two pools of exchangeable sterol have been identified in L fibroblast plasma membranes [3,56]. Again, essentially the same proportion of lateral sterol domains appears to occur in each leaflet of the L plasma membrane membrane (for reviews, see Refs. 3 and 56). Since the transbilayer migration rate is fast and since the sterol pools are similar in both leaflets, it seems very likely that the degree of equilibation of sterol between pools within each leaflet is similar for the outer and inner leaflets of the erythrocyte membrane. Thus, the presence of these sterol pools within each membrane leaflet would not be expected alter the transbilayer sterol distribution as measured with fluorescent sterol probes.

In conclusion, the results presented herein and by others [7] indicate that the majority of cholesterol is localized in the inner leaflet of the erythrocyte membrane. Although individual phospholipid species are also asymmetrically distributed across the human erythrocyte membrane, the overall distribution of the total phospholipid between the two surfaces is approx. 50:50. The molar ratio of cholesterol/phospholipid in erythrocytes is close to 1:1. Therefore, if 75% of the cholesterol is present on the cytoplasmic surface of the red cell membrane, this would imply that the cholesterol/phospholipid ratio in the inner leaflet is 1.5, a surprisingly high value. The role of such a high sterol/phospholipid ratio in the inner leaflet on cell function is not known. However, the following observations may be relevant. The cholesterol is segregated into cholesterol rich and poor domains [3,56]. Although most proteins appear to reside in cholesterol poor domains, a few such as the acetylcholine receptor reside in cholesterol rich domains (for reviews, see Ref. 56). It has been postulated that the effects of cholesterol on membrane proteins are not direct but rather are because phase separation of cholesterol promotes aggregation of the transport protein or membrane protein, where the active species is the aggregated protein or transporter (see review in Ref. 56). Such aggregation would be facilitated in the inner leaflet where the very high cholesterol/phospholipid ratio would result in a massive phase separation of cholesterol. In contrast, the outer leaflet would have a lower sterol/phospholipid ratio which might facilitate lateral mobility of receptor proteins.

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