Transbilayer movement of cholesterol in the human erythrocyte membrane

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Abstract The rate of transbilayer movement of cholesterol was measured in intact human erythrocytes. Suspended erythrocytes were incubated briefly with [^{3}H]cholesterol in ethanol at 4°C, or with liposomes containing [^{3}H]cholesterol over 6 hr at 4°C to incorporate the tracer into the outer leaflet of erythrocyte plasma membranes. The erythrocytes were then incubated at 37°C to allow diffusion of cholesterol across the membrane bilayer. Cells were treated briefly with cholesterol oxidase to convert a portion of the outer leaflet cholesterol to cholestenone, and the specific radioactivity of cholestenone was determined over the time of tracer equilibration. The decrease in specific radioactivity of cholestenone reflected transbilayer movement of [^{3}H]cholesterol. The transbilayer movement of cholesterol had a mean half-time of 50 min at 37°C in cells labeled with [^{3}H]cholesterol in ethanol, and 130 min at 37°C in cells labeled with [^{3}H]cholesterol exchanged from liposomes. The cells were shown, by the absence of hemolysis, to remain intact throughout the assay. The presence of 1 mM Mg^{2+} in the assay buffer was essential to prevent hemolysis of cells treated with cholesterol oxidase perturbed the cells, resulting in an accelerated rate of apparent transbilayer movement. Our data are also consistent with an asymmetric distribution of cholesterol in erythrocyte membranes, with the majority of cholesterol in the inner leaflet. — **Brasaemle, D. L., A. D. Robertson, and A. D. Attie.** Transbilayer movement of cholesterol in the human erythrocyte membrane. *J. Lipid Res.* 1988. **29:** 481–489.

Supplementary key words flip-flop • membranes • erythrocytes • cholesterol oxidase

Plasma cholesterol can enter cells through several pathways. Receptor-mediated and receptor-independent uptake of cholesterol carried on lipoproteins accounts for the majority of exogenous cholesterol entry (1–4). Additionally, lipoprotein cholesterol can enter cells through a pathway independent of endocytosis and in excess of concomitant entry of the corresponding apolipoproteins (5–8). The mechanisms underlying the entry of cholesterol into cells, independent of endocytosis, are unknown. One possible explanation is the association of cholesterol with the outer leaflet of the plasma membrane followed by transbilayer movement of cholesterol, and dissociation of cholesterol from the membrane into the cytoplasm. Previous studies indicate that cholesterol moves across membrane bilayers (9–17). There is, however, wide variance in the data describing the rate of transbilayer movement. For example, Lange, Dolde, and Steck (15) suggested a half-time of seconds for transbilayer movement of cholesterol in erythrocyte membranes, while other studies described a half-time of minutes or hours (10–12, 14). These experiments used a variety of probes of membrane cholesterol and employed different methods of measurement to determine the rate of transbilayer movement of cholesterol.

Several conditions are important to the accurate determination of the rate of transbilayer movement of cholesterol and the application of this measurement to intact cells. First, any tracers employed should be as similar to cholesterol as possible. Most spin-labeled and fluorescent analogs of cholesterol are more polar than cholesterol, and hence may not faithfully mimic its behavior in a membrane. Second, biologically relevant membranes should be used; the ideal systems are intact cells. Studies using cells should include a measure of membrane integrity. Third, the methods used should not perturb other features of the cell, such as the arrangement of membrane components.

In this study, we developed a new approach to measure the transbilayer movement of cholesterol in intact erythrocytes, using a radiolabeled cholesterol tracer. We followed the movement of [^{3}H]cholesterol across erythrocyte membranes at 37°C. Intact erythrocytes were labeled with [^{3}H]cholesterol under conditions that selectively radiolabel the outer leaflet of the plasma membrane. The radiolabeled cholesterol was then permitted to move freely across the membrane bilayer. The disappearance of [^{3}H]cholesterol from the outer leaflet was determined by treating cells briefly with cholesterol oxidase (18) under conditions that converted a small portion of outer leaflet cholesterol to cholestenone, and then measuring the rate of decline in the specific radioactivity of cholestenone. The lack of concurrent hemolysis with enzyme treatment demonstrated that the cells remained intact under these conditions.

EXPERIMENTAL PROCEDURES

Materials

[1,2-^{3}H(N)]cholesterol (53.0 Ci/mmol) was purchased from New England Nuclear. Radiochemical purity was determined by thin-layer chromatography (developed in benzene–ethyl acetate 2:3). Radioactive bands were located by scanning plates with a Bioscan System 200 Imaging Scanner interfaced with an Apple IIe computer. Cholesteryl [1-^{14}C]oleate (56.0 mCi/mmol) was obtained from New England Nuclear. Cholest-4-en-3-one was obtained from Steraloids; cholesterol, stigmasta-4,22-dienone, and egg phosphatidylcholine (Type V-E) were obtained from Sigma. Cholesterol oxidase (EC 1.1.3.6; *Brevibacterium* sp., 12.4 U/mg) was obtained from Beckman Instruments. Precoated thin-layer chromatography plates (250 μm silica gel G) were obtained from Analtech, Inc. Solvents used in lipid extractions were redistilled reagent grade.

Erythrocyte preparation

Human blood was collected in 0.32% citrate. Erythrocytes were collected by centrifugation (10 min; 2400 *g*) and were washed three times in four volumes of saline (145 mM NaCl, 5 mM KCl, 5 mM sodium phosphate, pH 7.4), and three times in assay buffer (310 mM sucrose, 1 mM $MgSO_4$, 5 mM sodium phosphate, pH 7.4) at 4°C. Erythrocytes were used within 10 hr of collection.

Labeling of erythrocytes with exogenous [^{3}H]cholesterol

Cells were suspended in 3 ml of saline in 15-ml sterile plastic centrifuge tubes; 50 μCi of [^{3}H]cholesterol (53.0 Ci/mmol) was added in 50 μl of ethanol, and the cells were incubated for 5 min at 4°C. Radiolabeled cells were then rinsed four times in four volumes of saline and three times in four volumes of assay buffer at 4°C in microfuge tubes; brief centrifugation (15 sec) at 13,000 *g* in a Fisher Microfuge Model 235A was used to pellet the cells. The entire rinsing procedure was completed in 15 min.

Labeling of erythrocytes with liposome [^{3}H]cholesterol

Liposomes were made by suspending 100 mg of egg phosphatidylcholine, 35 mg of cholesterol with 0.5 mCi [^{3}H]cholesterol (approximately 0.8 mol cholesterol/mol phosphatidylcholine), and cholesteryl [^{14}C] oleate as a nonexchangeable marker in 2 ml of saline with 0.01% EDTA, and then passing the suspension through an Aminco French Pressure cell three times at 20,000 psi (19). The solution was centrifuged 5 min in a microfuge (13,000 *g*), and the supernatant containing vesicles was eluted over a coarse Sephadex G50 column in saline with 0.01% EDTA. Erythrocytes were suspended in 3 ml of saline in 15-ml sterile plastic centrifuge tubes; 1 ml of liposome preparation (one-third of the preparation) was added, and the cells were incubated for 6 hr at 4°C. Radiolabeled cells were then rinsed four times in four volumes of saline and three times in four volumes of assay buffer at 4°C, as described above. The absence of ^{14}C radioactivity from cholesteryl [^{14}C] oleate, in the rinsed cell suspensions indicated that no measurable quantities of liposomes contaminated the cells.

Treatment of erythrocytes with cholesterol oxidase

The method of Lange and Ramos (18) for the treatment of cells with cholesterol oxidase was modified to allow more rapid measurements of plasma membrane cholesterol. Washed cells suspended in assay buffer (typically 20 to 50 μg of erythrocyte cholesterol/ml cell suspension) were treated with cholesterol oxidase (6 IU/ml) at 37°C or 10°C. Depending on the experiment, the cells were preincubated at 37°C, and then briefly treated with enzyme (20 sec), or briefly preincubated at 37°C or 10°C, and then treated continuously with enzyme. Aliquots of the reaction mixture were extracted in four volumes of chloroform–methanol 2:1. The organic phase was split into two portions: 80% for sterol mass analysis and 20% for determination of sterol radioactivity. The extracts were dried under nitrogen and stored at −20°C (1–4 days) in glass screw-topped test tubes until the lipid analysis was performed.

Sterol mass and radioactivity determinations

Mass determinations of cholesterol and cholestenone were made by gas chromatography using a Varian 3400 gas chromatograph with a Hewlett Packard 3390a Integrator. The samples were redissolved in isooctane, and injected into a 4-ft column of 1% Dexsil 300 on 100/120 Supelcoport, with nitrogen carrier gas (20 cm/min), and a temperature program of 250°C for 5 min, followed by a 6°C/min increase to 350°C. The mass of each sterol was determined by comparison of peak areas to known quantities of an internal standard, stigmasta-4,22-dienone.

Cholesterol and cholestenone were separated by thin-layer chromatography, and the radioactivity of each sterol was quantitated. Lipid extracts were redissolved in chloroform and spotted onto Silica Gel G plates with unlabeled standards. The plates were developed in hexanes–ethyl ether–acetic acid 80:25:1, and the lipid spots were visualized with I_2 vapor. Radioactivity was located by scanning the plates with a Bioscan System 200 Imaging Scanner interfaced with an Apple IIe computer, and the radioactive bands were scraped into scintillation vials. Scint A (United Technologies, Packard) was added, and the sample radioactivity was determined using a Beckman 100LS scintillation counter. Radioactivity counts typically ranged from 10^3 to 5×10^4 cpm; background counts were typically 40 to 70 cpm, and were determined by scintillation counting of a blank thin-layer chromatography band.

Other methods

Hemolysis of cholesterol oxidase-treated erythrocytes was determined by centrifuging aliquots of the reaction mixture for 15 sec at 13,000 *g* in a microfuge. The absorbance of the supernatant was determined at 540 nm, and was compared to the absorbance of the same quantity of cells lysed in a hypotonic buffer (5 mM sodium phosphate, pH 7.4) (15).

The rate of decrease of the specific activity of cholestenone and the rate of cholesterol oxidation by cholesterol oxidase were estimated using the Curve Fitter-PC program (Interactive Microware, Inc., State College, PA), which fits the data to a monoexponential curve using an IBM PC. The coefficients of correlation of the data were typically 0.90 to 0.99, but ranged from 0.78 to 0.99 (see Tables 1 and 2). The plateaus of oxidation of erythrocyte cholesterol by cholesterol oxidase at 10°C and 37°C were estimated using the HYPERO program (20), which fits the data into an equation for a hyperbola, $y = Ax/(B + x)$.

RESULTS

Oxidation of erythrocyte cholesterol

Cholesterol oxidase was used to convert plasma membrane cholesterol to cholest-4-en-3-one in erythrocytes suspended in assay buffer without Mg^{2+}. Cholesterol and cholestenone were then extracted, separated, and quantitated. The plasma membrane cholesterol of erythrocytes was rapidly oxidized by cholesterol oxidase at 37°C (**Fig. 1A**). Cholesterol oxidation was accompanied by hemolysis of the erythrocytes, indicating the permeabilization of the cells by enzyme treatment under these experimental conditions. The addition of 1 mM Mg^{2+} to the assay buffer allowed the complete oxidation of plasma membrane cholesterol without hemolysis (Fig. 1B). The rapid oxidation of 12.6 ± 3.2% (n = 8) of the erythrocyte cholesterol was followed by the slower oxidation of the remaining cholesterol ($t_{1/2}$ = 10.2 ± 2.7 min, n = 8). Cholesterol oxidation was complete by 40 min at 37°C.

The oxidation of erythrocyte cholesterol was measured at low temperature, a condition that restricts the movement of lipids in membrane bilayers (21–23). Approximately 14% of the total erythrocyte cholesterol was rapidly oxidized (14.4 ± 1.8%, n = 4), and no further oxidation was measured over 6 hr at 10°C (**Fig. 2**). The oxidation of erythrocyte cholesterol at 10°C was not accompanied by hemolysis, indicating that the cells were intact throughout the 6-hr treatment.

The cholesterol from cells disrupted by a hypotonic buffer (1 mM $MgSO_4$, 5 mM sodium phosphate, pH 7.4) was rapidly and completely oxidized while 10–14% of the cholesterol of intact cells was oxidized by cholesterol oxidase at 37°C (**Fig. 3**) and 10°C. The half-time for cholesterol oxidation in disrupted cells was 0.64 ± 0.43 min at 37°C (n = 5), and 0.84 ± 0.31 min at 10°C (n = 5). Hence, when all of the membrane cholesterol was available to cholesterol oxidase, rapid and complete oxidation occurred, while intact cell membranes prevented spontaneous oxidation of approximately 86% of the total cholesterol.

Rationale of the method; changes in the specific radioactivity of outer leaflet cholesterol at 37°C

Brief cholesterol oxidase treatments oxidize a small percentage of readily available cholesterol, without permeabilizing the cell. Hence, we postulate that the reactive cholesterol should represent a sampling of outer leaflet cholesterol. Erythrocytes briefly labeled with [^{3}H]cholesterol at 4°C (5 min) should incorporate the tracer selectively into the outer leaflet. The subsequent incubation of labeled cells at 37°C initiates the equilibration of inner

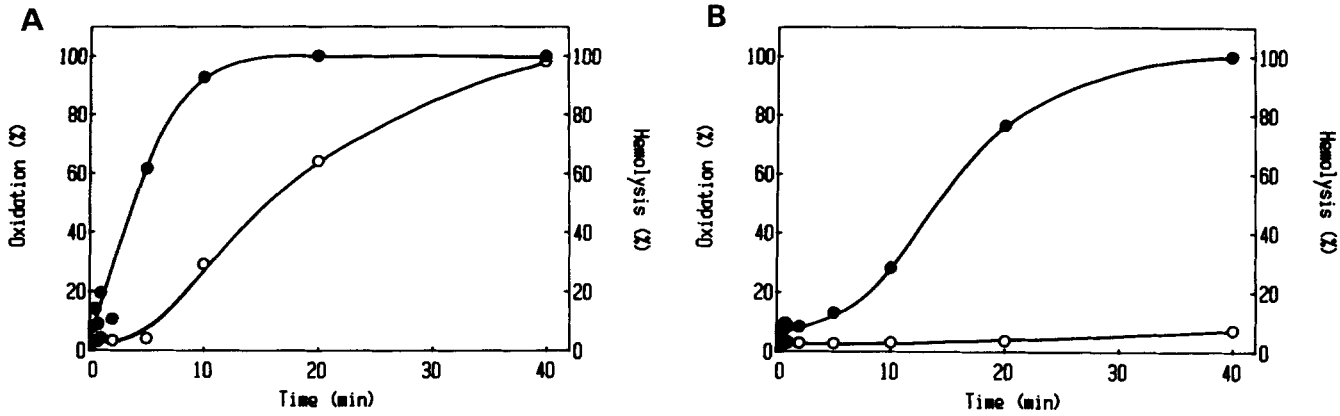

Fig. 1. Oxidation of erythrocyte cholesterol by cholesterol oxidase at 37°C (A) in the absence of Mg^{2+}, and (B) in the presence of Mg^{2+} (1 mM). The masses of cholesterol and cholestenone were determined, and the % oxidation (●) was calculated by the ratio of the mass of cholestenone to the total mass of free sterol (cholesterol and cholestenone). The % hemolysis (○) was determined as described in Experimental Procedures. The curves shown are representative curves from three experiments (A) and eight experiments (B).

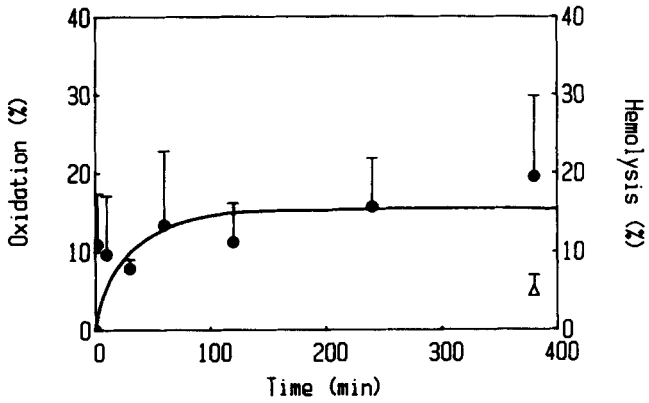

Fig. 2. Oxidation of erythrocyte cholesterol by cholesterol oxidase at 10°C. The % oxidation (●) and % hemolysis (△) were determined as described in Fig. 1. The data points represent the means of quadruplicate determinations.

and outer leaflet cholesterol through transbilayer movement. A decrease in the specific radioactivity of cholestenone with time at 37°C, therefore, represents mixing of the pool of radiolabeled outer leaflet cholesterol with a pool of unlabeled cholesterol, presumably inner leaflet cholesterol. During enzyme treatment, most cholesterol in the cell is not converted to cholestenone; the specific radioactivity of cholesterol represents unreacted outer leaflet cholesterol and unlabeled inner leaflet cholesterol, and remains unchanged throughout the 37°C incubation.

Suspended erythrocytes were labeled with [^{3}H]cholesterol at 4°C, and were then incubated at 37°C to allow transbilayer movement of plasma membrane cholesterol. At various times, aliquots of the suspended cells were removed and treated briefly (20 sec) with cholesterol oxidase at 37°C. During enzyme treatment, approximately 3% of the total erythrocyte cholesterol was oxidized to cholestenone (data not shown). The cell lipids were then extracted, and the specific radioactivities of cholesterol and cholestenone were determined. The specific radioactivity of cholestenone decreased with time of incubation at 37°C, and the specific radioactivity of cholesterol remained constant, as expected (**Fig. 4**). The half-time for the decrease of specific radioactivity of cholestenone was 49.6 ± 11.7 min (n = 4), suggesting a slow rate of transbilayer movement of cholesterol. The same experiments were conducted using erythrocytes labeled by [^{3}H]cholesterol exchange from liposomes over 6 hr at 4°C. Erythrocytes labeled by this method incorporated less than 1% the level of radioactivity attained by labeling cells with [^{3}H]cholesterol in ethanol. The half-time for the decrease of specific radioactivity of cholestenone was 130 ± 20 min (n = 4) (**Table 1**).

Effects of cholesterol oxidase on plasma membrane cholesterol at 37°C

To examine the consequences of continuous exposure of the plasma membrane to cholesterol oxidase, we incubated [^{3}H]cholesterol-labeled erythrocytes with cholesterol oxidase at 37°C. The cells were first labeled with [^{3}H]cholesterol at 4°C, and then warmed to 37°C. Cholesterol oxidase was added, and the cells were incubated at 37°C. Aliquots were removed at various times, extracted, and the specific radioactivities of cholesterol and cholestenone were determined. The specific radioactivity of cholestenone decreased rapidly (**Fig. 5**); the half-time for the process was 6.3 ± 2.0 min (n = 6), while the specific radioactivity of cholesterol remained constant throughout the incubation. The same experiments were conducted using erythrocytes labeled by [^{3}H]cholesterol exchange from liposomes. The half-time for the decrease of specific radioactivity of cholestenone was 18.1 ± 7.1 min (n = 4) (**Table 2**).

The equilibration of radiolabeled outer leaflet cholesterol with unlabeled cholesterol was accelerated in the continuous presence of cholesterol oxidase at 37°C. This could either reflect accelerated transbilayer movement of cholesterol or indicate that cholesterol oxidase gained access to cholesterol in the inner leaflet during the incubation. Measurements of cell integrity, however, demonstrated that hemoglobin was not leaking from the cells.

Erythrocyte membrane cholesterol asymmetry

Several of our observations suggested that the cholesterol distribution across the erythrocyte plasma membrane bilayer is asymmetric. First, the ratios of the initial specific activities of cholestenone to cholesterol were

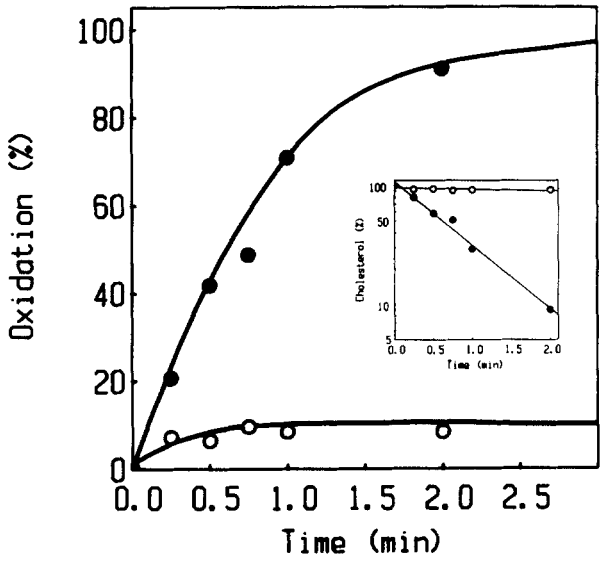

Fig. 3. Oxidation of the cholesterol of hypotonically disrupted (●) and intact (○) erythrocytes by cholesterol oxidase at 37°C. Erythrocytes were suspended in hypotonic buffer (●) or assay buffer (○) and then treated with cholesterol oxidase. The % oxidation was determined as described in Fig. 1. The inset shows a semi-logarithmic plot of the % cholesterol remaining in cells treated with cholesterol oxidase. The curves shown are representative curves of five experiments.

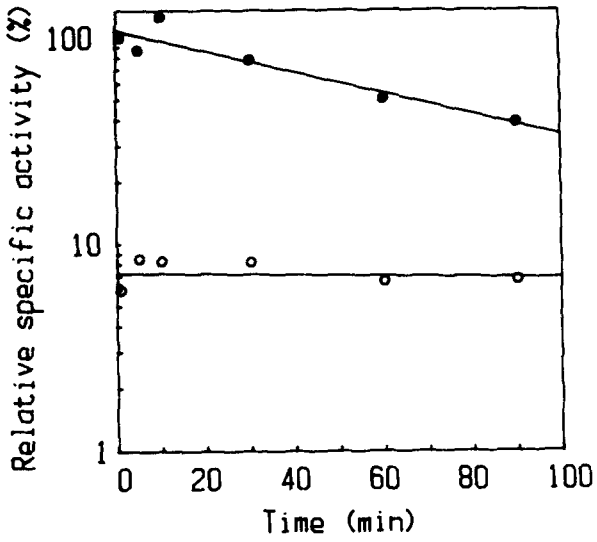

Fig. 4. Changes in specific radioactivities of cholestenone (●) and cholesterol (○) with time at 37°C. Erythrocytes were labeled with exogenous [^{3}H]cholesterol, and were then incubated at 37°C for the indicated times. Aliquots were removed and treated briefly with cholesterol oxidase. The specific radioactivities of cholesterol and cholestenone were determined as described in Experimental Procedures, and the data were expressed as the radioactivity of ^{3}H-labeled sterol/μg of sterol, relative to the initial specific radioactivity of cholestenone. Curves shown are representative curves from four experiments.

approximately 9:1 to 10:1 in experiments conducted on erythrocytes labeled with [^{3}H]cholesterol in ethanol (see Figs. 4 and 5), suggesting that approximately 10–11% of erythrocyte plasma membrane cholesterol is in the outer leaflet. Experiments conducted on cells labeled by [^{3}H]cholesterol exchange from liposomes indicate a ratio of the initial specific activities of cholestenone to cholesterol of 3:1, again suggesting an inner leaflet enrichment of cholesterol. Second, the oxidation profile of erythrocyte cholesterol at 37°C indicates that approximately 13% of the available cholesterol was rapidly oxidized, while the remaining 87% was oxidized more slowly by cholesterol oxidase (Fig. 1B). Additionally, the oxidation profile of erythrocyte cholesterol at 10°C showed rapid oxidation of 14% of the total cholesterol without further cholesterol oxidation over 6 hr (Fig. 2). The pattern of oxidation indicated two pools of cholesterol in intact erythrocytes with different rates of reaction with cholesterol oxidase. Cells that were hypotonically disrupted failed to exhibit this pattern of oxidation, and all available cholesterol was rapidly oxidized (Fig. 3).

DISCUSSION

The mechanism of entry of plasma cholesterol into cells, independently of lipoprotein receptors, is unknown. One hypothesis to explain entry of free cholesterol into the cell is the association of cholesterol monomers with the external face of the plasma membrane, followed by transbilayer movement of cholesterol to the cytoplasmic face, and subsequent dissociation of cholesterol into the cytoplasm. Data from several studies lend credibility to this hypothesis. Studies of the exchange of radiolabeled cholesterol between donor and acceptor membranes and vesicles demonstrated that cholesterol spontaneously diffuses between membranes separated by aqueous compartments (9–14, 16, 17, 24–27). The total equilibration of radiolabeled cholesterol between uniformly labeled donor and acceptor vesicles implies the ability of cholesterol to cross membrane bilayers. Our studies further establish transbilayer movement of cholesterol across the membranes of intact cells. The most important observation of this paper is that transbilayer movement of cholesterol in intact erythrocytes has a half-time of 50 to 130 min at 37°C. Thus, transbilayer movement of cholesterol may contribute to the slow entry of exogenous free cholesterol into cells in a receptor- and endocytosis-independent fashion.

We examined the equilibration of radiolabeled cholesterol between two distinct pools in the plasma membranes of erythrocytes. The equilibration of radiolabeled outer leaflet cholesterol with unlabeled cholesterol was slow in erythrocytes incubated at 37°C. The simplest explanation of this result is that the unlabeled pool of cholesterol represents cholesterol in the inner leaflet of the plasma membrane that equilibrates with the labeled outer leaflet cholesterol by transbilayer diffusion at 37°C. We cannot exclude the possibility that cholesterol from latent subdomains of the outer leaflet moves laterally, contributing to the observed dilution in the specific activity of cholestenone. However, this pool of cholesterol would need to meet several criteria: *1*) it would exhibit restricted lateral movement; *2*) it would not readily exchange with exogenously added [^{3}H]cholesterol; and *3*) it would be inaccessible to cholesterol oxidase. Diffusion coefficients for the lateral movement of lipids in natural and artificial

TABLE 1. Estimated rates of transbilayer movement of cholesterol at 37°C for cells labeled with [^{3}H]cholesterol in ethanol and by exchange from liposomes

	Ethanolic Labeling		Liposome Labeling	
Experiment	$t_{1/2}$	Coefficient of Correlation[a]	$t_{1/2}$	Coefficient of Correlation[a]
	min		*min*	
1	57.9	0.781	142	0.937
2	34.5	0.974	152	0.938
3	46.2	0.853	114	0.969
4	59.8	0.942	112	0.901
Mean ± SD	49.6 ± 11.7		130 ± 20	

[a]Coefficient of correlation from Curve Fitter-PC program for data fit to a monoexponential function.

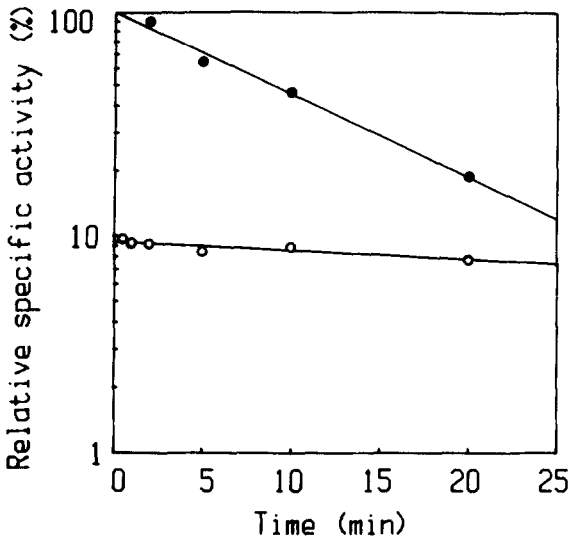

Fig. 5. Changes in specific radioactivities of cholestenone (●) and cholesterol(○) with time at 37°C in the continuous presence of cholesterol oxidase. Erythrocytes were labeled with exogenous [^{3}H]cholesterol, and were then treated continuously with cholesterol oxidase at 37°C for the indicated times. Curves shown are representative curves from six experiments.

membranes range from 10^{-9} to 10^{-8} cm^2s^{-1} (21, 22), indicating rapid lateral diffusion. This rate of lateral movement is too rapid to account for the observed dilution effects.

Two radiolabeling procedures were used in our studies: *1*) [^{3}H]cholesterol was introduced to suspended erythrocytes in an ethanolic dispersion; and *2*) liposomes were prepared with [^{3}H]cholesterol and incubated with erythrocytes to exchange the tracer into the cells. Erythrocytes labeled by the two methods gave different estimates for the rate of transbilayer movement of cholesterol in intact cells; however, both indicate a slow rate of movement. The reasons for the differences are unknown. Several experimental factors may contribute to the results: the length of time needed to incorporate [^{3}H]cholesterol into the cells and the resulting level of incorporated tracer varied considerably. The incorporation of tracer from ethanol required a very brief incubation (5 min) at 4°C, and resulted in a high level of cellular [^{3}H]cholesterol, while the incorporation of tracer into cells by exchange from liposomes required a much longer incubation (6 hr) at 4°C, and resulted in a very low level of cellular [^{3}H]cholesterol (less than 1% the incorporation of the former method). The data may indicate that the two methods label different subdomains within the outer leaflet, hence the more rapid equilibration of [^{3}H]cholesterol in cells labeled by the tracer in ethanol may represent two mixing processes, one between the subdomains and the other across the membrane. If there are two mixing processes, they would have to occur at similar rates, since the data fit best into a monoexponential model, and poorly into a biexponential function.

The rates of transbilayer movement of cholesterol estimated in our experiments in intact erythrocytes agree with the rates estimated from several experiments in which the exchange of radiolabeled cholesterol between natural and artificial membranes and vesicles was monitored (11, 12, 14, 16, 17), and one study where the transbilayer movement of sterophenol, a fluorescent cholesterol analog, was measured in phospholipid liposomes (28). However, other exchange experiments suggested that cholesterol moves across bilayers more slowly (13), or not at all (24–26). Variability in the results of exchange experiments may be due to a number of experimental factors. Lipid equilibration between donor and acceptor membranes requires transbilayer equilibration of radiolabeled lipids, followed by desorption of the lipid from the donor membrane, diffusion of the lipid through an aqueous medium, and association of the lipid with the acceptor membrane. Transbilayer diffusion rates are estimated from the time required for all of these steps to occur; transbilayer diffusion has been assumed to require less time than this equilibration process, hence, a range of probable rates can be determined.

Continuous incubation of erythrocytes with cholesterol oxidase resulted in the accelerated equilibration of radiolabeled cholesterol with unlabeled cholesterol. Cholesterol oxidase thus exerts a perturbing effect on plasma membrane cholesterol. The nature of the perturbation is unknown. Rapid oxidation of outer leaflet cholesterol resulted in a membrane enriched in cholestenone. Bruckdorfer and Sherry (27) have shown that cholestenone is less compatible with a lipid bilayer structure than cholesterol, resulting in a higher rate of desorption of cholestenone from membrane vesicles. Additionally, the presence of cholestenone in a lipid bilayer alters membrane fluidity (29), and membrane permeability (30). These factors may have increased the accessibility of inner leaflet cholesterol to cholesterol oxidase through increased rates of trans-

TABLE 2. Estimated rates for accelerated transbilayer movement of cholesterol by continuous cholesterol oxidase incubation at 37°C for cells labeled with [^{3}H]cholesterol in ethanol and by exchange from liposomes

	Ethanolic Labeling		Liposome Labeling	
Experiments	$t_{1/2}$	Coefficient of Correlation[a]	$t_{1/2}$	Coefficient of Correlation[a]
	min		*min*	
1	3.29	0.996	21.3	0.926
2	5.68	0.905	25.6	0.873
3	7.79	0.995	16.3	0.914
4	7.58	0.974	9.08	0.958
5	8.57	0.864		
6	4.97	0.972		
Mean ± SD	6.3 ± 2.0		18.1 ± 7.1	

[a]Coefficient of correlation from Curve Fitter-PC program for data fit to a monoexponential function.

bilayer movement of cholesterol, or by penetration of the enzyme into the inner leaflet. Prolonged incubations of cells in the presence of cholesterol oxidase resulted in alterations of membrane properties. For this reason, we chose to measure changes in the specific activity of outer leaflet cholesterol using very brief enzyme treatments (20 sec) to determine the rate of transbilayer movement of cholesterol.

The hemolysis of erythrocytes treated with cholesterol oxidase was prevented by the addition of Mg^{2+} to the assay buffer. The cholesterol oxidase treatment of intact cells requires that the cells be suspended in a low ionic strength buffer for maximal enzyme activity (31). Lange, Dolde, and Steck (15) measured the oxidation of erythrocyte cholesterol by cholesterol oxidase in low ionic strength buffer without Mg^{2+}, and found that all erythrocyte cholesterol was rapidly oxidized. Since cholesterol oxidase reacts with outer leaflet cholesterol, they concluded that rapid oxidation of all erythrocyte cholesterol is a consequence of very rapid transbilayer movement of cholesterol across the bilayer, with a half-time of less than 1 min. We conducted similar experiments in the absence of Mg^{2+} and obtained rapid oxidation of erythrocyte cholesterol (Fig. 1A); however, in our studies, hemolysis of the erythrocytes followed after a brief lag. The rapid oxidation of approximately 60% of erythrocyte cholesterol occurred before permeabilization of the membranes. Our data suggest that the continuous presence of cholesterol oxidase accelerates the apparent transbilayer movement of cholesterol, hence the early rapid oxidation of erythrocyte cholesterol in the absence of Mg^{2+} is likely a result of accelerated transbilayer movement and/or increased permeability of the membranes. When intact cells were treated with cholesterol oxidase without accompanying hemolysis (in the presence of 1 mM Mg^{2+}, Fig. 1B), the oxidation of erythrocyte cholesterol was much slower. Additionally, we have presented evidence that the apparent rate of transbilayer movement of cholesterol is accelerated in the continuous presence of cholesterol oxidase; hence, we believe that the rate of oxidation of cholesterol is not an accurate indication of the rate of transbilayer movement.

Our results also suggest that cholesterol has an asymmetrical orientation in erythrocyte membranes. Cholesterol in intact erythrocyte plasma membranes was unequally available to cholesterol oxidase, and to labeling with [^{3}H]cholesterol at 4°C. Movement of lipids in bilayers is restricted at low temperatures (21–23); therefore, the incorporation of radiolabeled cholesterol into membranes at 4°C preferentially labels the outer leaflet when very brief labeling procedures are used. We have shown that approximately 10–30% of the total erythrocyte cholesterol is *1*) preferentially labeled by [^{3}H]cholesterol at 4°C; and *2*) rapidly oxidized by cholesterol oxidase at 37°C and 10°C. Furthermore, only 14% of erythrocyte cholesterol was oxidized over 6 hr under conditions where transbilayer movement of membrane components is restricted (10°C). All of the cholesterol of hypotonically disrupted cells was rapidly oxidized by cholesterol oxidase ($t_{1/2} < 1$ min). These results suggest an asymmetric distribution of cholesterol in the erythrocyte plasma membrane with the majority of cholesterol in the inner leaflet.

Other types of experiments have demonstrated cholesterol asymmetry in erythrocyte membranes. Experiments using freeze-fractured erythrocytes indicated enrichment of cholesterol in the outer leaflet of human erythrocytes; however, the percent enrichment varied with different experimental conditions (32). Schroeder (33) and Hale and Schroeder (34) demonstrated preferential incorporation of dehydroergosterol into the outer leaflet of mouse and rat erythrocyte membranes. Several factors may contribute to differences between their experimental system and native conditions: *1*) dehydroergosterol was used as a cholesterol analog; and *2*) quenching of fluorescence following trinitrobenzenesulfonic acid (TNBS) treatment was used to determine the distribution of dehydroergosterol. Sterol feeding experiments in *Mycoplasma capricolum* indicate that alkyl-substituted sterols exhibit different localization properties than cholesterol in biological membranes (35, 36). Furthermore, TNBS treatment of membranes results in destabilization of membranes and altered transbilayer distribution and mobility of phospholipids (37, 38). Interestingly, Schroeder (33) and Hale and Schroeder (34) noted a preferential incorporation of dehydroergosterol into the inner leaflet of plasma membranes of mouse LM fibroblasts. They described a high asymmetry of sterol in these membranes with as much as 80% (a fourfold enrichment) of the sterol in the inner leaflet.

In this report, we have demonstrated that transbilayer movement of cholesterol occurs with a half-time of 50 to 130 min in erythrocytes incubated at 37°C. If the rate of transbilayer movement of cholesterol in erythrocytes is comparable to the corresponding rates in other cell types in vivo, then the transbilayer entry of free cholesterol into cells may be quantitatively important. The uptake of lipoprotein-free cholesterol in excess of uptake of the entire lipoprotein particle has been demonstrated in several systems (5–8, 39–41). High density lipoproteins that were enriched in cholesterol (5), depleted in phospholipids (7, 39, 41), or reconstituted with cholesterol and cholesteryl esters (40) delivered free cholesterol to cultured rat hepatoma and granulosa cells in excess of cholesteryl esters and apolipoproteins. The rates of transbilayer movement of cholesterol that we measured are compatible with the rates of cholesterol uptake reported in the latter studies and support the entry of free cholesterol into cells by diffusion across the plasma membrane bilayer.

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