Hydrogen Peroxide Oxidation Induces the Transfer of Phospholipids from the Membrane into the Cytosol of Human Erythrocytes†

Linda S. Brunauer,‡ Michael S. Moxness,§ and Wray H. Huestis*,§

Department of Chemistry, Santa Clara University, Santa Clara, California 95053, and Department of Chemistry, Stanford University, Stanford, California 94305

Received May 6, 1993; Revised Manuscript Received February 14, 1994•

ABSTRACT: The effects of oxidative damage on membrane phospholipid organization were examined in human erythrocytes. Exposure to H_2O_2 induced shape changes in these cells; normal discocytes became echinocytic, and stomatocytes generated by foreign phosphatidylserine incorporation reverted to discoid morphology. H_2O_2 treatment also inhibited phosphatidylserine transport from the outer to inner membrane monolayer, consistent with earlier reports on oxidative sensitivity of the aminophospholipid translocator. The morphological changes are consistent with movement of inner monolayer lipids to the outer monolayer, as might be expected if aminophospholipid sequestration is compromised. However, lipid extraction and prothrombinase activation assays showed no increased exposure of phosphatidylserine on the cell surface. Instead, phosphatidylserine was found associated with the cytosolic fraction of H_2O_2-treated cells. These observations suggest that oxidative damage alters the lipid organization of erythrocyte membranes, not by randomizing the lipid classes within the bilayer, but by inducing extraction of inner monolayer components into the cytosol.

The phospholipids of the erythrocyte membrane are asymmetrically distributed across the bilayer (Bretscher, 1972); phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) are found primarily in the inner monolayer while the outer monolayer is composed mainly of phosphatidylcholine (PC) and sphingomyelin (SM). This aminophospholipid distribution is maintained by an active translocator (flippase) that efficiently sequesters all of the PS and most of the PE in the inner monolayer (Schroit & Zwaal, 1991; Seigneuret & Devaux, 1984). Incubation with foreign PS results in a biphasic shape change characterized by a transient echinocytosis followed by reversion to discocytes and, ultimately, to stomatocytic cells. These changes in red blood cell (RBC) morphology are consistent with uptake of the exogenous PS into the outer monolayer and subsequent flip of the lipid to the inner monolayer (Daleke & Huestis, 1985, 1989). Thus the activity of the flippase can be conveniently monitored by following shape changes induced by exogenous PS incorporation into the bilayer: intercalation of lipid into the outer monolayer produces echinocytes, and inner monolayer intercalation produces stomatocytes. The activity of the translocator and attendant shape changes are inhibited by vanadate, low temperatures, and sulfhydryl-active reagents (Connor & Schroit, 1987; Daleke & Huestis, 1985; Seigneuret & Devaux, 1984; Tilley et al., 1986).

In this study, we have used erythrocyte shape changes to examine the effects of oxidative damage on translocator activity and phospholipid distribution. Oxidatively damaged components accumulate in erythrocytes during their life span in circulation. This damage, which appears in the form of protein aggregation (Kannan et al., 1991), lipid peroxidation (Jain, 1988), and enzyme deactivation (Jimeno et al., 1991), has been demonstrated to occur at an accelerated rate in erythrocytes isolated from individuals with diseases such as sickle cell anemia (Hebbel, 1990; Rice-Evans et al., 1986) and diabetes (Rajeswari et al., 1991; Wolff et al., 1991). Oxidation also has been associated with rapid removal of erythrocytes by the reticuloendothelial system (Pradhan et al., 1990; Snyder et al., 1985). The trigger that initiates red cell phagocytosis by macrophages has not been elucidated fully. Loss of PS asymmetry (Schroit et al., 1985) and the ensuing alteration in lipid packing (Schlegel & Williamson, 1987) have been implicated as the events that signal destruction. However, there are conflicting reports on loss of PS asymmetry as a result of cell oxidation (Herrmann & Devaux, 1990; Jain, 1984; Wali et al., 1987). We have explored the transbilayer distribution of exogenous and endogenous phospholipids in RBC after treatment with hydrogen peroxide, which is frequently used to mimic the effects of oxidative damage in senescent RBC or cells damaged by disease. We have also characterized the echinocytosis that develops during oxidation and demonstrated a unique interaction between PS and a cytosolic component of the erythrocyte.

MATERIALS AND METHODS

Materials. Dimyristoylphosphatidylserine (DMPS), dipalmitoylphosphatidylserine (DPPS), and dioleoylphosphatidylserine (DOPS) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Coagulation factors [factors II (prothrombin), factor V, and factor Xa] were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). L-[3-^{3}H]Serine (specific activity 20 Ci/mmol) and [^{14}C]methyl

† This work was supported by NIH Grant HL 23787, a William and Flora Hewlett grant of Research Corporation, and an Arthur Vining Davis Junior Faculty Fellowship awarded by Santa Clara University.

• To whom correspondence should be addressed.

‡ Santa Clara University.

§ Stanford University.

• Abstract published in *Advance ACS Abstracts,* March 15, 1994.

[1] Abbreviations: BSA, bovine serum albumin; DLPC, dilauroylphosphatidylcholine; ^{14}C-DLPC, dilauroylphosphatidyl-[1-^{14}C]choline; DLPE, dilauroylphosphatidylethanolamine; DLPS, dilauroylphosphatidylserine; ^{3}H-DLPS, dilauroylphosphatidyl-L-[3-^{3}H]serine; DMPS, dimyristoylphosphatidylserine; DMPC, dimyristoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; DPPS, dipalmitoylphosphatidylserine; Hct, hematocrit; lyso-PS, lysophosphatidylserine; MI, morphological index; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, red blood cells; SM, sphingomyelin; TLC, thin layer chromatography.

0006-2960/94/0433-4527$04.50/0

iodide (specific activity 50 Ci/mol) were purchased from Amersham Corporation (Arlington Heights, IL). Dilauroylphosphatidylcholine (DLPC), dilauroylphosphatidylethanolamine (DLPE), dimyristoylphosphatidylcholine (DMPC), bovine serum albumin (BSA), *Streptomyces* species phospholipase D (type VII), serine, and Sar-Pro-Arg-*p*-nitroanilide were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Dilauroylphosphatidylserine (DLPS) was synthesized as previously described by (Hermetter et al., 1982) and Hall (personal communication). Radiolabeled DLPS (^{3}H-DLPS) was synthesized by phospholipase D catalyzed head group exchange (Comfurius et al., 1990a) of DLPC with L-[3-^{3}H]-serine (1 Ci/mol). DLPS and ^{3}H-DLPS were purified chromatographically using CM52 cellulose (Whatman, Inc., Clifton, NJ). Radiolabeled DLPC (^{14}C-DLPC) was synthesized (Stockton et al., 1974) by methylation of DLPE with [^{14}C]methyl iodide (6 Ci/mol) and purified by thin layer chromatography on silica gel G plates.

Cells. Human RBC were obtained from healthy adult volunteers by venipuncture and collection into citrate anticoagulant. RBC were washed by repeated centrifugations (3000*g*, 5 min) in 150 mM NaCl. RBC were stored at 5 °C in 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.4 (PBS) and used within 24 h.

Vesicle Preparation. Lipid stocks were prepared in chloroform and stored at −20 °C under argon. Small unilamellar vesicles were prepared by removing the chloroform under a gentle stream of argon, suspending the lipid in magnesium-free PBS, and subsequently sonicating the suspension to clarity, under argon, in a bath sonicator.

Cell–Vesicle Incubations. Incubations were initiated by mixing RBC with suspensions of lipid vesicles to yield a final hematocrit (Hct) of 50%; the concentration of lipid in these mixtures is indicated in the figure legends. Incubations were carried out at 37 °C and unincorporated lipid vesicles were removed by washing in PBS.

Oxidation. RBC were pretreated for 5 min with PBS containing 1 mM NaN_3 to inhibit catalase. Cells were then incubated at room temperature with H_2O_2 (concentrations as noted in figure legends) at a final Hct of 5%. Peroxide treatments were carried out at room temperature to retard echinocytosis in controls (Snyder et al., 1985). ATP levels remained constant under these incubations conditions, as determined by luciferin–luciferase assay (Lundin et al., 1976); treatment with PBS-azide alone had no effect on cell shape (data not shown).

Cell Morphology. Samples were prepared for morphological analysis by fixation in 10–20 volumes of buffered glutaraldehyde (1% glutaraldehyde prepared in PBS). Cells were examined by light microscopy and scored using a previously described grading scale (Bessis, 1973; Ferrell et al., 1985). Echinocytes were assigned scores of +1 to +5, reflecting the degree of crenation; discocytes were scored 0; and stomatocytes were assigned scores of −1 to −4, depending on the degree of invagination. The average score of a field of 100 cells is defined as the morphological index (MI). The errors involved in counting replicate fields of cells was typically 0.1–0.15 MI unit.

Calcium Loading. Lipid scrambling, and attendant exposure of PS on the outer monolayer of RBC, was induced in cells by treatment at Hct 5% with calcium and 1 μM A23187 for 1 h at 37 °C, as previously described (Comfurius et al., 1990b). Treated cells were employed as controls to determine the effectiveness of the prothrombinase enzyme complex and ^{3}H-DLPS back extraction assays (Tables 2 and 3).

Prothrombinase Assay. Procoagulant activity, an indication of PS exposure on the surface of RBC, was assayed by a modification of a previously described assay (Bevers et al., 1983; Connor et al., 1989). Briefly, RBC were incubated for 3 min at 37 °C, at a final Hct of 0.1%, in buffer containing 50 mM Tris-HCl, 120 mM NaCl, 6 mM $CaCl_2$, 0.33 unit/mL of each factor Xa and factor Va, and 1.3 unit/mL prothrombin (factor II), pH 7.8. The reaction was terminated by the addition of EDTA (final concentration 15 mM), and the cells were removed by centrifugation. The amount of thrombin formed was determined by transferring an aliquot of the supernatant into a cuvette and measuring the $\Delta A_{405\ nm}$ upon addition of the chromogenic thrombin substrate Sar-Pro-Arg-*p*-nitroanilide (final concentration: 1.3 mM).

Extraction of ^{3}H-DLPS from RBC Membrane Outer Monolayer. Erythrocytes containing radiolabeled DLPS (10 μCi/L RBC in a typical experiment) were incubated at 5% Hct with 10% (w/v) BSA for 30 min at room temperature. The BSA was separated from cells by centrifugation (3000*g*, 10 min), and the supernatant and pellet were assayed for radioactivity as described below.

Separation of Membrane and Cytosol. Erythrocytes containing radiolabeled DLPS or DLPC were incubated at 0 °C for 30 min in 20 vol of lysis buffer (5 mM Na_2HPO_4, pH 8) and centrifuged (10000*g*, 30 min). The bulk of the supernatant was removed from the pellet, and aliquots of the two fractions were assayed for radioactivity. Quantitative removal of supernatant from the pellet was not attempted in order to avoid contamination of the cytosolic fraction (supernatant) with membrane components (pellet). The radiolabeled DLPS was extracted along with heme from the cytosolic fraction using acetone/HCl (Ascoli et al., 1981). The erythrocyte lipids were extracted from the membrane with chloroform/methanol (Mitchell et al., 1986) or acetone/HCl; both procedures gave similar results. The solvent was evaporated, and the samples were resuspended in chloroform/methanol and applied to Analtech silica gel TLC plates in equivalent portions. The chromatograms were developed in chloroform/methanol/formic acid (55:25:5), sprayed with 0.5% fluorescamine in acetone, and photographed on a UV light box with Polaroid type 57 film. Each lane was scraped in five sections of equal area and assayed for radioactivity.

Assay for Radioactivity. Samples containing hemoglobin and radiolabeled lipids were bleached overnight in 0.1 vol of 30% H_2O_2 at 60 °C; 10 mM sodium azide was added 10 min prior to bleaching in order to prevent foaming. Samples were counted in Ecolite (ICN Biomedicals, Inc., Irvine, CA) with a model LS 3801 scintillation counter (Beckman Instruments, Inc., Palo Alto, CA) using standard quench curves to calculate disintegrations per minute.

Non-Lipid-Induced Stomatocytes. RBC were incubated with benzoyl alcohol (Bassé et al., 1992) or chlorpromazine (Sheetz & Singer, 1974) or washed repeatedly with a low pH buffer (155 mM NaCl, 40 mM MES, pH 5.5; M. Gedde, personal communication) until the cells reached a stable stomatocytic morphology. They were subsequently treated with NaN_3 and H_2O_2, and the morphology was monitored.

RESULTS

Effect of H_2O_2 on the Aminophospholipid Translocation. Oxidative damage was induced in human RBC by brief treatment with low levels of H_2O_2 (<1 mM). Aminophospholipid translocase activity was monitored by observing

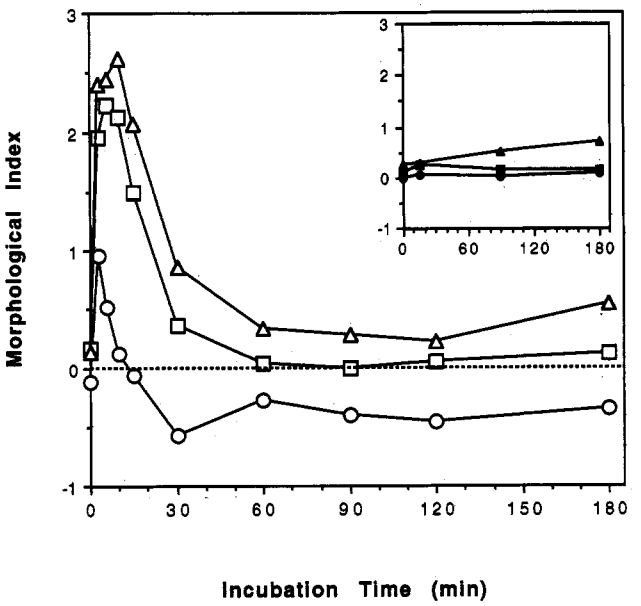

FIGURE 1: Effect of peroxide treatment on the uptake and translocation of DLPS. After a 15-min preincubation with 0 (circles), 0.4 (squares), or 0.8 mM (triangles) H_2O_2, cells were washed rapidly and then incubated with 65 (open symbols) or 0 μM (closed symbols; inset) DLPS. At the times indicated, aliquots of cells were fixed and MI's were calculated. The dotted line indicates a morphological index of 0.

changes in cell morphology following the addition of DLPS (Figure 1). As described previously (Daleke & Huestis, 1985), addition of DLPS to untreated RBC resulted in a biphasic shape change, a transient crenation followed by reversion to discocytes and, ultimately, to sphering or stomatocytosis. After approximately 60 min, DLPS-treated cells achieved a stable stomatocytic morphology; the degree of stomatocytosis was dependent on the concentration of DLPS employed.

Oxidation modified both the kinetics and the extent of the PS-induced shape change (Figure 1). The degree of the initial crenation was greater in oxidized cells, and they reached stable morphology at higher MI values than control cells. These differences are consistent with partial inhibition of the PS translocator. At the highest concentrations tested, H_2O_2 induced slight crenation in control cells (Figure 1, inset). Oxidant-linked inhibitory effects on PS translocation were still evident after normalization for this response.

Effect of H_2O_2 on Maintenance of Lipid-Induced Stomatocytes. RBC were incubated with DLPS and allowed to reach a steady-state morphology. Cells were then treated with H_2O_2, and subsequent shape transformations were monitored (Figure 2). DLPS-induced stomatocytes reverted rapidly ($t_{1/2}$ = 3 min) to discoid shape upon oxidation; the rate of the reversion was dependent on the oxidant concentration (Figure 2). Peroxide induced similar reversions in stomatocytes generated by the longer acyl chain lipids (DMPS, DPPS, and DOPS), but at slower rates (M. Moxness and L. Brunauer, unpublished observations). At the highest concentrations tested, prolonged incubation with H_2O_2 induced slight crenation in control cells (Figure 1, inset).

Flippase Activity and H_2O_2 Induced Shape Reversion. The relationship between oxidant-linked shape reversion and translocase activity was investigated. DLPS-induced stomatocytes were treated with varying concentrations of peroxide in concert with sodium orthovanadate (Figure 3), a species demonstrated to induce rapid inhibition of translocase activity (Bitbol et al., 1987). Vanadate treatment did not affect the stable stomatocytic shape of control PS-treated cells or alter

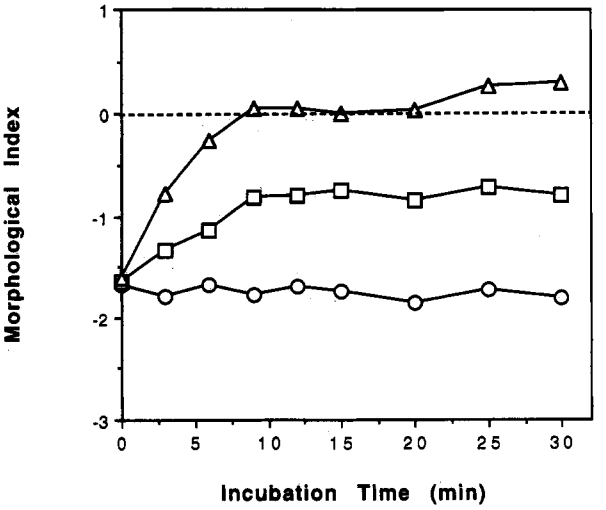

FIGURE 2: Effect of peroxide treatment on the morphology of RBC preloaded with DLPS. Erythrocytes were pretreated at 37 °C with 125 μM DLPS for 1.5 h. Cells were then treated with 0 (circles), 0.2 (squares), or 0.8 mM (triangles) H_2O_2 at room temperature. At the times indicated, aliquots were fixed and MI's were calculated. The dotted line indicates a morphological index of 0.

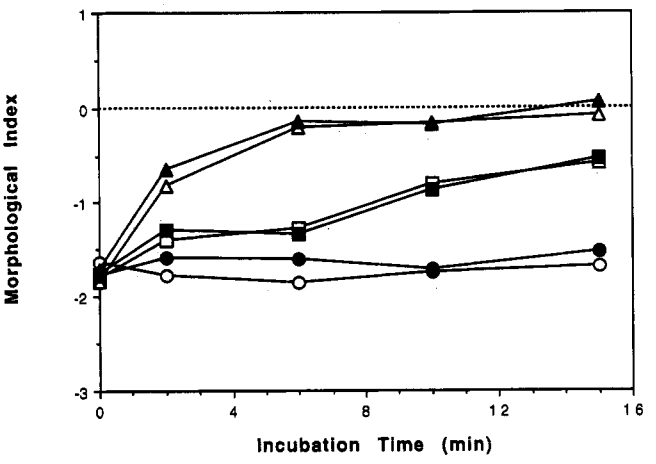

FIGURE 3: Effect of translocase inhibition on peroxide-induced shape reversion. Erythrocytes were pretreated at 37 °C with 120 μM DLPS for 1.5 h. Cells were then treated at room temperature with 0 (open symbols) or 100 μM (closed symbols) Na_3VO_4 for 5 min followed by 0 (circles), 0.2 (squares), or 0.8 mM (triangles) H_2O_2. At the times indicated, aliquots were fixed and MI's were calculated. The dotted line indicates a morphological index of 0.

the rate and extent of oxidation-induced reversion. These results indicated that a functioning translocase was not required for maintenance of stomatocytic shapes in unoxidized cells or for oxidation-induced shape reversion.

Effect of H_2O_2 on Echinocytes and Stomatocytes. The effects of H_2O_2 on RBC steady-state morphology were examined in lipid-induced echinocytes and in stomatocytes generated by methods other than PS incorporation. Stomatocytes were generated by incubation at low pH (pH 5.5) or exposure to the non-lipid amphipaths chlorpromazine and benzyl alcohol. In contrast to PS-induced stomatocytes, these cells maintained stable invaginated shapes upon oxidation (Table 1). Lipid-induced echinocytes, prepared by incubation of RBC with either DLPC or DMPC, similarly displayed steady-state morphologies that were not altered by exposure to H_2O_2. Parallel experiments were performed with echinocytes generated by treatment with vanadate followed by exposure to DLPS, a procedure that restricts incorporated PS to the outer leaflet of the bilayer. Subsequent treatment of

Table 1: H_2O_2-Induced Shape Changes in Echinocytes and Stomatocytes[a]

treatment	morphological index	
	0 mM H_2O_2	0.8 mM H_2O_2
stomatocytes		
125 μM DLPS	−2.2	+0.4
350 μM DMPS	−3.0	−2.0
250 μM chlorpromazine	−2.5	−2.4
pH 5.5	−1.4	−1.5
105 mM benzyl alcohol	−2.4	−2.2
discocytes		
control	0.0	+0.4
echinocytes		
75 μM DLPC	+3.0	+3.1
375 μM DMPC	+2.7	+2.9
vanadate + 63 μM DLPS	+1.4	+1.7

[a] RBC were treated with various amphipaths at the given concentrations until the cell morphology had reached a steady state. Each sample was then treated with either buffer or buffer containing 0.8 mM H_2O_2 for 30 min at room temperature, and MI values were determined.

Table 2: Back-Extraction of ^{3}H-DLPS from Oxidized or Calcium-Treated RBC[a]

H_2O_2 concentration (mM)	% of ^{3}H-DLPS extracted into BSA	calcium concentration (μM)	% of ^{3}H-DLPS extracted into BSA
0.0	4.0	0.0	2.3
0.2	4.2	50	5.0
0.8	4.0	100	5.6
2.0	4.0	500	14.2

[a] ^{3}H-DLPS was incorporated into RBC followed by treatment with H_2O_2 for 1 h at room temperature or with calcium and ionophore for 1 h at 37 °C. ^{3}H-DLPS present in the outer monolayer was extracted with 10% (w/v) BSA and analyzed by liquid scintillation counting.

these echinocytes with oxidant again had no effect on the stability of the echinocytosis (Table 1). The shape alterations seen in PS stomatocytes did not occur.

^{3}H-DLPS Back Extraction Assay for Outer Monolayer PS. The results of the above studies are consistent with an oxidation-linked diffusion of PS from the inner to outer membrane leaflet. The appearance of PS on the exofacial membrane surface was examined using two complementary procedures. In the first, a back-extraction assay, ^{3}H-DLPS was introduced into RBC by cell–vesicle incubation. Unincorporated lipid was washed away, and the cells were treated with varying concentrations of H_2O_2. The cells were then treated with a 10% solution of BSA, a procedure demonstrated to remove all extractable lipids from the outer monolayer (Williamson et al., 1992; Morrot et al., 1989). Previous experiments showed quantitative extraction of outer monolayer ^{3}H-DLPS with dioleoylphosphatidylcholine vesicles (Daleke & Huestis, 1989) or BSA (data not shown). As shown in Table 2, BSA extraction revealed no increased accessible DLPS in oxidized cells. In contrast, treatment with Ca^{2+} and ionophore, a procedure demonstrated to induce lipid reorganization and exposure of PS on the exofacial side of RBC membranes (Comfurius, et al., 1990b), did result in a measurable increase in accessible DLPS (Table 2). Complete randomization of PS is not expected at these ionophore/calcium concentrations (Williamson et al., 1992; Henseleit et al., 1990), which were chosen to minimize membrane vesiculation.

Prothrombinase Assay for Outer Monolayer PS. Exposure of endogenous PS on the surface of oxidized cells was examined by measuring procoagulant activity of treated cells in a prothrombinase assay. RBC pretreated with Ca^{2+} and ionophore exhibited increased procoagulant activity (Table 3). Oxidation of RBC, in contrast, did not result in a detectable increase in procoagulant activity, indicating no increase in PS exposure in the outer monolayer.

Table 3: Prothrombinase Assay of Endogenous PS Exposure in Oxidized or Calcium-Treated RBC[a]

H_2O_2 concentration (mM)	Δ absorbance at 405 nm (Δ abs/min × 10^4)	calcium concentration (μM)	Δ absorbance at 405 nm (Δ abs/min × 10^4)
0.0	9.48 ± 0.03	0.0	8.28 ± 0.10
0.2	8.61 ± 0.03	50	9.75 ± 0.07
0.8	9.53 ± 0.03	100	10.7 ± 0.06
2.0	9.95 ± 0.02	500	70.0 ± 0.03

[a] RBC were treated for 1 h with H_2O_2 at room temperature or with calcium and ionophore for 1 h at 37 °C. The treated cells were incubated with coagulation factors for 5 min, and the reaction was stopped with EDTA as described under Materials and Methods. Cells were removed by centrifugation and procoagulant activity present in the supernatant was determined by measuring the rate of ΔA_{405} upon addition of the chromagenic thrombin substrate Sar-Pro-Arg-*p*-nitroanilide. Errors represent the standard deviation of the data.

Table 4: Assay for Lipids in the Cytosol of Oxidized RBC[a]

sample	H_2O_2 concentration (mM)	% radioactivity in cytosolic aliquot
^{3}H-DLPS	0.0	8.9
^{3}H-DLPS	0.8	49.7
^{14}C-DPLC	0.0	4.7
^{14}C-DLPC	0.8	12.2

[a] Radiolabeled lipid was incorporated into RBC, followed by treatment with H_2O_2 at the given concentrations. Cells were lysed and centrifuged to separate the cytosol from the membrane. Each fraction was analyzed by liquid scintillation counting.

Table 5: Assay for Radioactivity in TLC Sections[a]

TLC section	control RBC		oxidized RBC	
	membrane	cytosol	membrane	cytosol
heme	1	1	0	1
PS/PE	6	2	2	11
DLPS/PC	75	4	29	47
Lyso-PS/SM	5	2	3	3
origin	1	3	0	3
total (% dpm)	88	12	34	66

[a] ^{3}H-DLPS was incorporated into RBC followed by treatment with 2 mM H_2O_2 for 30 min at room temperature. Samples were prepared as described under Materials and Methods. Chromatograms were developed, scraped and assayed as described under Materials and Methods. Data are expressed as a percentage of total dpm ^{3}H-DLPS recovered from the chromatogram.

Assay of Cytosol for DLPS. The possibility that oxidation induces removal of inner monolayer PS to the cytosol was examined. ^{3}H-DLPS was introduced into RBC by cell–vesicle incubation. Unincorporated lipid was removed by washing and the cells were treated with H_2O_2. Osmotic lysis of these RBC yielded a cytosolic fraction and a membrane fraction that were separated by centrifugation. An aliquot of membrane-free cytosol from oxidized RBC contained a 5-fold increase in radiolabeled lipid compared with an equivalent aliquot of cytosol from control RBC (Table 4). Analogous experiments with ^{14}C-DLPC showed that the cytosol from oxidized RBC could also extract a small amount of an outer monolayer intercalator, possibly as the membrane is permeabilized during lysis. Similar extraction results were obtained in experiments employing ^{3}H-DLPS in concentrations sufficient to induce shape change (Table 5).

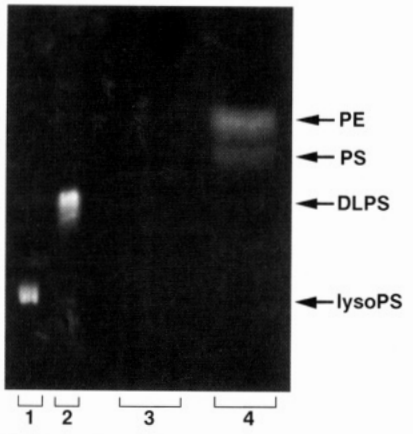

FIGURE 4: Thin layer chromatography of the membrane and cytosolic fractions from oxidized RBC. ^{3}H-DLPS was incorporated into RBC followed by treatment with 2 mM H_2O_2 at room temperature for 30 min. Samples and chromatograms were prepared as described under Materials and Methods. Chromatograms were stained with fluorescamine to detect free amines. Lane 1, lyso-PS standard; lane 2, DLPS standard; lane 3, cytosolic fraction; lane 4, membrane fraction.

Contamination of the cytosolic fraction with membrane buds would explain the presence of ^{3}H-DLPS; therefore, quantitative recovery of cytosolic radiolabel was not attempted. Extraction of the cytosolic fraction with acetone/HCl afforded the complete separation of heme (Ascoli et al., 1981) and lipid from the protein material, which precipitated. Thin layer chromatography indicated that no detectable endogenous PE or PS was present in the cytosol of oxidized erythrocytes (Figure 4). These data indicate that the method used to separate cytosol from membrane fragments, which involved analysis of aliquots of cytosol, was successful. Thin layer chromatography of the cytosolic and membrane fractions also showed that radiolabeled DLPS was not altered by H_2O_2 treatment; most of the radioactivity comigrated with DLPS (Table V).

DISCUSSION

The bilayer couple model states that excess lipid in the red cell outer membrane leaflet will induce evagination, while excess lipid present in the inner leaflet will induce invagination. Measurement of shape changes associated with incorporation of foreign lipid has been demonstrated to be an accurate method to determine the transbilayer distribution of these lipids (Daleke & Huestis, 1985, 1989). These workers used this method to demonstrate inhibition of the aminophospholipid translocator by diamide. In the present work, oxidative damage by another oxidant, H_2O_2, also appears to compromise PS sequestration. The translocator in H_2O_2-treated cells has adequate activity to transform PS-echinocytes to discocytes, but the oxidized RBC never attain stomatocytic shapes. Additionally, peroxide-treated control cells slowly become echinocytic, and stomatocytes generated by exogenous PS uptake revert to discocytes on peroxide treatment.

Vanadate inhibition does not affect the rate of reversion, indicating that shape reversion is not mediated by the aminophospholipid translocator. H_2O_2 reversion is specific for PS-induced stomatocytes; stomatocytes induced by low pH or treatment with either benzyl alcohol or chlorpromazine do not undergo reversion on oxidation. In addition, PC and vanadate-inhibited PS echinocytes do not revert to discoid shape upon oxidation.

One process that would generate the observed shape changes is passive redistribution of PS to the outer monolayer. However, the prothrombinase assay of control cells indicates that there is no exposure of endogenous PS after H_2O_2 treatment. This conclusion is supported by back-extraction assays of cells containing exogenous PS: oxidation does not increase ^{3}H-DLPS accessibility to BSA extraction. These findings are in agreement with experiments utilizing spin-labeled PS (Herrmann & Devaux, 1990), which demonstrated no loss of PS asymmetry in RBC during oxidation. These findings do not support previous studies that used phospholipase A_2 to assay for outer monolayer PS (Arduini et al., 1989; Jain, 1984; Wali et al., 1987). These latter studies employed higher oxidant concentrations (i.e., up to 4 mM) than were used in most of the present work; however, cells treated with 2–4 mM H_2O_2 also showed no increased procoagulant activity (data not shown). Such conflicting reports may reflect bilayer destabilization by the lipase action (Franck et al., 1986), compounding the instability of oxidatively compromised cells.

The bilayer couple model also accounts for the observed morphology changes if inner monolayer components are extracted into the cytosol; removal of inner monolayer lipid would alter the bilayer balance in favor of echinocytic forms. Accordingly, ^{3}H-DLPS is found in significant amounts in the cytosol of oxidized cells. The slow H_2O_2-induced echinocytosis of discocytes might similarly be due to the loss of endogenous, long chain PS from the bilayer. The radiolabeled lipid is extracted readily along with heme from the cytosolic fraction by acetone/HCl; this demonstrates that the lipid is not covalently associated with protein. Thin layer chromatography shows that the DLPS is not converted to lyso-PS or other degradation products upon H_2O_2 treatment. Fluorescamine staining of the TLC chromatogram reveals no detectable endogenous PE or PS in the cytosol; thus, oxidation does not induce endovesiculation of the cell membrane.

We have shown that H_2O_2 oxidative damage does not cause exofacial exposure of PS but may instead cause an accumulation of lipids in the cytosol over time. The absence of reversion in echinocytes indicates that lipid extraction can occur only from the inner leaflet, thereby implicating a cytosolic component as the extracting agent. A possible candidate is hemoglobin, which degrades to methemoglobin and hemichrome upon oxidation (Chiu & Lubin, 1989). Further studies will address the identity of the extracting species and the physiological significance of this phenomenon.

ACKNOWLEDGMENT

We thank our colleagues for many helpful discussions regarding the evolution of this project and the preparation of this paper. We gratefully acknowledge the excellent technical assistance of Ms. Renee Chun, Ms. Heidi Geissler, Mr. Stephan Gutzeit, and Mr. Patrick O'Brien. We also thank Ms. Sansan Lin and Ms. Eun Yang for preparation of the ^{14}C-DLPC used in these studies.

REFERENCES

Arduini, A., Stern, A., Storto, S., Belfiglio, M., Mancinelli, G., Scurti, R., & Federici, G. (1989) *Arch. Biochem. Biophys. 273*, 112–120.

Ascoli, F., Rossi Fanelli, M. R., & Antonini, E. (1981) *Methods Enzymol. 76*, 72–87.

Bassé, F., Sainte-Marie, J., Maurin, L., & Bienvenüe, A. (1992) *Eur. J. Biochem. 205*, 155–162.

Bessis, M. (1973) in *Red Cell Shapes* (Bessis, M., Weed, R. I., & Leblond, P. F., Eds.) pp 1–25, Springer-Verlag, New York.

Bevers, E. M., Comfurius, P., & Zwaal, R. F. (1983) *Biochim. Biophys. Acta 736*, 57–66.

Bitbol, M., Fellmann, P., Zachowski, A., & Devaux, P. F. (1987) *Biochim. Biophys. Acta 904*, 268–282.

Bretscher, M. S. (1972) *Nature (London), New Biol. 236*, 11–12.
Chiu, D., & Lubin, B. (1989) *Semin. Hematol. 26*, 128–135.
Comfurius, P., Bevers, E. M., & Zwaal, R. F. (1990a) *J. Lipid Res. 31*, 1719–1721.
Comfurius, P., Senden, J. M. G., Tilly, R. H. J., Schroit, A. J., Bevers, E. M., & Zwaal, R. F. A. (1990b) *Biochim. Biophys. Acta 1026*, 153–160.
Connor, J., & Schroit, A. J. (1987) *Biochemistry 26*, 5099–105.
Connor, J., Bucana, C., Fidler, I. J., & Schroit, A. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3184–3188.
Daleke, D. L., & Huestis, W. H. (1985) *Biochemistry 24*, 5406–5416.
Daleke, D. L., & Huestis, W. H. (1989) *J. Cell Biol. 108*, 1375–1385.
Ferrell, J. E., Lee, K. J., & Huestis, W. H. (1985) *Biochemistry 24*, 2849–2857.
Franck, P. F., Op den Kamp, J. A. F., Roelofsen, B., & Van Deenen, L. L. M. (1986) *Biochim. Biophys. Acta 857*, 127–130.
Hebbel, R. P. (1990) *Semin. Hematol. 27*, 51–69.
Henseleit, U., Plas, G., & Haest, C. (1990) *Biochim. Biophys. Acta 1029*, 127–135.
Hermetter, A., Paltauf, F., & Hauser, H. (1982) *Chem. Phys. Lipids 30*, 35–45.
Herrmann, A., & Devaux, P. F. (1990) *Biochim. Biophys. Acta 1027*, 41–46.
Jain, S. K. (1984) *J. Biol. Chem. 259*, 3391–3394.
Jain, S. K. (1988) *Biochim. Biophys. Acta 937*, 205–210.
Jimeno, P., Garcia-Perez, A. I., Luque, J., & Pinilla, M. (1991) *Biochem. J. 279*, 237–243.
Kannan, R., Yuan, J., & Low, P. S. (1991) *Biochem. J. 278*, 57–62.
Lundin, A., Rickardsson, A., & Thore, A. (1976) *Anal. Biochem. 75*, 611.
Mitchell, K. T., Ferrell, J. J., & Huestis, W. H. (1986) *Anal. Biochem. 158*, 447–453.
Morrot, G., Herve, P., Zachowski, A., Fellmann, P., & Devaux, P. F. (1989) *Biochemistry 28*, 3456–3462.
Pradhan, D., Weiser, M., Lumley, S. K., Frazier, D., Kemper, S., Williamson, P., & Schlegel, R. A. (1990) *Biochim. Biophys. Acta 1023*, 398–404.
Rajeswari, P., Natarajan, R., Nadler, J. L., Kumar, D., & Kalra, V. K. (1991) *J. Cell. Physiol. 149*, 100–109.
Rice-Evans, C., Omorphos, S. C., & Baysal, E. (1986) *Biochem. J. 237*, 265–269.
Schlegel, R. A., & Williamson, P. (1987) *J. Cell. Physiol. 132*, 381–384.
Schroit, A. J., & Zwaal, R. F. (1991) *Biochim. Biophys. Acta 1071*, 313–329.
Schroit, A. J., Madsen, J. W., & Tanaka, Y. (1985) *J. Biol. Chem. 260*, 5131–5138.
Seigneuret, M., & Devaux, P. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751–3755.
Sheetz, M. P., & Singer, S. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4457.
Snyder, L. M., Fortier, N. L., Trainor, J., Jacobs, J., Leb, L., Lubin, B., Chiu, D., Shohet, S., & Mohandas, N. (1985) *J. Clin. Invest. 76*, 1971–1977.
Stockton, G. W., Polnaszek, C. F., Leitch, L. C., Tulloch, A. P., & Smith, I. C. P. (1974) *Biochem. Biophys. Res. Commun. 60*, 844–850.
Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J. A. F., & Van Deenen, L. L. (1986) *FEBS Lett. 194*, 21–27.
Wali, R. K., Jaffe, S., Kumar, D., Sorgente, N., & Kalra, V. K. (1987) *J. Cell. Physiol. 133*, 25–36.
Williamson, P., Kulick, A., Zachowski, A., Schlegel, R. A., & Devaux, P. F. (1992) *Biochemistry 31*, 6355–6360.
Wolff, S. P., Jiang, Z. Y., & Hunt, J. V. (1991) *Free Radicals Biol. Med. 10*, 339–352.