Schlessinger, J., Schechter, Y., Willingham, M. C., & Pastan, I. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2659–2663.
Schreiber, A. B., Lax, I., Yarden, Y., Eshhar, S., & Schlessinger, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7535–7539.
Schrieber, A. B., Liberman, T. A., Lax, I., Yarden, Y., & Schlessinger, J. (1983) *J. Biol. Chem. 258*, 846–853.
Shimizu, K., Birnbaum, D., Ruley, M. A., Fasano, O., Suard, Y., Edlund, L., Taparocosky, E., Goldfarb, M., & Wigler, M. (1983) *Nature 304*, 497–500.
Ullrich, A., Bell, J. R., Chen, E. A., Herrera, R., Petruzzeli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y. C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature 313*, 756–761.
Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., LeBon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., & Fujita-Yamaguchi, Y. (1986) *EMBO J. 5*, 2503–2512.
Yanagita, Y., Abdel-Ghany, M., Raden, D., Nelson, N., & Racker, E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 925–959.

Fluorescence Assay for Phospholipid Membrane Asymmetry†

Jonathan C. McIntyre‡ and Richard G. Sleight*

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524

Received July 17, 1991; Revised Manuscript Received September 19, 1991

ABSTRACT: Highly fluorescent 7-nitro-2,1,3-benzoxadiazol-4-yl-lipid (NBD-lipid) analogues are widely used to examine lipid transport and membrane structure. We have developed a method for chemically modifying NBD-labeled lipids in both artificial and biological membranes. This was achieved by treating fluorescently labeled membranes with dithionite ($S_2O_4^{-2}$). When small unilamellar vesicles containing NBD-labeled phospholipids were reacted with dithionite, only the fluorescent lipid located on the outer leaflet of the vesicles' bilayer was reduced. Seven different NBD-lipid analogues, including a fluorescent sterol, were reduced by treatment with dithionite to nonfluorescent 7-amino-2,1,3-benzoxadiazol-4-yl-lipid derivatives. To assess the feasibility of using this reagent in biological systems, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine was inserted into the outer leaflet of the plasma membrane of CHO-K1 cells. Subsequent incubation of these cells with a nontoxic concentration of dithionite resulted in the complete loss of fluorescence from the plasma membrane. In contrast, when cells were permitted to endocytose some of their fluorescently labeled plasma membrane and then treated with dithionite, fluorescence at the plasma membrane was eliminated, while intracellular labeling was not affected. These data suggest that dithionite reacts with NBD-labeled lipids in the outer leaflet of membrane bilayers, producing nonfluorescent derivatives. We demonstrate how reduction of NBD-lipids with dithionite can be used to prepare asymmetrically labeled liposomes and to measure transverse-membrane asymmetry in vesicles. This method should be useful in many biochemical investigations, including the measurement of phospholipid translocase activity.

Phospholipids have an asymmetric transbilayer distribution across the plasma membranes of a variety of cell types (Op den Kamp, 1977). Membrane phospholipid asymmetry has been most vigorously studied in human erythrocytes. Aminophospholipids are located primarily in the inner leaflet of erythrocyte membranes while phosphatidylcholine and sphingomyelin are found primarily in the outer leaflet (Bretscher, 1972; Gordesky & Marinetti 1973; Verkleij et al., 1973). Maintenance of phospholipid asymmetry in erythrocytes is of physiological significance. For example, the appearance of phosphatidylserine on the outer leaflet of the membrane may be a signal for erythrocyte removal from the circulation by the reticuloendothelial system (Tanaka & Schroit, 1983; Schroit et al., 1985). Formation and maintenance of phospholipid asymmetry in erythrocytes probably involves an ATP-dependent *N*-ethylmaleimide-sensitive transport protein called aminophospholipid translocase (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986). Aminophospholipid translocase activity has been demonstrated in several other cell types, including platelets (Sune et al., 1987), lymphocytes (Zachowski et al., 1987), and fibroblasts (Sleight & Pagano, 1985; Martin & Pagano 1987). This activity has also been observed in synaptosomes (Zachowski et al., 1990) and chromaffin granules (Zachowski et al., 1989).

The most successful methods for monitoring aminophospholipid translocase activity require first labeling the outer leaflet of the test membrane with either a spin-labeled or fluorescent phospholipid analogue (Morrot et al., 1989; Connor & Schroit, 1988). The analogue is then allowed to be acted upon by the translocase for a specific time. To remove any analogue that remains at the outer leaflet, the cells are incubated with bovine serum albumin (BSA).[1] After separation

† This work was supported in part by NIH Grant GM39035 and in part by BRSG S07 RR05408-29 awarded by the Biomedical Research Support Grant Program, National Center for Research Resources, National Institutes of Health.
* To whom correspondence should be addressed.
‡ J.C.M. was supported by Molecular and Cellular Training Grant HL07527.

0006-2960/91/0430-11819$02.50/0

of membranes from BSA/analogue complexes by centrifugation, the amount of analogue associated with BSA is determined spectrophotometrically. The fraction of the lipid analogue transported to the membranes' inner leaflet is defined as the difference between the original amount of analogue inserted and the amount of analogue removed by the BSA treatment. This method of analysis is very time consuming, and large experimental errors are commonly associated with the results obtained. Translocase activity measurements have less error when the amount of translocated lipid is determined by the fluorescence remaining in the cells. However, a more rapid and precise assay for determining lipid asymmetry would greatly aid efforts to reconstitute and purify phospholipid translocases.

Fluorescent lipid derivatives containing a 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) moiety are probes used to study membrane lipid biochemistry (Chattopadhyay, 1990). Conner and Schroit (1988) have shown that C_6-NBD-PS is a substrate for aminophospholipid translocase in human erythrocytes. Utilizing a fluorescent microscopic technique, it has been demonstrated that C_6-NBD-PS and C_6-NBD-PE are substrates for translocase in several types of nucleated cells (Sleight & Pagano, 1985; Martin & Pagano 1987). NBD-lipid derivatives have a bright yellowish-green fluorescence and high quantum yields. The electron-withdrawing nitro functional group at position 4 (see Figure 1) most likely aids in delocalization of the π electrons in the conjugated ring system. We postulated that reduction of the nitro group to an electron-donating amine would alter the electronic transitions of this analogue and alter its fluorescence properties. Sodium dithionite (hydrosulfite) has been used to reduce nitrotyrosine residues to aminotyrosine in protein modification studies without disruption of protein structure (Sokolovsky et al., 1967; McIntyre et al., 1990) and has been shown to reduce a variety of aryl–nitro compounds to the corresponding amine (Wasmuth et al., 1964; Kolker & Waters, 1963). In aqueous solution dithionite ion ($S_2O_4^{-2}$) is in equilibrium with SO_2^- radical. This radical is a reaction intermediate in nitro reduction (Wasmuth et al., 1964; Gutch & Water, 1964). Since the parent ion and radical are both charged, it seemed likely they would not permeate lipid bilayers. In this paper we present a characterization of the reaction of dithionite with NBD-lipid analogues and describe a simple fluorescence assay for measuring membrane lipid asymmetry.

Materials and Methods

Reagents. Miscellaneous organic solvents, chloroform, methanol, ethyl acetate, and diethyl ether were purchased from Fisher Scientific, Pittsburgh, PA. NBD chloride, aminohexanoic acid, and sodium dithionite were obtained from Aldrich Chemical, Milwaukee, WI. Stock solutions of dithionite were prepared in 1.0 M Tris (pH 10.0) because the reagent is highly susceptible to spontaneous breakdown in acidic solutions. Dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, (palmitoyl,C_6-NBD)-PC, C_6-NBD-PE, C_{12}-NBD-PC, *N*-NBD-PE, and *N*-Rh-PE were purchased from Avanti Polar Lipids, Pelham, AL. (Palmitoyl,C_6-NBD)-PS and (palmitoyl,C_6-NBD)-PA were synthesized by transphosphatidylation using a modified version of the method of Comfurius and Zwaal (1977). Thin layer chromatography plates were purchased from VWR Scientific, Swedesboro, NJ. Cholesteryl [1-^{14}C]oleate was obtained from New England Nuclear, Wilmington, DE. The fluorescent sterol 22-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol) was purchased from Molecular Probes, Eugene, OR. Sepharose-6B-100 and sodium azide were obtained from Sigma Chemical, St. Louis, MO. NBD-aminohexanoic acid was synthesized from NBD-Cl and aminohexanoic acid as described previously (Longmuir et al., 1985).

All lipids were stored at −20 °C in chloroform/methanol (1:1). Phospholipid concentrations were determined by lipid phosphate analysis (Rouser et al., 1966). Unless otherwise stated, lipid vesicles were prepared by ethanol injection using 13.3 μmol of lipid/mL of ethanol, producing a 7.5% ethanolic solution (Kremer et al., 1977). The vesicles were dialyzed for at least 8 h against 10 mM HEPES, 0.9% NaCl, pH 7.4, prior to use. Vesicles having four different distributions of NBD-lipid labeling were prepared. One set of labeled vesicles was produced by including the fluorescent lipid analogue during vesicle formation. The fraction of fluorescent lipid present in the inner vs outer leaflet of these vesicles is proportional to the total amount of lipid present in these leaflets (Connor & Schroit, 1987). We call these vesicles "symmetrically labeled", as the distribution of the fluorescent label reflects the normal distribution of lipids across the bilayer. A second set of vesicles was prepared in which the fluorescent lipid analogue was present in only the outer leaflet of the bilayer. These "outside only" labeled vesicles were produced by adding the NBD-lipid in ethanol (1% of total volume) to preformed vesicles. The third set of vesicles, labeled exclusively on their inner leaflet, were prepared as described by Dao et al. (1991). Briefly symmetrically labeled vesicles were incubated with BSA to remove acyl-chain-labeled lipid from the outer leaflet. The asymmetrically labeled vesicles were then separated from BSA by column chromatography. In later experiments, dithionite treatment was used to produce vesicles having inside only labeling. The procedure for producing these vesicles is described under Results. A fourth set of vesicles was prepared having NBD-labeled lipids in both leaflets, but with various levels in inner vs outer leaflet labeling. These vesicles were prepared by adding NBD-lipid in ethanol to symmetric or inside only labeled vesicles.

Lipid Transfer Assay. In several experiments, the amount of (palmitoyl,C_6-NBD)-PC in the outer leaflet of liposomes was determined by a modified version of the lipid transfer assay described previously (Struck et al., 1981; Dao et al., 1991). In these experiments, liposomes were prepared containing 1 mol % (palmitoyl,C_6-NBD)-PC, 1 mol % *N*-Rh-PE, and 98 mol % DOPC (donor vesicles). A 100-fold excess of unlabeled DOPC liposomes (acceptor vesicles) was incubated with donor vesicles for 10 min. Spontaneous transfer of (palmitoyl,C_6-NBD)-PC from the outer leaflet of the donors to the acceptors results in dequenching of the NBD-labeled lipid. This increase in NBD-lipid fluorescence was recorded. The fraction of

[1] Abbreviations: ABD, 7-amino-2,1,3-benzoxadiazol-4-yl; BSA, bovine serum albumin; C_6-NBD-FA, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminocaproic acid; C_6-NBD-PE, 1-acyl-2-(C_6-NBD)phosphatidylethanolamine; C_{12}-NBD-PC, 1-acyl-2-*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminododecanoylphosphatidylcholine; CHO, Chinese hamster ovary; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; HCMF, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered Puck's saline without calcium and magnesium; HMEM, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered Eagle's minimal essential medium, pH 7.4, without indicator; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; NBD-cholesterol, 22-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-23,24-bisnor-5-cholen-3β-ol; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-dioleoylphosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; (palmitoyl,C_6-NBD)-PA, 1-palmitoyl-2-(C_6-NBD)phosphatidic acid; (palmitoyl,C_6-NBD)-PC, 1-palmitoyl-2-(C_6-NBD)phosphatidylcholine; (palmitoyl,C_6-NBD)-PS, 1-palmitoyl-2-(C_6-NBD)-phosphatidylserine.

(palmitoyl,C_6-NBD)-PC in the outer leaflet of the symmetrically labeled liposomes was determined by comparing the amount of NBD-lipid transferred from outside only the symmetrically labeled vesicles. The following equations were used for the calculation:

$$F_{tr} = F_{eq} - F_i - F_{ac} \quad (1)$$

percent label in outer leaflet =

$$[F_{tr(sym)}/F_{tr(all\ outside)}] \times 100 \quad (2)$$

In eq 1, F_{tr} is the fluorescence of NBD-lipid transferred from the donor to the acceptor. This is obtained by subtracting the apparent fluorescence of the acceptor vesicles alone, F_{ac}, and the fluorescence of the donor vesicles alone, F_i, from the fluorescence at equilibrium of donor and acceptor vesicles together, F_{eq}. Equation 2 is the ratio of lipid transferred from all outside labeled vesicles compared to symmetrically labeled vesicles. When identical concentrations of NBD-lipid are used in the preparation of the vesicles, this ratio is equal to the fraction of NBD-lipid originally present in the outer leaflet of the symmetrically labeled vesicles.

Dithionite-Based Assay for Measuring Lipid Asymmetry. The assay was performed by the following procedure: (i) Lipid vesicles containing 0.5–1.8 mol % NBD-labeled lipid were prepared by ethanol injection (Kremer et al., 1977). (ii) Two milliliters of a solution containing 10 μM lipid vesicles, 10 mM HEPES, 0.9% NaCl (pH 7.4) was placed in a fluorescence cuvette. (iii) The fluorescence emission of this solution was recorded over time at an excitation wavelength of 470 nm and an emission wavelength of 540 nm. (iv) After a constant baseline fluorescence was obtained, 20 μL of 1.0 M $Na_2S_2O_4$ in 1.0 M Tris (pH 10.0) was added, and the reaction was followed by the loss of fluorescence emission.

To calculate the amount of NBD-lipid reacted in liposomes and cells, the following equation was used:

$$\text{percent reacted} = [1 - [(F_r - F_{ap})/(F_o - F_{ap})]] \times 100 \quad (3)$$

Where F_r is the fluorescence after the reaction is complete, F_{ap} is the apparent fluorescence of the cells or liposomes without NBD-lipid, and F_o is the fluorescence of the cells or liposomes with NBD-lipid before reaction.

Instrumentation. Fluorescence measurements were obtained using a Perkin-Elmer LS-5 fluorescence spectrophotometer. Absorbance readings were obtained using a Beckman Instruments DU-65 spectrophotometer or a Gilford RESPONSE spectrophotometer. A Zeiss IM 35 microscope equipped with a 100× planapochromat objective was used to visualize NBD fluorescence in cells grown on glass coverslips.

Cells. Chinese hamster ovary cells (line K1) were obtained from the American Type Culture Collection, Rockville, MD. Cells were grown in Eagle's minimal essential medium supplemented with 5% fetal calf serum in a water-saturated atmosphere of 5% CO_2 in air. For microscopy experiments, the cells were grown in 35-mm culture dishes containing glass coverslips. The cells were then washed three times with ice-cold HMEM and covered with 2 mL HMEM. For experiments with cells in suspension, 150-mm culture dishes of CHO-K1 cells were grown to 80% confluence and harvested by treatment with 0.05% trypsin. *N*-NBD-PE was inserted into the cells by the addition of 20 μL of a 1.67 mM stock solution in ethanol to the bathing medium. After the cells were allowed to incubate 5 min at 2 °C, they were washed three times with bathing medium. In some experiments, the cells were subsequently incubated at 37 °C for 45 min.

Preparation of ABD-Labeled Lipids. In a round bottom flask, 2.0 mg (2.6 μmol) of (palmitoyl,C_6-NBD)-PC was dissolved in 25 mL of 50% ethanol with stirring. Nine milligrams (52.0 μmol) of solid sodium dithionite was added to the orange-yellow solution, causing it to immediately turn a deep red color. The reaction was allowed to proceed for 30 min at room temperature and was stopped by removing the solvent using a rotary evaporator. Lipid was extracted from the pasty red solid (Bligh & Dyer, 1959) and stored in 2 mL of chloroform/methanol (1:1). These same conditions were used at a larger scale to produce ABD-aminohexanoic acid. The fatty acid analogue was used for chemical analysis because of its availability and simpler structure relative to the phospholipid.

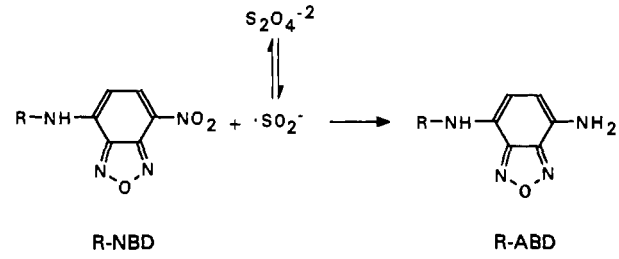

FIGURE 1: Reduction of 7-nitro-2,1,3-benzoxadiazol-4-yl-labeled lipids to 7-amino-2,1,3-benzoxadiazol-4-yl-labeled lipids with dithionite.

Dithionite reduces aryl–nitro compounds to their corresponding amines (Wasmuth et al., 1964; Kolker & Waters, 1963; Fieser & Fieser, 1934). Dithionite also catalyzes the reduction of diazo compounds, *C*- and *N*-nitroso compounds, O_2, H_2O_2, I_2, and many heavy metal ions (Fieser & Fieser, 1967; Greenwood & Earnshaw, 1984). The resistance of furazan (1,3-diazoles) rings to ring opening by reduction is well established. For example, furoxans (furazan *N*-oxides) are reduced by trialkyl and triaryl phosphines and phosphites without ring opening, producing intact furazans (Grundmann, 1964). From this information, we predicted that the reaction of NBD with dithionite would result in reduction of the nitro group at position 4 to an amine without any side reactions or ring opening. We have examined the UV/visible, IR, and ^1H NMR spectra of C_6-NBD-FA before and after treatment with dithionite. The data obtained (not shown) are consistent with the reaction shown in Figure 1. Since nitrobenzodiazole-containing compounds are called NBD analogues, we refer to the aminobenzodiazole-containing products produced by treatment of NBD-labeled lipids with dithionite as ABD-labeled lipids.

Results

As described under Materials and Methods, incubation of NBD-labeled lipids with dithionite in 50% ethanol produces 7-amino-2,1,3-benzoxadiazol-4-yl-lipid (ABD-lipid) derivatives. To determine if this reaction could be used to measure lipid asymmetry, we characterized the ABD-labeled lipids and examined properties of the reaction with labeled liposomes and cells.

Spectral Properties of (Palmitoyl,C_6-ABD)-PC. The UV/visible absorbance and fluorescence emission spectra of (palmitoyl,C_6-NBD)-PC and (palmitoyl,C_6-ABD)-PC were obtained in chloroform/methanol (2:1). The UV/visible spectra of the NBD derivative was dominated by a strong sharp visible absorbance with a maximum at approximately 460 nm and another transition centered at 340 nm (Figure 2). The spectrum of an equal concentration of the ABD derivative was dramatically different. The visible absorbance was very broad, with a maximum at 435 nm that extended far into the longer wavelength, giving the compound its reddish color. Whereas the (palmitoyl,C_6-NBD)-PC absorbance was

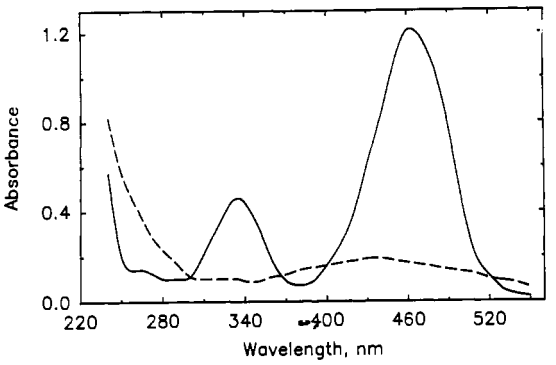

FIGURE 2: UV/visible absorbance spectra of (palmitoyl,C_6-NBD)-PC and (palmitoyl,C_6-ABD)-PC. Spectra were obtained using a Gilford RESPONSE spectrophotometer with 1-nm slit widths at a scan speed of 30 nm/min. The concentration of lipid was 50 μM, and the solvent was chloroform/methanol (2:1). (Solid line) (palmitoyl,C_6-NBD)-PC, (dashed line) (palmitoyl,C_6-ABD)-PC.

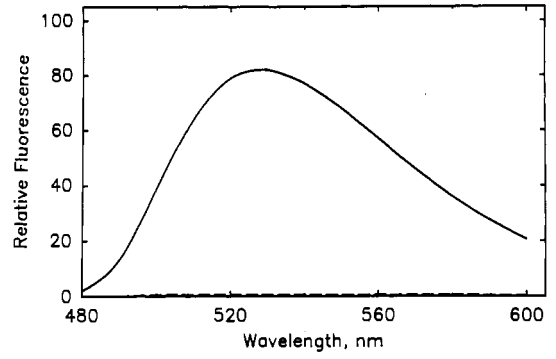

FIGURE 3: Fluoresence emission spectra of (palmitoyl,C_6-NBD)-PC and (palmitoyl,C_6-ABD)-PC. Fluorescence emission spectra were obtained at an excitation wavelength of 470 nm with a 5-nm slit width and an emission slit width of 5 nm. The concentration of lipid was 6.0 μM in chloroform/methanol (2:1). (Solid line) (palmitoyl,C_6-NBD-)-PC, (dashed line) (palmitoyl,C_6-ABD)-PC.

very strong in the visible region, (palmitoyl,C_6-ABD)-PC absorbance was much weaker. However, the ABD derivative had an increased UV absorbance compared to the NBD-derivative.

The fluorescence emission spectra of the two compounds were obtained in chloroform/methanol (2:1) with an excitation wavelength of 470 nm (Figure 3). We were able to detect less than 0.2% fluorescence emission from (palmitoyl,C_6-ABD)-PC as compared to (palmitoyl,C_6-NBD)-PC. This was also true for the comparison of ABD- and NBD-labeled six-carbon fatty acids.

Reaction of Dithionite with Lipid Vesicles Containing NBD-Labeled Lipids. Having established that the reaction of NBD-labeled lipids in ethanolic solutions with dithionite produced a nonfluorescent product, the same reaction was investigated using fluorescent analogues incorporated in liposomal membranes. "Symmetrically labeled" small unilamellar lipid vesicles containing 1 mol % (palmitoyl,C_6-NBD)-PC were prepared by including the fluorescent lipid during vesicle preparation. Under this condition, the fluorescent lipid analogue distributes between inner and outer leaflets of the liposomal membrane according to the distribution of total lipid across the bilayer (Connor & Schroit, 1987). These vesicles were reacted with varying amounts of dithionite. The reaction was monitored by the loss of fluorescence emission at 540 nm (λ_{ex} = 470 nm), and the extent of reaction was determined as described under Materials and Methods. A typical reaction profile is presented in Figure 4A. Upon the addition of dithionite, a rapid decrease in

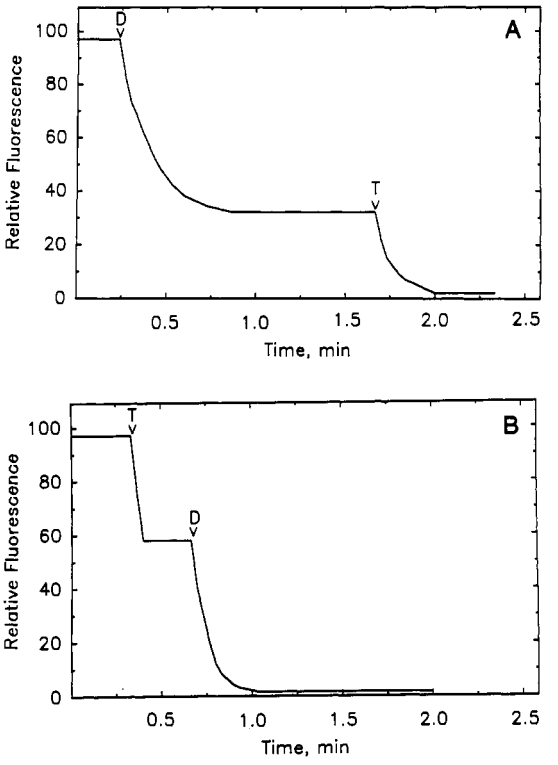

FIGURE 4: Reaction of dithionite with lipid vesicles containing NBD-labeled lipids. A 2-mL solution of 10 μM lipid vesicles containing 1 mol % (palmitoyl,C_6-NBD)-PC and 99 mol % DOPC in 10 mM HEPES, 0.9% NaCl, pH 7.4 was placed in a cuvette and fluorescence (λ_{ex} = 470 nm; λ_{em} = 540 nm) was measured. In panel A, 20 μL of 1 M $Na_2S_2O_4$ in 1 M Tris, pH 10.0, was added at the time indicated by the arrow labeled D. After the reaction was complete (i.e., no change in fluorescence over time), Triton X-100 was added to a final concentration of 1.25% detergent (v/v) at the time indicated by the arrow labeled T. In panel B, Triton X-100 was added at the time indicated by the arrow labeled T. After a new baseline was established, 20 μL of 1 M dithionite was added at the time indicated by the arrow labeled D.

fluorescence was observed. Within 50–70 s after the addition of dithionite, no further decrease in fluorescence was detected. At that time 67% of the original fluorescence was destroyed. When Triton X-100 was added to the cuvette, the remaining fluorescence was destroyed. When the vesicles were disrupted with Triton X-100 prior to reaction with dithionite (Figure 4B), a 40% decrease in fluorescence was observed due to detergent quenching. After the addition of the reducing agent, all of the remaining fluorescence was lost within 30 s. The vesicles used in this experiment have approximately 63–70% of their total lipid located in the outer leaflet (Dao et al., 1991). Therefore, the results obtained are consistent with dithionite reacting only with lipid at the vesicles' surface, until the vesicles are disrupted, making all of the lipid available for reaction.

The reaction of dithionite with symmetrically labeled lipid vesicles was repeated using a wide range of dithionite concentrations (Figure 5). The concentrations of dithionite used represent a (2.5×10^3)–(1.4×10^6) molar excess of the reducing agent relative to (palmitoyl,C_6-NBD)-PC. At room temperature, the very rapid initial rates of these reactions could not be accurately determined; therefore, the data are presented as dithionite concentration vs the minimum time required for completion of the reaction. At concentrations of dithionite above 25 mM, the reaction was completed in less than 30 s. Regardless of the concentration of dithionite used, the fraction of fluorescent lipid reacted remained constant. An average of 67.3 ± 1.3% of the fluorescent lipid was reacted for the 36 separate determinations. We routinely used 9.9 mM dithionite

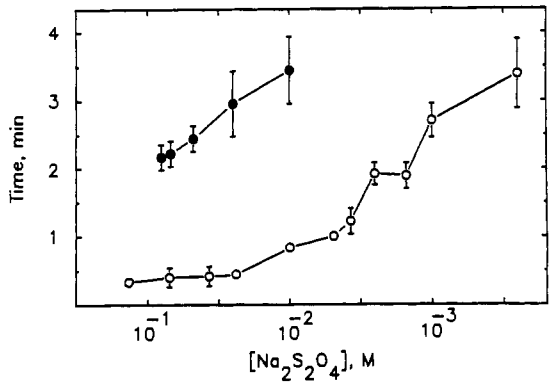

FIGURE 5: Time required for complete reaction of (palmitoyl,C_6-NBD)-PC with dithionite. The minimum time required to complete the reaction of (palmitoyl,C_6-NBD)-PC with dithionite (e.g., 1.0 min in Figure 4A) was determined. Assays were performed as described in the legend to Figure 4A. Lipid vesicles were composed of 1 mol % (palmitoyl,C_6-NBD)-PC and 99 mol % DOPC and were present in the assay at a concentration of 10 μM. (Open circles) reaction at room temperature; (closed circles) reaction at 2 °C.

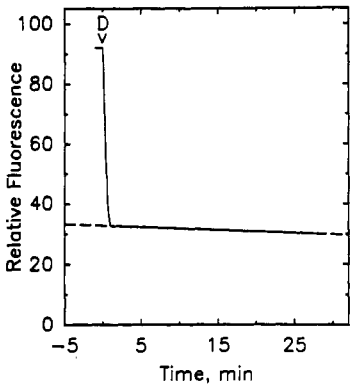

FIGURE 6: Reaction of dithionite with (palmitoyl,C_6-NBD)-PC labeled liposomes. The reaction was performed as described in the legend to Figure 4A and allowed to continue for 28 min. The arrow labeled D indicates the time at which dithionite was added to the cuvette. The dashed line is the extrapolation of the slow reaction rate to time zero.

for assaying lipid asymmetry because this was the lowest concentration which allowed completion of the reaction within 60 s.

The reaction was also performed at 2 °C to assess the possibility of using it with cells labeled with NBD-lipid analogues, which are routinely handled at 2 °C. Although the reaction occurred more slowly at 2 °C, the fraction of lipid reacted was the same as that determined at room temperature. At 2 °C, a higher concentration of dithionite is required to complete the reaction rapidly (Figure 5).

Analysis of the Slow Reaction of NBD-Labeled Lipid Vesicles with Dithionite. In Figure 4A, a typical example of the reaction of dithionite with symmetrically labeled lipid vesicles is presented. In an identical experiment, the reaction was followed for much longer times (Figure 6). At times originally thought to be indicative of the completion of the reaction (i.e., between 0.7 and 1.7 min, Figure 4A), a very slow reduction of fluorescence was occurring. The slow rate of reaction observed after the initial fast reaction was not the result of lipid vesicles settling in the cuvette. When outside only labeled vesicles were treated with dithionite, only the fast reaction rate was observed. In contrast, when inside only labeled vesicles were treated, only the slow reaction rate was seen. Given sufficient incubation time with dithionite, all of the fluorescence in the various liposome preparations was lost (data not shown). These data suggested that the slow rate fluorescence loss represents passage of either $S_2O_4^{-2}$ or SO_2^- radical across DOPC membranes and reaction of labeled lipid in the inner leaflet.

The rate of reaction at the inner leaflet was investigated by measuring initial velocities at room temperature and at 2 °C. A rate of 0.25% reacted/min at the inner leaflet with 9.9 mM dithionite at room temperature was determined. Since the fast reaction was complete between 50 and 70 s (9.9 mM dithionite), only 0.25–0.28% of the total NBD-lipid reacted at these times was due to reaction at the inner leaflet. Therefore, measurements of the fraction of (palmitoyl,C_6-NBD)-PC present in the outer leaflet, derived from the reaction of dithionite with lipid vesicles, were valid when determined 50–70 s after the start of the reaction. The V_{max} for reaction at the inner leaflet, derived from double-reciprocal plots, was 0.417% reacted/min for DOPC liposomes at room temperature and 0.227% reacted/min at 2 °C (data not shown).

A very slow rate of inner leaflet reaction at room temperature was also observed using liposomes symmetrically labeled with (palmitoyl,C_6-NBD)-PC but composed of 99 mol % DPPC. A 4-fold increase in the rate of the inner leaflet reaction was observed when the reaction was performed at 45 °C (T_m for DPPC = 41 °C) (McIntyre and Sleight, unpublished data). However, this rate was still negligible compared to the rate of reaction of the probe in the outer leaflet.

Reaction of Dithionite with Symmetrically Labeled Vesicles of Varying Size. The size of the vesicles produced by ethanol injection is controlled by the ratio of lipid to ethanol (Kremer et al., 1977). The greater the ratio, the larger the vesicles produced. Light-scattering studies have shown that vesicles having outer radii of 25–65 nm can be produced by ethanol injection (Kremer et al., 1977). The fraction of the total lipid in the outer leaflet of a liposome is proportional to the size of the vesicle. Small vesicles have a small radius of curvature, which restricts the packing geometry and results in more lipid molecules being located in the outer leaflet (New, 1990). Therefore, altering the injection conditions produces vesicles of differing size, each size having different amounts of lipid in their outer leaflet. We used vesicles of varying size, containing a symmetric distribution of (palmitoyl,C_6-NBD)-PC, to assess the ability of the dithionite reaction to measure membrane asymmetry. As a control in this experiment, the amount of lipid in the outer leaflet of these vesicles was also tested using an established lipid transfer assay (Dao et al., 1991).

A series of liposomes were produced having 25–60-nm outer radii (Kremer et al., 1977). These vesicles were prepared having two different lipid compositions. The first set (A) contained a symmetric distribution of 1 mol % (palmitoyl-,C_6-NBD)-PC, the second (B) contained 1 mol % (palmitoyl,C_6-NBD)-PC and 1 mol % *N*-Rh-PE. Set A were treated with dithionite, and the fraction of NBD-lipid reacted was determined (Figure 7). Set B were analyzed using the lipid transfer assay. The reaction of dithionite with vesicles of increasing size indicated a decrease in the fraction of fluorescent lipid in the outer leaflet of larger vesicles, as predicted. The lipid transfer assays also indicated the fraction of (palmitoyl,C_6-NBD)-PC transferred decreased with increasing vesicle size. Both methods produced similar results. These data further verify that the amount of NBD-lipid analogue in the outer leaflet of liposomes can be determined by reaction with dithionite.

Reaction of Dithionite with a Variety of NBD-Labeled Lipids. The dithionite reaction was used to determine NBD-labeled lipid distribution across the membranes of liposomes

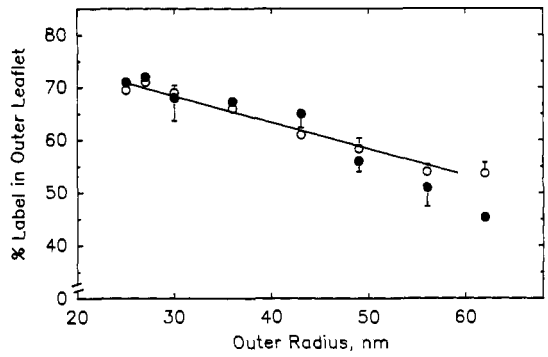

FIGURE 7: Fraction of (palmitoyl,C_6-NBD)-PC in symmetrically labeled liposomes decreases with increasing vesicle size. Lipid vesicles of varying size were produced by ethanol injection (Kremer et al., 1977) with various ratios of lipid/ethanol. The predicted size of the vesicles were taken from Kremer et al. (1977). For dithionite assays (open circles), vesicles were prepared containing 1 mol % (palmitoyl,C_6-NBD)-PC and 99 mol % DOPC. The final concentration of dithionite in the assays was 9.9 mM. For resonance energy transfer assays (closed circles) (Dao et al., 1991), vesicles containing 1 mol % (palmitoyl,C_6-NBD)-PC, 1 mol % *N*-Rh-PE and 98 mol % DOPC were incubated with a 100-fold excess of 100 mol % DOPC vesicles for 10 min. Data points represent the mean ± standard deviation of at least three determinations.

Table I: Dithionite Reacts with a Variety of NBD-Labeled Lipid Analogues[a]

	location of fluorescent label	
	outer leaflet only	symmetric
NBD-lipid	% reacted	% reacted
(palmitoyl,C_6-NBD)-PA	97	69
(palmitoyl,C_6-NBD)-PC	96	67
C_6-NBD-PE	97	69
(palmitoyl,C_6-NBD)-PS	98	68
C_{12}-NBD-PC	98	69
N-NBDH-PE	98	69
NBD-cholesterol	95	66

[a] Lipid vesicles contained 1 mol % NBD-lipid and 99 mol % DOPC; 10 μM lipid vesicles and 5 mM dithionite were used in the assays.

prepared with seven different NBD-lipid analogues. The vesicles were labeled by either adding the lipid analogue to preformed vesicles (outer leaflet labeled) or by including the lipid analogue in the original lipid preparation (symmetrically labeled). When vesicles with label localized to the outer leaflet were reacted with dithionite, 95–98% of the NBD-lipid was reacted (Table I). We have recently determined that increasing the amount of ethanol used for adding the NBD-lipid increases the fraction of label incorporated into the inner leaflet (McIntyre and Sleight, unpublished observation). Thus, the small amount of NBD-lipid not reacted with dithionite may be located in the inner leaflet.

Reaction of dithionite with vesicles symmetrically labeled with NBD-lipids resulted in reduction of 66–69% of the analogue (Table I). This value is consistent with the predicted amount of lipid in the outer leaflet of these vesicles (Dao et al., 1991). A third set of vesicles were produced, by the method of Dao et al. (1991), having (palmitoyl,C_6-NBD)-PC localized almost exclusively to the inner leaflet. This method produces vesicles that have 87–94% of the labeled lipid in the inner leaflet. When these vesicles were reacted with dithionite, 5–15% of NBD-lipid was accessible to the reagent.

The possibility of using the dithionite reaction to chemically eliminate NBD-labeled lipids in the outer leaflet of liposomes, producing vesicles labeled only at the inner leaflet, was explored. Vesicles symmetrically labeled with NBD-lipid were reacted with dithionite and isolated using a Sepharose-6B-100

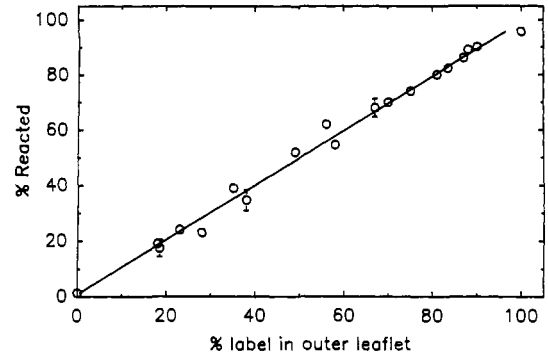

FIGURE 8: Fraction of (palmitoyl,C_6-NBD)-PC present in the outer leaflet of lipid vesicles prepared with varying label asymmetries. A series of lipid vesicles were produced containing (palmitoyl,C_6-NBD)-PC at varying labeling asymmetries as described under Materials and Methods. The fraction of NBD-lipid present in the outer leaflet of these vesicles was determined by reacting a 10 μM solution of the liposomes with 9.9 mM dithionite. Data points represent the mean ± standard deviation of at least three determinations.

column. The distribution of the NBD-lipid in these vesicles was tested, and values ranging from 0 to 2% NBD-lipid in the outer leaflet were obtained. This suggested that inside only labeled vesicles prepared by reaction with dithionite are superior to those produced by other methods. Dithionite treatment of symmetrically labeled liposomes was used throughout the remainder of this study to produce inside only labeled lipid vesicles.

Reaction of Dithionite with Lipid Vesicles Having (Palmitoyl,C_6-NBD)-PC Distributed in Varying Asymmetries. When NBD-lipid analogues are included during liposome formation, they distribute symmetrically (Connor & Schroit, 1987). In the previous paragraph and under Materials and Methods, we describe methods for the production of lipid vesicles that contain NBD-lipid derivatives localized exclusively to the inner leaflet of liposomal membranes. Insertion of the lipid probe into the outer leaflet of the membrane can be achieved by adding NBD-lipids in ethanol to preformed vesicles (Connor & Schroit, 1987). By a combination of these three methods, it is possible to produce lipid vesicles having known amounts of NBD-lipids present in the inner and outer leaflets of the membrane (see Materials and Methods). We produced a series of vesicles having various amounts of NBD-lipid in the inner vs outer leaflet. The predicted asymmetry of these vesicles were then compared to the asymmetry measured using the dithionite reaction (Figure 8). The fraction of NBD-lipid present in the outer leaflet of these liposomes as determined by the dithionite reaction was almost identical with that predicted by the method of preparation. This result demonstrates the accuracy of the dithionite reaction in measuring small changes in lipid asymmetry.

Reaction of Dithionite with CHO-K1 Cells Labeled with N-NBD-PE. Our laboratory as well as others have had great success in using NBD-labeled lipids to monitor intracellular lipid trafficking (Sleight & Pagano, 1985; Sleight & Abanto, 1989; Kobayashi & Pagano, 1989). If dithionite is nontoxic and does not leak into cells, it could be used to measure asymmetry at the plasma membrane. Many of the NBD-lipid probes label the external leaflet of the plasma membrane and luminal leaflet of endocytic vesicles. Treatment of living cells with dithionite may destroy the fluorophore at the plasma membrane, permitting enhanced viewing and biochemical analysis of labeled intracellular organelles.

To determine the cytotoxicity of dithionite treatment, cells were plated at low density on 100-mm plastic dishes. After 24 h, the cells were washed three times with HCMF and once

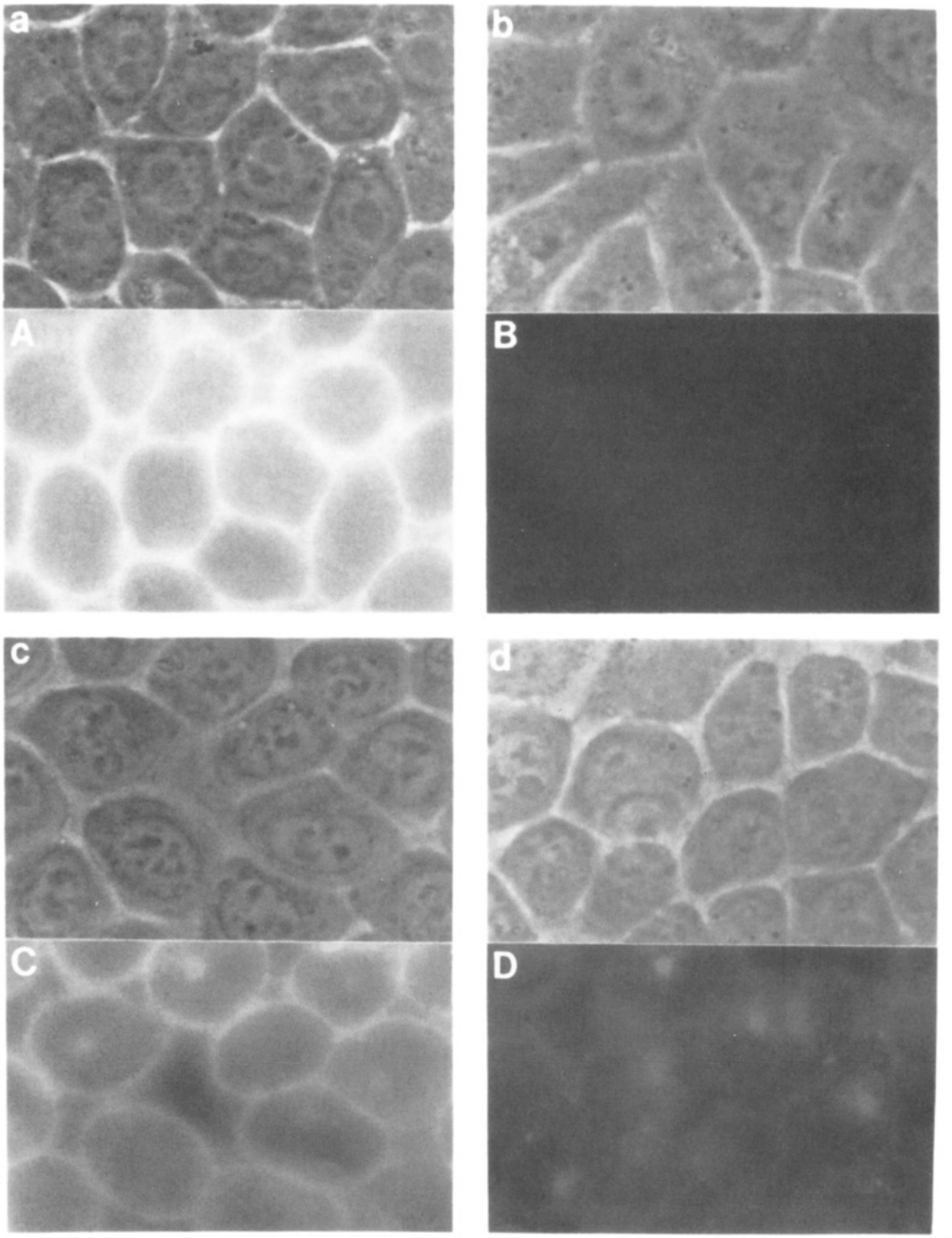

FIGURE 9: Microscopic changes in *N*-NBD-PE-labeled CHO-K1 cells after treatment with dithionite. CHO-K1 cells were labeled with *N*-NBD-PE at 2 °C as described under Materials and Methods. Photographic exposure and printing times were identical for all fluorescence photomicrographs. (A, a) Cells labeled at 2 °C. (B, b) Cells reacted with dithionite after initial labeling with *N*-NBD-PE. The final dithionite concentration was 88 mM, and the reaction was allowed to proceed for 3.5 min at 2 °C. (C, c) Cells labeled at 2 °C and then incubated at 37 °C for 45 min. (D, d) Cells incubated as in (C, c) and then brought to 2 °C and treated with dithionite. Upper case letters indicate fluorescence photomicrographs and lower case letters indicate the corresponding phase contrast photomicrographs.

with 10 mM HEPES–saline (pH 7.4). After the cells were incubated at room temperature with 0–100 mM dithionite in the same HEPES–saline solution for 5 min, they were washed three times with HCMF. Complete culture medium was then added to the dishes and the cells placed in a 37 °C CO_2 incubator. Three days later the cells were stained and the number of colonies counted. The number of colonies/plate was identical (±5%) for all samples, indicating that, at the concentrations of dithionite tested, the reagent was not toxic.

CHO-K1 cells grown on glass coverslips were labeled at 2 °C with *N*-NBD-PE, as described under Materials and Methods. Immediately after labeling, the cells had highly fluorescent plasma membranes (Figure 9A). The dithionite reaction was performed using similar samples at 2 °C to minimize any toxic effects of the reagent and to inhibit metabolism of the fluorescent probe. After the dithionite was removed by washing the cells with cold HCMF, no fluorescence was observed at the plasma membrane (Figure 9B). When CHO-K1 cells labeled at 2 °C with *N*-NBD-PE were incubated at 37 °C for 45 min, some internalization of the probe occurred (Figure 9C). However, the internalized fluorescence was difficult to visualize due to the brilliant labeling of the plasma membrane. As described previously, the internalization of the fluorescent probe at 37 °C is the result of endocytosis (Sleight & Pagano, 1984). After permitting endocytosis to occur at 37 °C, some samples were cooled to

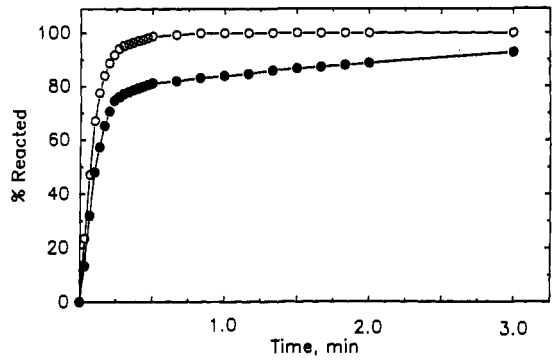

FIGURE 10: Reaction of dithionite with CHO-K1 cells labeled with *N*-NBD-PE in suspension. Cells were harvested from 150-mm culture dishes by treatment with 0.05% trypsin, washed three times with HCMF, and then labeled with 33.4 nmol of *N*-NBD-PE at 2 °C. (Open circles) cells after labeling and the addition of 88 mM dithionite at room temperature; (closed circles) cells incubated at 37 °C for 45 min after insertion of *N*-NBD-PE at 2 °C, followed by reaction with dithionite at room temperature. Dithionite was added to the cells at time 0.

2 °C and treated with dithionite. As shown in Figure 9D, brilliant fluorescence at the plasma membrane was again eliminated by the dithionite treatment. The intracellular labeling was not affected and was much easier to visualize. These results demonstrated that dithionite could be used specifically to eliminate the fluorescence of NBD-lipids at the plasma membrane.

To quantitate the reaction of dithionite with cells labeled with *N*-NBD-PE, a similar experiment was performed using cells in suspension. Cells maintained at 2 °C after labeling with *N*-NBD-PE were reacted with dithionite at room temperature. Within 30 s of the addition of dithionite, all of the NBD-fluorescence was destroyed (Figure 10). Cells labeled with *N*-NBD-PE were incubated at 37 °C for 45 min, to permit endocytosis, and then reacted with dithionite. Within 30 s of the addition of dithionite, 75–80% of the NBD-fluorescence was destroyed. The fluorescence that was not destroyed within the first 30 s was slowly lost with time (Figure 10). We believe this slow reaction rate results from passage of dithionite through the plasma membrane. We determined that the slow intracellular reaction rate was approximately 4% reacted/min. Thus, 30 s after treatment with dithionite, 2% of the initial *N*-NBD-PE is destroyed inside the cells, and all of the plasma membrane label is destroyed. Taking into account the rate of dithionite reaction inside the cells, we calculated that after a 45-min incubation at 37 °C, approximately 23–28% of the fluorescent lipid at the plasma membrane was internalized. This observation agrees with a previous examination of the rate of NBD-lipid endocytosis into CHO-K1 cells (Sleight & Abanto, 1989).

Discussion

We have presented a method for preferentially eliminating the fluorescence of NBD-labeled lipids in the outer leaflet of lipid bilayers. This method can be used to measure the distribution of fluorescent lipid analogues in the inner vs outer leaflets of membranes. Besides measuring lipid asymmetry, reduction of NBD-labeled lipids with dithionite can be used to produce lipid vesicles containing labeled phospholipids exclusively located in their inner leaflet. These vesicles should be useful for studies examining transmembrane movement and membrane fusion. Another application of the dithionite-based method is preferential elimination of NBD-analogues in the outer leaflet of the plasma membrane of living cells. This technique should greatly simplify microscopic studies of lipid trafficking, as removal of the fluorescent signal from the plasma membrane facilitates examination of fluorescent lipid intracellularly.

A similar chemical modification assay for measuring membrane asymmetry using spin-labeled lipids has been reported (Seigneruet & Devaux, 1984). In this assay, the ESR signal of spin-labeled lipids is eliminated by reduction with ascorbate (Kornberg & McConnell, 1971). Ascorbate is impermeable to cell membrane at low temperatures and therefore preferentially eliminates spin labels at the cell surface. However, cell cytosol also reduces spin-labeled probes. Since spin-labeled lipid may move between membrane bilayers several times prior to treatment with ascorbate, some spin-labeled lipid is prematurely reduced. Therefore, inaccurate values for the amount of label accessible to reduction are obtained. Consequently, assays based on the reduction of spin-labeled probes are rarely used to measure transmembrane distribution in cells. Unlike spin-labeled probes, NBD-lipid analogues are not reduced in the cytosol. Even after long periods of incubation with cells, complete recovery of the NBD label can be obtained from cells and their bathing medium (Sleight & Pagano, 1984, 1985). A second advantage of NBD-labeled lipids is the ability to follow transport in living cells by fluorescence microscopy.

We are currently attempting to apply the dithionite assay for the measurement of protein-mediated transmembrane lipid movement. The measurement of phospholipid flippase activity requires determination of phospholipid distribution between the inner and outer leaflets of membranes. Selective removal of either spin-labeled or fluorescently labeled lipid analogues from membranes by incubation in the presence of albumin is the most commonly used method for measuring flippase activity (Morrot et al., 1988; Connor & Schroit, 1988). Typically, the outer leaflet of cell or organelle membranes are tagged by insertion of labeled lipid. After incubation of the samples under conditions favoring transmembrane movement, the lipid probe is removed from the outer leaflet by transfer to fatty acid free albumin. This "back-exchange" occurs because the lipid analogues have an increased solubility relative to their native counterparts. The amount of lipid analogue associated with the membranes and albumin are then measured, providing an estimate of inner and outer leaflet labeling respectively.

We have recently characterized the removal of acyl-chain-labeled NBD-lipid analogues from liposomes by back-exchange with BSA (Dao et al., 1991). We determined that a large molar excess of BSA relative to NBD-lipid (450:1) is required for efficient removal of the probe. However, even after treatment with a large quantity of BSA, some NBD-lipid remains in the outer leaflet. The amount of BSA used to remove labeled lipids by back-exchange in previous asymmetry assays was far less than we have found necessary. Incomplete removal of labeled lipids from the outer leaflet of membranes would result in an overestimation of flippase activity. Back-exchange assays require separation of albumin from the membranes. This process is time consuming and may add to the large experimental error associated with these assays.

The dithionite reaction method for measuring membrane asymmetry has several advantages over back-exchange methods. Reaction with dithionite is very fast. Depending on the concentration of dithionite used, the reaction can be completed in less than 60 s. Thus, a large number of assays can be performed quickly. The dithionite method is accurate. We are able to routinely assay fluorescent lipid asymmetry across liposomal membranes with less than 2% error. In

contrast, back-exchange methods often have greater than 13% error associated with them (Connor & Schroit, 1988). One disadvantage of the dithionite method is that dithionite and/or the reactive SO_2^- radical may leak across some membranes.

We have found that dithionite and SO_2^- radicals are only slightly permeable to DOPC liposomes at room temperature. The maximum velocity of the reaction at the inner leaflet was determined to be 0.417% reacted/min. Approximately 9-fold higher concentrations of dithionite were used to treat labeled cells. This was necessary due to the larger amounts of fluorescent lipid present and to ensure that the reaction went to completion quickly. At this higher concentration, the reagent was about 10 times more permeable to CHO-K1 cells. The reaction rate of NBD-labeled lipids inside the cells was 4% reacted/min at room temperature. Complete reaction of labeled lipid at the surface of CHO-K1 cells occurred within 30 s of incubation. Because the rate of reaction at the cell surface is much greater than inside the cells (Figure 10), it is possible to determine the amount of NBD-lipid present at the cell surface by extrapolation. The permeability of dithionite and SO_2^- radicals may vary greatly with membrane lipid and protein composition and membrane structure. Therefore, careful characterization of the rate of the dithionite reaction at the membrane surface and after leakage must be performed with each new membrane tested.

In summary, we have devised a method for selectively abolishing the fluorescence of NBD-labeled lipids in the outer leaflet of lipid bilayers. This method can be applied to investigate several aspects of lipid transport and membrane structure. We presume that dithionite treatment can also be used to distinguish NBD-labeled proteins present at cell, organelle, or liposome surfaces.

Acknowledgments

We thank Chad Hagedorn, Mike Gunther, Faith Burnham, and Dr. Meena Mehta for excellent technical assistance and helpful suggestions. We are also thankful to Curt Balch, Cameron Sheeler, and Peter Hug for critical evaluation of the manuscript.

References

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Bretscher, M. S. (1972) *J. Mol. Biol. 71*, 523–528.

Chattopadhyay, A. (1990) *Chem. Phys. Lipids 53*, 1–15.

Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45.

Chattopadhyay, A., & London, E. (1988) *Biochim. Biophys. Acta 938*, 24–34.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Connor, J., & Schroit, A. J. (1987) *Biochemistry 26*, 5099–5105.

Connor, J., & Schroit, A. J. (1988) *Biochemistry 27*, 848–851.

Daleke, D. L., & Huestis, W. H. (1985) *Biochemistry 24*, 5406–5416.

Dao, H-N. T., McIntyre, J. C., & Sleight, R. G. (1991) *Anal. Biochem. 196*, 46–53.

Fieser, L. F., & Fieser, M. A. (1934) *J. Am. Chem. Soc. 56*, 1565.

Fieser, L. F., & Fieser, M. A. (1967) *Reagents for Organic Synthesis*, pp 1081–1082, John Wiley and Sons, New York.

Gordesky, S. E., & Marinetti, G. V. (1973) *Biochem. Biophys. Res. Commun. 50*, 1027–1031.

Greenwood, N. N., & Earnshaw. A. (1984) *Chemistry of the Elements*, pp 853–854, Pergamon Press, New York.

Grundmann, C. (1964) *Berichte 97(2)*, 575–578.

Gutch, C. J. W., & Waters, W. A. (1966) *Chem. Commun. 2*, 39–41.

Kobayashi, T., & Pagano, R. E. (1989) *J. Biol. Chem. 264*, 5966–5973.

Kolker, P. L., & Waters, W. A. (1963) *Proc. Chem. Soc., London*, 55.

Kornberg, R. D., & McConnell, H. M. (1971) *Biochemistry 10*, 1111–1120.

Kremer, J. M. H., Esker, M. W. J., Pathmamanohan, K., & Wiersema, P. H. (1977) *Biochemistry 16*, 3932–3935.

Longmuir, K. J., Martin, O. C., & Pagano, R. E. (1985) *Chem. Phys. Lipids 36*, 197–207.

Martin, O. C., & Pagano, R. E. (1987) *J. Biol. Chem. 262*, 5890–5898.

McIntyre, J. C., Schroeder, F. S., & Behnke, W. D. (1990) *Biochemistry 29*, 2092–2101.

Morrot, G., Herve, P., Zachowski, A., Fellman, P., & Devaux, P. F. (1989) *Biochemistry 28*, 3456–3462.

New, R. R. C. (1990) *Liposomes, A Practical Approach*, p 22, Oxford University Press, New York.

Op den Kamp, J. A. F. (1977) *Annu. Rev. Biochem. 48*, 47–71.

Rouser, G., Siakotos, A. N., & Fleisher, S. (1966) *Lipids 1*, 85–86.

Schroit, A. J., Madsen, J., & 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.

Sleight, R. G., & Pagano, R. E. (1984) *J. Cell Biol. 99*, 742–751.

Sleight, R. G., & Pagano, R. E. (1985) *J. Biol. Chem. 260*, 1146–1154.

Sleight, R. G., & Abanto, M. N. (1989) *J. Cell Sci. 93*, 363–374.

Sokolovsky, M., Riordan, J. F., & Vallee, B. L. (1967) *Biochem. Biophys. Res. Commun. 27*, 20–25.

Struck, D. S., Hoekstra, D., & Pagano, R. E. (1991) *Biochemistry 30*, 4093–4099.

Sune, A., Bette-Bobillo, P., Bienvenue, A., Fellman, P., & Devaux, P. F. (1987) *Biochemistry 26*, 2972–2978.

Tanaka, Y., & Schroit, A. J. (1983) *J. Biol. Chem. 258*, 11335–11343.

Tilley, L., Cribier, S., Roelfen, B., Op den Kamp, J. A. F., & van Deenen, L. L. M. (1986) *FEBS Lett. 194*, 21–27.

Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 323*, 178–193.

Wasmuth, C. R., Edwards, C., & Hutcherson, R. (1964) *J. Phys. Chem. 68*, 423–425.

Zachowski, A., & Devaux, P. F. (1989) *Nature 340*, 75–76.

Zachowski, A., & Morot Gaudry-Talarmain, Y. (1990) *J. Neurochem. 55*, 1352–1356.

Zachowski, A., Herrmann, A., Paraf, A., & Devaux, P. F. (1987) *Biochim. Biophys. Acta 897*, 197–200.