Radioiodinated, Photoactivatable Phosphatidylcholine and Phosphatidylserine: Transfer Properties and Differential Photoreactive Interaction with Human Erythrocyte Membrane Proteins†

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ABSTRACT: An isotopically labeled cross-linking reagent, succinimido 3-(3-[^{125}I]iodo-4-azidophenyl)propionate, has been synthesized and coupled to 1-acyl-2-(aminocaproyl)phosphatidylcholine according to previously described procedures [Schroit, A. J., & Madsen, J. (1983) *Biochemistry 22*, 3617–3623]. ^{125}I- and N_3-labeled phosphatidylserine (^{125}I-N_3-PS) was produced from the phosphatidylcholine (PC) analogue by phospholipase D catalyzed base exchange in the presence of L-serine. These phospholipid analogues are photoactivatable, are labeled with ^{125}I at high specific activity, completely incorporate into synthetic vesicles, and spontaneously transfer between membranes. When an excess of acceptor vesicles or red blood cells (RBC) was mixed with a population of donor vesicles containing the ^{125}I-N_3-phospholipids, approximately 40% of the analogues transferred to the acceptor population. After transfer in the dark to RBC, all of the ^{125}I-N_3-PC incorporated into the cells could be removed by washing with serum, whereas the ^{125}I-N_3-PS could not. After photolabeling of intact RBC, ~50% of the PC and 20% of the PS cross-linked to membrane proteins as determined by their insolubility in $CHCl_3$/MeOH. Analysis of probe distribution by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that ^{125}I-N_3-PS preferentially labeled a M_r 30000 peptide which contained ~30% of the protein-bound label.

Studies of lipid–lipid and lipid–protein interactions are important to understanding both the functional and structural properties of biological membranes. While there are a variety of methodologies that may be employed to study these phenomena, the most direct approach involves the labeling of membrane components with site-directed ligands bearing chemically reactive, photoactivatable groups (Staros, 1980; Middaugh et al., 1983; Bayley, 1983). Although a variety of "lipophilic probes" have been used for this purpose (Klip & Gitler, 1974; Hu & Wisnieski, 1979; Bayley & Knowles, 1980), a more specific and unambiguous approach to studying "nearest-neighbor" moieties has been to attach photoreactive groups to phospholipid acyl chains (Chakrabarti & Khorana, 1975; Gupta et al., 1977; Bisson & Montecucco, 1981, 1985) or their polar head groups (Burnett et al., 1985). Indeed, such reagents have proved useful in determining intermolecular cross-linking between phospholipids (Gupta et al., 1979; Brunner & Richards, 1980), the location of membrane proteins in lipid bilayers (Takagaki et al., 1983; Brunner et al., 1983; Burnett et al., 1985), and the lipid binding site in phosphatidylcholine exchange protein (Moonen et al., 1979; Westerman et al., 1983).

Recent studies employing fluorescent-labeled (Tanaka & Schroit, 1983; Schroit et al., 1984, 1985) and spin-labeled (Seigneuret & Devaux, 1984; Seigneuret et al., 1984) analogues of PS[1] have been used to study the possible role of PS in homeostasis and its translocation in RBC membranes, respectively. Indeed, the translocation of PS from the inner to outer leaflet in activated platelets (Bevers et al., 1983) has been shown to be important in the prothrombin-converting activity of these cells (Bevers et al., 1982), and its translocation in sickled RBC has been implicated as an important factor in the pathogenesis of sickle cell anemia (Franck et al., 1985; Schwartz et al., 1985). Since PS does not appear to spontaneously translocate between leaflets in synthetic model membranes (Tanaka & Schroit, 1986), it seems reasonable to assume the existence, in biological membranes, of specific lipid transporters (Bretscher, 1973) that control lipid movement and maintain a particular transbilayer distribution of membrane phospholipids. Indeed, Bishop and Bell (1985) have recently presented strong evidence that such "flipases" are intimately involved in the translocation of phosphatidylcholine in microsomal membranes. In addition, Seigneuret and Devaux (1984) have reported that the transport of exogenously supplied spin-labeled PS from the outer to inner leaflet of RBC is ATP-dependent.

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[1] Abbreviations: BOC-ON, 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile; DOPC, dioleoylphosphatidylcholine; DTT, dithiothreitol; ^{125}I-N_3-PC, -PS, -PA, and -DG, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-azidophenyl)propionyl]amino]caproyl]phosphatidylcholine, -phosphatidylserine, -phosphatidic acid, and -diglyceride; ^{125}I-PE, *N*-[3-(3-[^{125}I]-iodo-4-hydroxybenzyl)propionyl]dipalmitoylphosphatidylethanolamine; LUV, large unilamellar vesicle(s); *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.4; RBC, red blood cell(s); SDS, sodium dodecyl sulfate; SN, supernatant; SUV, small unilamellar vesicle(s); *t*-Boc, *tert*-butoxycarbonyl; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid.

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In principle, it should be possible to isolate and characterize such proteins by using appropriately designed, chemically reactive phospholipid analogues. For example, the proteins involved in the intracellular sorting and transport of fluorescent phospholipid analogues (Pagano & Sleight, 1985) might be directly determined by appropriate assays for intracellular proteins bearing photoactivatable phosphatidic acid and/or its metabolic products.

In this study we present a series of new, isotopically labeled and photoactivatable phospholipid analogues that readily transfer into membranes. The rationale for the design of these compounds was the observation that certain lipids containing terminal aromatic moieties attached to the 2-position fatty acid will spontaneously transfer between populations of lipid vesicles and between vesicles and cells (Monti et al., 1977; Roseman & Thompson, 1980; Schroit & Madsen, 1983; Brunner et al., 1983). In addition, these compounds contain an azido photoactivatable group and are labeled with ^{125}I at high specific activities, which greatly facilitates their detection and quantification by nondestructive techniques as compared to other "transferable" photoactivatable lipids (Bisson & Montecucco, 1985). We demonstrate that ^{125}I-N_3-lipid analogues readily transfer between vesicles and between vesicles and cells and present our observations that ^{125}I-N_3-PS preferentially labels a M_r 30000 protein in RBC membranes following photolysis.

Experimental Procedures

Materials. DOPC, lyso-PC, *N*-NBD-PE, and *N*-Rho-PE were purchased from Avanti Polar Lipids (Birmingham, AL). BOC-ON was obtained from Pierce Chemical Co. (Rockford, IL). Na^{125}I (specific activity ~2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Phospholipase A_2 (hog pancreas) and phospholipase D (cabbage) were products of Boehringer Mannheim. Phospholipase C (*Clostridium perfringens*) was obtained from Calbiochem. TLC was carried out with heat-activated (120 °C for 1–2 h) silica gel 60 thin-layer plates from Merck. All reagents for SDS–polyacrylamide gel electrophoresis were obtained from Bio-Rad. RBC were obtained from fresh human blood collected by venipuncture into heparinized tubes. The presence of ^{125}I-labeled compounds was assessed by autoradiography using Kodak XAR-5 film. Radiation was measured by scintillation counting on a Packard auto γ counter. Fluorescence was quantified with a Farrand Mark II spectrophotofluorometer.

Chemical Synthesis. (A) Synthesis of 1-Acyl-2-(aminocaproyl)phosphatidylcholine (NH_2-PC). NH_2-PC was prepared as described previously (Schroit & Madsen, 1983) according to W. Shaw (personal communication). Briefly, the reactive amino group of aminocaproic acid was protected by *tert*-butoxycarbonylation in water/methanol/tetrahydrofuran. The blocked aminocaproic acid was then mixed with dicyclohexylcarbodiimide (Selinger & Lapidot, 1983) to yield the [(*t*-Boc)amino]caproic anhydride, which was then used to acylate lyso-PC in the presence of *N*,*N*-dimethyl-4-aminopyridine (Gupta et al., 1977). The product [[(*t*-Boc)-amino]caproyl]-PC was stored at −20 °C and deblocked with anhydrous HCl before use.

(B) Synthesis of ^{125}I-N_3-PC. The complete description of the synthesis, purification, and characterization of succinimido 3-(3-[^{125}I]iodo-4-azidophenyl)propionate ([^{125}I]AIPPS) will appear elsewhere.[2] Briefly, [^{125}I]AIPPS was prepared by radioiodination of (4-aminophenyl)propionic acid using carrier-free Na^{125}I in the presence of thallium trichloride

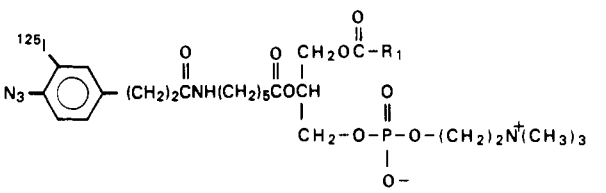

FIGURE 1: Structure of ^{125}I-N_3-PC.

(Rashidbaigi & Ruoho, 1982), followed by the formation of the azide and subsequent conversion to the *N*-hydroxysuccinimide ester. ^{125}I-N_3-PC (Figure 1) was prepared by mixing ~1 mCi of carrier-free [^{125}I]AIPPS (specific activity 2200 Ci/mmol) with 100 μg of NH_2-PC in 200 μL of $CHCl_3$/MeOH (1:2) containing 1 μL of triethylamine for 18 h at 2 °C. Five milliliters of $CHCl_3$/MeOH/H_2O (2:2:1.8) was then added, and the mixture was vigorously vortexed. After centrifugation, the lower organic phase was washed with water, and ^{125}I-N_3-PC was purified by preparative thin-layer chromatography using $CHCl_3$/MeOH/acetic acid/acetone/H_2O (5:1:1:2:0.5). The product (R_f = 0.31) was recovered as described previously (Schroit, 1982). Typical yields of ^{125}I-N_3-PC were ~40% with respect to the initial amount of ^{125}I used.

(C) Preparation of ^{125}I-N_3-PS. ^{125}I-N_3-PS was prepared from ^{125}I-N_3-PC by phospholipase D catalyzed base exchange in the presence of L-serine (Comfurius & Zwaal, 1977). The derivative was purified by sequential thin-layer chromatography using $CHCl_3$/MeOH/28% NH_4OH (65:35:5) [which did not completely separate ^{125}I-N_3-PS (R_f = 0.31) from ^{125}I-N_3-PA (R_f = 0.29)] and $CHCl_3$/MeOH/acetic acid/acetone/H_2O (5:1:1:2:0.5). Positive identification of ^{125}I-N_3-PS was accomplished by derivatization with TNBS, which resulted in its complete conversion to a faster migrating trinitrophenyl derivative [R_f = 0.8, in $CHCl_3$/MeOH)/acetic acid/acetone/H_2O (5:1:1:2:0.5), vs. R_f = 0.33 for ^{125}I-N_3-PS].

Lipid Vesicles. Small unilamellar vesicles (SUV) were prepared by ethanol injection (Kremer et al., 1977) or by ultrasonication (Huang, 1969). Briefly, ethanol-injected SUV were made by drying 0.5 mg of total lipid (DOPC with trace amounts of the indicated ^{125}I-N_3-lipid analogue and *N*-NBD-PE or *N*-Rho-PE) under a stream of dry nitrogen gas and by further drying under vacuum. The lipids were then dissolved in 167 μL of absolute ethanol and injected with stirring into 2.2 mL of PBS. LUV were prepared by the same general procedure using 5 mg of lipid in 167 μL of ethanol injected into 2.2 mL of PBS. The resulting vesicle suspensions were then dialyzed overnight against several liters of the same buffer.

Vesicle–Vesicle Exchange. Vesicle–vesicle exchange was carried out by mixing donor vesicles with a 7-fold excess (w/w) of acceptor vesicles for 30 min at room temperature. The mixed vesicle populations were then separated by chromatography on Bio-Gel A-15m or by centrifugation (Pagano et al., 1981) in a Beckman Airfuge for 90 min (~130000*g* at 20 °C).

Vesicle–RBC Incubations. Washed RBC (the buffy coat was discarded) were resuspended in the indicated vesicle suspensions [2 × 10^8 RBC/(20 μg of donor vesicles·mL)] for various periods of time at 37 °C. The cells were then washed with PBS and/or PBS containing 25% fetal calf serum and resuspended in PBS. TLC analysis of the cell-associated ^{125}I-N_3-lipid analogues extracted from RBC 6 h after vesicle treatment revealed a single radioactive component that cochromatographed with the original standard preparations (see also Figure 7).

[2] J. Lowndes, M. Hoken-Neaverson, and A. E. Ruoho, submitted for publication.

The amount of ^{125}I-N_3-lipid uptake was determined by scintillation counting. In some experiments *N*-NBD-PE was included in the donor vesicle population. To determine the amount of RBC-associated fluorescent lipid, aliquots of 2×10^7 RBC in 1 mL of PBS were lysed by the addition of 0.1 mL of 20% Triton X-100. The relative fluorescence was then compared to a standard curve of relative NBD fluorescence at 525 nm (λ_{ex} 470 nm) with known amounts of lipid in 1.1 mL of PBS/Triton X-100 containing 2×10^7 RBC.

Photolysis of RBC. RBC were incubated with the ^{125}I-N_3-lipids for 30 min at 37 °C, washed with PBS, and adjusted to 2×10^8 RBC/mL of PBS. The cells were then chilled on ice, transferred to 1-cm quartz fluorometer cuvettes, and irradiated under constant mixing for the indicated period of time 12 cm from an Osram HBO 100W/2 super-pressure mercury lamp with or without a 0–54 high-pass filter (>320 nm). The use of filtered light did not alter the results. The cells were then resuspended in PBS or in 25% serum for 30 min at 20 °C and washed with PBS. Aliquots of the cells were then used to determine the fraction of protein-bound radiation. This was done by resuspending 1 mL of the washed RBC in 3 mL of $CHCl_3$/MeOH (1:2) for 30 min on ice. The suspension was then centrifuged, and the radiation in the washed pellets and in the supernatant was determined. Partitioning of the $CHCl_3$/MeOH/PBS (1:2:1) supernatants by adding 1 mL of $CHCl_3$ and 1 mL of 0.1 N HCl showed that >90% of the radiation partitioned to the lower phase irrespective of photolysis time or the ^{125}I-N_3-lipid employed.

Effect of Thiols on ^{125}I-N_3-PC Cross-Linking to Red Blood Cells. Concentrated solutions of reduced glutathione and DTT were prepared immediately before use in degassed, N_2-purged PBS titrated to pH 7.5 with sodium hydroxide. Stock RBC were diluted to the indicated concentrations, and photolysis was carried out as described above.

Separation of Photolysis Products. (A) SDS–Polyacrylamide Gel Electrophoresis. Ghosts were prepared from the photolyzed RBC in 5 mM phosphate buffer (Steck & Kant, 1974) and solubilized in SDS electrophoresis sample buffer (Laemmli, 1970). Electrophoresis was carried out on a 10% acrylamide separating gel with a 6% acrylamide stacking gel by using the discontinuous buffer system of Laemmli (1970). A constant current of 50 mA per slab was applied until the bromophenol blue tracking dye was about 1.5 cm from the bottom of the gel. The gels were then stained with Coomassie blue R-250, dried, autoradiographed, and cut into 2-mm slices for direct scintillation counting.

(B) Thin-Layer Chromatography. After photolysis, the reaction mixtures were extracted as described above and an aliquot of the organic phase was chromatographed on TLC plates in $CHCl_3$/MeOH/28% NH_4OH (65:35:5). The distribution of radiation was determined by autoradiography.

Results

Degradation of ^{125}I-N_3-PC by Phospholipases and Base-Catalyzed Transesterification. To verify the labeling specificity of [^{125}I]AIPPS to 1-acyl-2-(aminocaproyl)-PC (see Experimental Procedures), the purified product was subjected to hydrolysis with phospholipases (Kates, 1972). Figure 2 shows a thin-layer autoradiogram of the three reaction mixtures. It can be seen that the ^{125}I-N_3-PC was completely degraded to its expected products of ^{125}I-N_3-PA, ^{125}I-N_3-aminocaproic acid, and ^{125}I-N_3-diglyceride by phospholipase D, A_2, and C, respectively. In addition, only minor amounts of radiation (<10%) could be detected in the water-soluble, phosphocholine-containing fraction obtained by hydrolysis with phospholipase C.

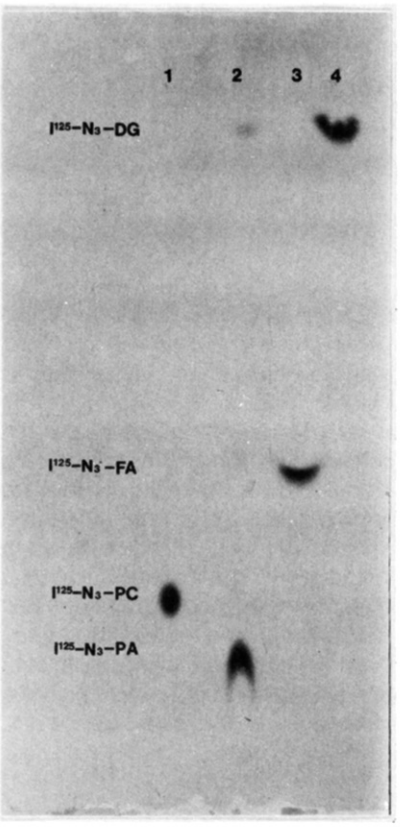

FIGURE 2: Thin-layer autoradiograph of ^{125}I-N_3-PC (lane 1) hydrolyzed with phospholipase D (^{125}I-N_3-PA; lane 2), phospholipase A_2 (^{125}I-N_3-fatty acid; lane 3), and phospholipase C (^{125}I-N_3-diglyceride; lane 4) chromatographed in $CHCl_3$/MeOH/28% NH_4OH (65:35:5).

Table I: Products Obtained by Phospholipase D Catalyzed Base Exchange of ^{125}I-N_3-PC in the Presence of L-Serine

^{125}I-N_3 product	yield[a] (%)	R_f[b]	^{125}I-N_3 product	yield[a] (%)	R_f[b]
DG	29	0.78	PS	16	0.31
unidentified	16	0.63	PA	17	0.26
PC	23	0.39			

[a] Yields are based on the total amounts of radiation recovered from the TLC plates from a representative experiment. [b] In $CHCl_3$/MeOH/28% NH_4OH (65:35:5).

Incubation of ^{125}I-N_3-PC with phospholipase D in the presence of L-serine (Comfurius & Zwaal, 1977) produced acceptable yields of ^{125}I-N_3-PS, although the major byproduct was diglyceride and not phosphatidic acid (Table I). The reason for the production of such large amounts of ^{125}I-N_3-diglyceride is not known but may be related to our previous observations on the transesterification of other ^{125}I-labeled lipids containing *N*-(phenylpropionyl)aminocaproyl side chains (Schroit & Madsen, 1983).

Transfer of ^{125}I-N_3-lipids between Vesicles. To determine whether the ^{125}I- and N_3-labeled lipids possess transfer properties similar to other "aromatic side chain" containing phospholipid analogues [i.e., acyl-chain NBD labeled (Struck & Pagano, 1980) and phenylpropionyl-labeled (Schroit & Madsen, 1983) lipids], vesicles containing ^{125}I-N_3-PC were formed, and the ability of the lipid to transfer to acceptor vesicles was determined (Figure 3 and Table II). Although separation and quantitative recovery of the LUV and SUV (labeled with nonexchangeable *N*-Rho-PE and *N*-NBD-PE, respectively) populations were readily achieved by column chromatography on Bio-Gel A-15m (Figure 3), recovery of the ^{125}I-N_3-lipids was poor due to adsorption of ~20% of the

Table II: Transfer of ^{125}I-N_3-lipids between SUV Donor and LUV Acceptor Vesicle Populations[a]

donor vesicles	^{125}I-N_3-lipid		SUV donors[b]		LUV acceptors[b]		SUV donor[c] (^{125}I-N_3-lipid/ μg of SUV)	lipid transferred[d] (%)
	total (cpm)	SN (cpm)	total (μg)	SN (μg)	total (μg)	SN (μg)		
^{125}I-N_3-PC + acceptors	12037	8234	66.2	65.0	413.6	142.4	95.9	48.0
^{125}I-N_3-PC alone	11629	11105	63.0	60.2	0	1.7	184.5	
^{125}I-N_3-PS + acceptors	2332	1679	62.7	60.6	420.4	159.7	21.1	43.6
^{125}I-N_3-PS alone	2091	2174	57.7	58.2	1.6	0.3	37.4	
acceptors alone	0	0	0.1	0	417.0	165.4		

[a] SUV donor vesicles containing ^{125}I-N_3-lipid/*N*-Rho-PE/DOPC (trace:1:99) were prepared as described (see Experimental Procedures) and incubated for 30 min at 30 °C with a 6.7-fold excess (w/w) of DOPC/DOPE/*N*-NBD-PE (50:50:1) acceptor LUV. The vesicles were then separated by centrifugation at 130000*g* for 90 min. The extent of ^{125}I-N_3-lipid transferred between the SUV and LUV was determined by the amount of ^{125}I in each of the populations as assessed by the relative rhodamine (SUV) and NBD (LUV) fluorescence remaining in the supernatants. [b] The amounts of SUV and LUV were determined by measuring the relative fluorescence of rhodamine (λ_{ex} 568 nm; λ_{em} 589 nm) and NBD (λ_{ex} 468 nm; λ_{em} 530 nm) in the mixed populations and in the supernatants after centrifugation. [c] The ratio of ^{125}I-N_3-lipid/μg of donor SUV was calculated by using the following formula: total cpm added − {[(cpm pelleted × μg of LUV added)/μg of LUV pelleted]/μg of SUV added}, where the cpm and μg of LUV precipitated = (total cpm − SN cpm) and (total LUV − SN LUV), respectively. [d] The percent lipid transferred = 100 × (1 − fraction of ^{125}I-N_3-lipid/μg of SUV) in the mixed vesicle population and control SUV population, respectively.

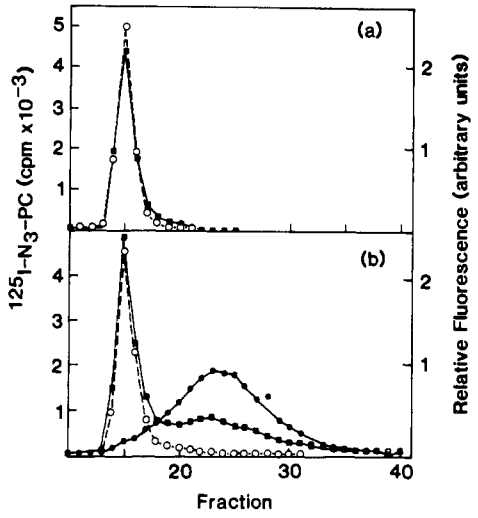

FIGURE 3: Vesicle–vesicle transfer of ^{125}I-N_3-PC between large (LUV) and small (SUV) vesicle populations. LUV were formed from DOPC/*N*-Rho-PE/^{125}I-N_3-PC (99:1:trace) and SUV from DOPC/*N*-NBD-PE (99:1). Bio-Gel A-15m chromatography of LUV alone (a) and a mixture of LUV and SUV (1/7 w/w) after a 30-min incubation at 20 °C (b) is shown. (■) ^{125}I-N_3-PC; (○) *N*-Rho-PE; (●) *N*-NBD-PE.

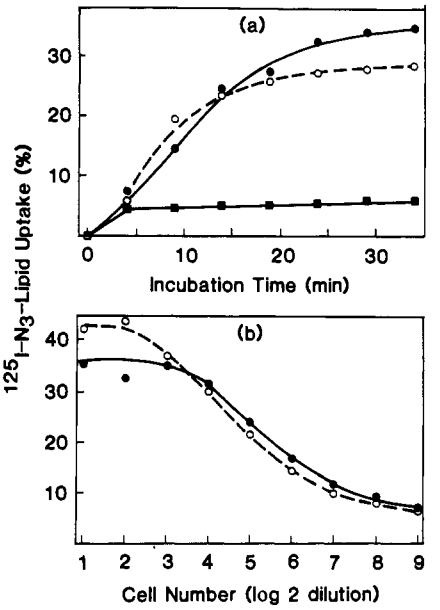

FIGURE 4: Uptake of ^{125}I-N_3-PC and ^{125}I-N_3-PS by RBC. (a) Donor vesicles containing DOPC/*N*-NBD-PE/indicated ^{125}I-N_3-lipid analogue (99:1:trace) were incubated with RBC at 37 °C [20 μg of lipid/(2 × 10^8 RBC·mL)]. At appropriate intervals aliquots were removed and washed by centrifugation at 2 °C, and the amount of cell-associated ^{125}I was determined by scintillation counting. (b) Various dilutions (2-fold) of RBC (from 2 × 10^9 RBC/mL) were incubated with a constant amount of donor vesicles for 30 min and washed, and the amounts of cell-associated ^{125}I and fluorescence were determined. (○) ^{125}I-N_3-PC; (●) ^{125}I-N_3-PS; (■) *N*-NBD-PE.

lipid to the gel. The results obtained, however, did show that ~30–40% of the total recoverable ^{125}I did redistribute to the SUV acceptor population, indicating that these lipids are transferable.

In order to better quantify lipid transfer, mixed LUV acceptor (*N*-NBD-labeled) and SUV donor (*N*-Rho-labeled) populations were separated by centrifugation. As can be seen in Table II, when donor SUV containing ^{125}I-N_3-PC or ^{125}I-N_3-PS were mixed with acceptor LUV, virtually all of the donor SUV remained in the supernatant, whereas only ~70% of the LUV precipitated. After correction for nonprecipitable LUV in the supernatant (assuming that the ^{125}I-N_3-lipid distribution between the various LUV subpopulations at equilibrium is constant; see Figure 3 legend), the ratio of ^{125}I-N_3-lipid/μg of SUV was approximately half that of the initial SUV preparation, indicating that 48% of the ^{125}I-N_3-PC and 44% of the ^{125}I-N_3-PS transferred from the SUV to the LUV acceptor population (Table II). These results suggest that most of the ^{125}I-N_3-lipid analogues were removed from the external leaflets of the vesicle bilayers and that the remaining radiation probably represents lipid confined to the vesicles' inner leaflets.

Transfer of ^{125}I-N_3-lipids from Vesicles to Cells. When RBC were incubated with DOPC vesicles containing *N*-NBD-PE and ^{125}I-N_3-lipids, only the iodinated analogues were transferred to the cells in significant amounts (Figure 4). The fraction of the probes partitioning into the RBC membranes was determined from the fraction of radiation pelleting with the cells upon centrifugation. It can be seen that increasing the incubation time or increasing the number of cells increased the amount of ^{125}I incorporated into the RBC. Uptake was rapid and equilibrated after 20 min (Figure 4a), with ~40% of the total ^{125}I added becoming cell associated (Figure 4b). In contrast to these large amounts of ^{125}I-N_3-lipids, the amount of *N*-NBD-PE that became cell associated was low and remained relatively constant over the time course of the experiment (Figure 4a). In addition, the ratio of ^{125}I/*N*-NBD-PE (cpm/fluorescence) in the donor vesicle population decreased ~5–10-fold in the washed vesicle treated RBC. Since *N*-NBD-PE is a "nontransferable" lipid (Struck & Pagano, 1980), these results strongly suggest that the uptake of ^{125}I-N_3-lipid analogues by RBC was due primarily to lipid transfer

Table III: Uptake and Cross-Linking of ^{125}I-N_3-lipids with Red Blood Cells[a]

^{125}I-N_3-lipid	lipid added[b] (cpm)	lipid uptake[b] cpm	lipid uptake[b] %	lipid remaining after serum back-exchange, irradiation time: 0 min cpm	0 min %[c]	0 min % C/M soluble[d]	3 min cpm	3 min %[c]	3 min % C/M soluble[d]
^{125}I-N_3-PC (reduced[e])	7567	1999	26.4	64	3.2		102	5.1	
^{125}I-N_3-PC	6408	2508	39.1	220	8.8	86.1	1150	47.8	47.8
^{125}I-N_3-PS	3382	1394	41.1	1150	82.5	97.1	1195	85.7	79.0

[a] RBC were mixed with the indicated vesicle suspension for 30 min at 37 °C [2 × 10^8 RBC/(20 μg of SUV·mL)]. The cells were then washed twice in ice-cold PBS and irradiated for 3 min. The RBC were then resuspended in 25% serum, spun, and washed again in PBS. The amount of ^{125}I-N_3-lipids in the RBC was monitored in each step by scintillation counting. [b] cpm/2 × 10^7 RBC. [c] Percentage of lipid uptake (determined after PBS wash) remaining after back-exchange with 25% serum. [d] Percentage of ^{125}I-N_3-lipids remaining after serum back-exchange that was $CHCl_3$/MeOH (1:2) soluble. [e] The azide groups were inactivated by incubating the lipid analogues in 10 mM DTT/0.1% Triton X-100 for 30 min at 20 °C. The reduced product (one spot on TLC) was extracted, purified by TLC, and incorporated into the donor liposomes as were the other lipid analogues.

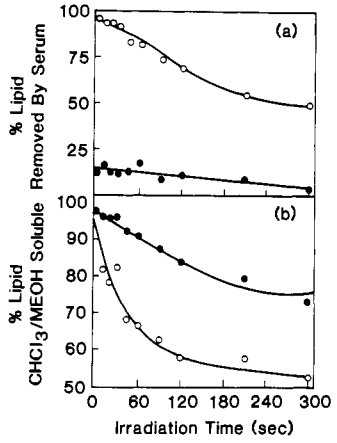

FIGURE 5: Effect of irradiation time on immobilization of ^{125}I-N_3-PC and ^{125}I-N_3-PS in RBC. RBC were incubated with donor vesicles containing ^{125}I-N_3-PC (○) or ^{125}I-N_3-PS (●) [20 μg of lipid/(2 × 10^8 RBC·mL)] for 30 min at 37 °C. The cells were then washed with PBS, irradiated for the indicated times, and washed again with PBS or with 25% serum in PBS. Aliquots of the serum-washed cells were then suspended in $CHCl_3$/MeOH (2:1), and the fraction of $CHCl_3$/MeOH-soluble lipids was determined by counting aliquots of the total suspension and aliquots of the supernatants after centrifugation. (a) Fraction of total cell-associated radiation (determined in the PBS-washed cells) removed by serum. (b) Fraction of the residual (after serum wash) total cell-associated radiation soluble in $CHCl_3$/MeOH.

Table IV: Effect of Thiols on ^{125}I-N_3-PC Cross-Linking to Red Blood Cells[a]

thiol	lipid uptake[b] (cpm)	lipid remaining after serum back-exchange[c]: cpm	%	% C/M soluble[d]
PBS control	5888	3022	51.6	63.9
glutathione				
30 mM	5236	2229	41.9	65.8
20 mM	5042	2315	46.5	62.5
15 mM	5137	2426	46.9	62.4
10 mM	5263	2522	45.9	63.7
5 mM	5187	2676	52.8	61.3
DTT				
30 mM	5717	887	15.6	69.5
20 mM	5251	995	18.5	71.7
15 mM	5297	1278	24.0	71.4
10 mM	5459	1375	24.3	63.9
5 mM	5366	1704	31.7	57.9

[a] RBC were treated with vesicles as described in Table III. After washing, the cells were mixed with appropriate concentrations of thiols, incubated for 5 min, and then irradiated for 3 min. The RBC were then resuspended in PBS or 25% serum, spun, and washed again in PBS. The amount of ^{125}I-N_3-PC in the RBC was monitored in each step by scintillation counting. [b] Lipid uptake is defined as the residual radiation in irradiated RBC remaining after a PBS wash. (The actual counts per minute were essentially identical to control nonirradiated cells.) [c] Percentage of lipid remaining was determined after back-exchange with 25% serum. [d] Percentage of ^{125}I-N_3-lipids remaining after serum back-exchange that were $CHCl_3$/MeOH (1:2) soluble.

and not to fusion or binding of intact vesicles to the RBC.

Photoactivation and Covalent Attachment of ^{125}I-N_3-lipids to RBC. RBC were incubated with DOPC donor vesicles containing ^{125}I-N_3-PC or ^{125}I-N_3-PS for 30 min at 37 °C, washed, and subsequently irradiated for various times. The relationship between irradiation time and the inability to remove the probes from the cells by serum-mediated "back-exchange" is shown in Figure 5 and Table III. Increasing the duration of irradiation was accompanied by a concomitant decrease in the fraction of cell-associated ^{125}I-N_3-PC that could be removed by serum (Figure 5a). In contrast, most of the ^{125}I-N_3-PS (>80%) could not be removed from the cells irrespective of irradiation (see Discussion). To determine the fraction of incorporated probe covalently attached to membrane proteins, irradiated RBC were washed with serum and the fraction of the residual (after serum treatment), cell-associated ^{125}I-N_3-lipid soluble in $CHCl_3$/MeOH (1:2) was determined. The results shown in Figure 5b indicate that increasing irradiation time resulted in decreased amounts of $CHCl_3$/MeOH-soluble ^{125}I-N_3-lipids. However, the relative amounts of the cell-associated ^{125}I-N_3-PC and ^{125}I-N_3-PS that became covalently attached to proteins ($CHCl_3$/MeOH-insoluble lipids) were dramatically different with the two probes. About 50% of the ^{125}I-N_3-PC and only ~20% of the ^{125}I-N_3-PS present in the cells after a 3–5-min irradiation time precipitated during extraction with $CHCl_3$/MeOH (Figure 5b and Table III).

To test for the labeling specificity of the probes, analogous experiments were performed by using ^{125}I-N_3-PC treated with DTT, which reduces the photoreactive azide group (see Table III, footnote *e*). The results summarized in Table III show that reduced ^{125}I-N_3-PC transferred to the RBC as well as did the nonreduced lipids. Unlike the nonreduced probes, all of the reduced ^{125}I-N_3-PC that transferred to the RBC could be removed by serum treatment irrespective of irradiation time. This strongly suggests that photolabeling was the result of nitrene-mediated reactions and not due to incorporation of free ^{125}I.

Effect of Thiols on Covalent Attachment of ^{125}I-N_3-PC to RBC. Due to the electrophilic character of photogenerated phenylnitrenes and their long lifetimes, it is possible that these probes label proteins outside the bilayer. Indeed, it has been shown by Bayley and Knowles (1978, 1980) that photogenerated phenylnitrenes can label hydrophobic and hydrophilic domains of membrane proteins as well as exogenously adsorbed proteins (e.g., lysozyme) possibly via water-soluble, free-radical intermediates. Since phenylnitrenes can be scavenged by thiols (Bayley & Knowles, 1978), we performed the experiments described above in the presence of reduced glutathione and DTT to determine the extent to which labeling occurred via

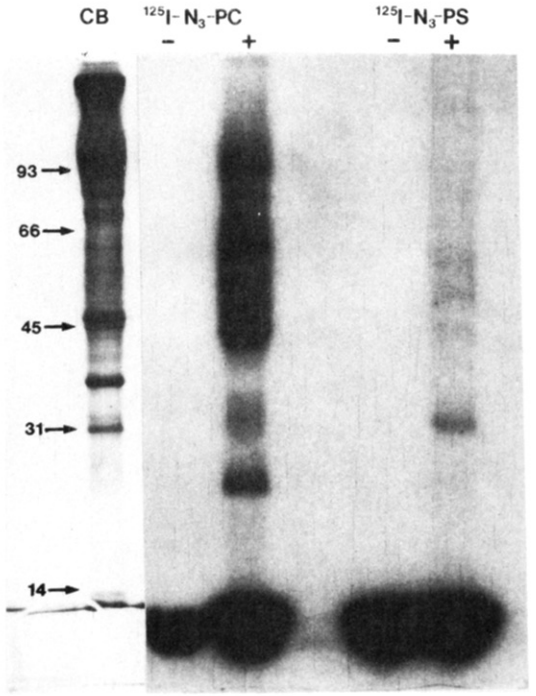

FIGURE 6: Electrophoretic distribution of ^{125}I-N_3-lipids in photoaffinity-labeled RBC. Ghosts were prepared from vesicle-treated, irradiated, and serum-washed RBC (see Table III, footnote *a*). The ghosts were solubilized in SDS/mercaptoethanol, and $\sim 10^8$ RBC equivalents were applied to each lane. The gels were fixed, stained with Coomassie blue (CB), and subsequently autoradiographed. (−) Nonirradiated (control) RBC; (+) RBC irradiated for 3 min.

Table V: Distribution of ^{125}I-N_3-lipids in Electrophoretically Separated RBC Proteins

$10^{-3}M_r$[a]	^{125}I-N_3-PC (%)[b]	^{125}I-N_3-PS (%)[b]	$10^{-3}M_r$[a]	^{125}I-N_3-PC (%)[b]	^{125}I-N_3-PS (%)[b]
100–80	22.8	11.7	30 (7)	8.4	30.2
73–53	43.9	44.7	22	9.2	
48 (5)	15.7	13.7			

[a] Colocalizing bands in parentheses. Nomenclature based on the classification by Steck (1974). [b] The amount of label present in each fraction is the percentage of the total protein-bound label (label not running at the lipid front) determined by densitometry and verified by scintillation counting of cut gels (2-mm slices).

the aqueous phase (Table IV). The amount of residual cell-associated lipid (i.e., label not removed by serum) was only marginally altered at the highest concentration of glutathione (30 mM), whereas DTT inhibited labeling in a concentration-dependent fashion. Interestingly, the fraction of chloroform/methanol-soluble lipid recovered after photolysis was unchanged irrespective of the presence of thiols.

SDS–Polyacrylamide Gel Electrophoresis. To determine which RBC proteins become labeled with the ^{125}I-N_3-lipids, ghosts were prepared from irradiated, serum-washed RBC, and the distribution of the RBC proteins (Coomassie blue staining) and that of the covalently bound probes (autoradiography) were analyzed by SDS gel electrophoresis. Figure 6 shows the electrophoretic profiles of Coomassie blue stained and comigrating ^{125}I-labeled products. Approximately half of the ^{125}I-N_3-PC-derived radiation and 85% of the ^{125}I-N_3-PS-derived radiation migrated at the gel dye front, probably representing labeled lipid products (see below). Besides the relatively low coupling efficiency of ^{125}I-N_3-PS, other major differences in the labeling patterns of the probes were observed. As can be seen from the autoradiographs (Figure 6) and the estimated distribution of radiation (compiled in Table V), the majority of the radiation (~40%) was found in the M_r 73 000–53 000 fraction. Although probe distribution could not be assigned to any distinct RBC bands in this region, both probes appeared to migrate with band 5, whereas ^{125}I-N_3-PS appeared to preferentially comigrate with band 7, with ~30% of the radiation being present in the M_r 30 000 fraction.

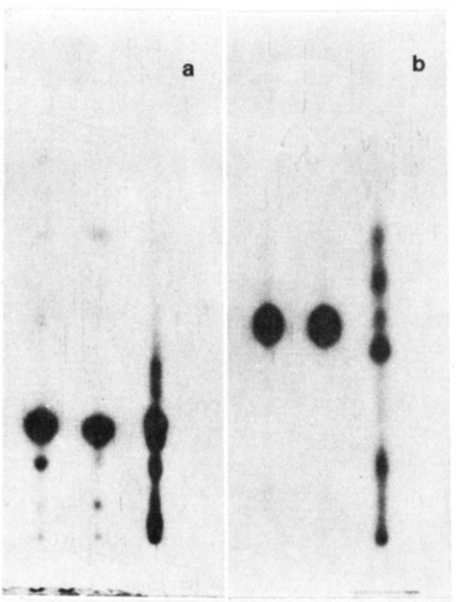

FIGURE 7: Thin-layer chromatography of $CHCl_3$/MeOH-soluble ^{125}I-N_3-lipids extracted from RBC. RBC were treated with ^{125}I-N_3-lipids and processed as described in Table III. The $CHCl_3$/MeOH-soluble ^{125}I-N_3-PS (a) and ^{125}I-N_3-PC (b) were partitioned, and the lower organic phase was removed, dried under N_2, resuspended in a small amount of $CHCl_3$, and spotted (~5000 cpm/sample). Lanes from left to right: ^{125}I-N_3-lipid standard (lane 1), extracted (nonirradiated and non-serum-treated) control RBC (lane 2), and RBC irradiated (and serum treated) for 3 min (lane 3).

Thin-Layer Chromatography of Extracted, Photolyzed RBC. In an attempt to determine whether photolyzed lipid products were formed, the organic phase from partitioned $CHCl_3$/MeOH-soluble RBC extracts was chromatographed on thin-layer plates, and the distribution of radiation was determined by autoradiography. The results presented in Figure 7 show that approximately half of the ^{125}I-N_3-PS migrated as slower moving products, whereas the majority of the ^{125}I-N_3-PC ran slower than the corresponding standard ^{125}I-labeled lipids. Since virtually all of the non-cross-linked PC analogue was removed from the intact red cells by washing with serum, it seems reasonable that all of the residual cell-associated radiation be associated with RBC membrane components. In the case of the PS analogue, which, irrespective of radiation, cannot be removed from the cells, a substantial fraction of the probe remained non-cross-linked. However, due to the uncertainty of whether the radiation represents ^{125}I-N_3-lipids cross-linked to endogenous phospholipids (thereby possibly conferring an altered mobility) or unbound photolyzed byproducts, it is difficult to estimate the distribution of the probes among the various lipid species.

Discussion

In this paper, we have described an efficient method for the synthesis of transferable, radioiodinated, and photoactivatable phospholipid analogues. The technique involves radioiodination of (4-aminophenyl)propionic acid using carrier-free Na^{125}I in the presence of thallium trichloride (Rashidbaigi & Ruoho, 1982), followed by the formation of the azide and subsequent conversion to the *N*-hydroxysuccinimide ester. The activated ester is then reacted with phosphatidylcholine containing a terminal primary amine in the 2-position fatty acid as de-

scribed for phospholipid alkylation with Bolton–Hunter reagent (Schroit & Madsen, 1983). The product ^{125}I-N_3-PC is obtained in high yield (~40% of maximum based on the amount of ^{125}I) with the high specific activity of Na^{125}I (~2200 Ci/mmol). Through the use of phospholipase D catalyzed base exchange, acceptable yields of the PS analogue (and the PA analogue as a byproduct) were obtained. In addition, the PC analogue also serves as a precursor for the synthesis of the ^{125}I-N_3-phosphatidylethanolamine and -phosphatidylglycerol analogues with phospholipase D in the presence of appropriate bases (unpublished observations). Positive identification of ^{125}I-N_3-PC was obtained by hydrolysis of the product with phospholipase A_2 and phospholipase C, which resulted in its complete conversion to the corresponding ^{125}I-N_3-fatty acid and ^{125}I-N_3-diglyceride, respectively. Although conversion of ^{125}I-N_3-PC to ^{125}I-N_3-PS with phospholipase D was straightforward, relative large amounts of diglyceride were formed (Table I), a result similar to our previous observations on the preparation of ^{125}I-labeled PS (Schroit & Madsen, 1983).

Both lipid analogues could be completely incorporated into synthetic bilayer membranes and efficiently transferred to acceptor vesicle populations. The maximum amount of lipid that could be removed from the donor vesicles in the presence of excess acceptor membranes was ~40% of the total amount of lipid analogue present (Tables II and III), strongly suggesting that only those molecules residing in the outer leaflet were transferred. In addition, removal of the analogues from donor vesicles or from RBC was absolutely dependent on the presence of acceptor membranes (or serum), and no radiation could be detected in the supernatants of centrifuged LUV or RBC.

Previous work from this laboratory has shown that once NBD-PS (acyl chain labeled) is transferred to RBC, it can no longer be removed from the cells, in contrast to other similarly labeled lipids such as NBD-PC and NBD-PG (Schroit et al., 1985). The reason for the stability of these fluorescent PS analogues in RBC membranes is not known. It may be attributed, in part, to an energy-dependent translocation to the cells' inner leaflet (Seigneuret & Devaux, 1984) and/or to an apparent specific immobilization of the lipid in the cells' outer leaflet (Tanaka & Schroit, 1986). The observation that the transfer of ^{125}I-N_3-PC but not ^{125}I-N_3-PS to RBC is reversible (Figure 5 and Table III) does suggest that both the fluorescent and photoactivatable probes localize in an analogous manner in RBC membranes. Thus, photoaffinity labeling of the latter may provide some indication of which moieties might be responsible for the stability or transport of PS in RBC membranes.

Irradiation of RBC resulted in a substantial increase in the fraction of irreversibly bound ^{125}I-N_3-PC. This increase could not be detected, however, in the case of ^{125}I-N_3-PS since it was a priori irreversibly bound irrespective of irradiation. Indeed, the only indication that ^{125}I-N_3-PS was covalently coupled to the RBC membrane was from the observation of a concomitant increase in the fraction of protein-bound probe (decreased solubility in organic solvent). This was probably due to specific labeling and not to free iodide since photolysis of RBC that contained reduced ^{125}I-N_3-PC did not produce significant amounts of irreversibly bound ^{125}I (Table III). In addition, it should be noted that irradiation per se did not result in detectable alterations in the polyacrylamide profiles of the RBC proteins (for up to 5 min), nor did it lyse the RBC as demonstrated by the lack of ^{51}Cr release into the supernatants of spun, ^{51}Cr-labeled RBC (results not shown).

The distribution of radiolabel among the components of labeled RBC is somewhat heterogeneous as are the distributions obtained after photolysis with other hydrophobic probes such as azidobenzene (Wells & Findlay, 1979) and adamantanediazirine (Bayley & Knowles, 1980). Although this observation could be interpreted as being due to cross-linking that occurred at the membrane surface [as opposed to within the bilayer (Bayley & Knowles, 1978)], the inability to inhibit labeling with glutathione (Table IV) suggests that the probe did label the membrane from within the bilayer. Therefore, it would seem that the inhibition of labeling obtained with DTT might be a result of its partitioning within the bilayer since DTT is freely soluble in a variety of organic solvents whereas glutathione is not. These observations are further supported by the results of Malatesta et al. (1983), who have shown that similarly labeled phosphatidylcholine selectively labels cytochrome *c* oxidase in its hydrophobic membrane-spanning stretch.

There were several significant differences in the relative amounts and distribution of ^{125}I-N_3-PC and ^{125}I-N_3-PS that became cross-linked to proteins and lipids. First, since apparently all of the noncovalently bound ^{125}I-N_3-PC was removed by pretreatment with serum, only very small amounts of the initial material could be detected by TLC (Figure 7). On the other hand, a significant amount of what appeared to be ^{125}I-N_3-PS was detectable after photolysis by TLC (Figure 7). We assume, therefore, that all the noncovalently bound ^{125}I-N_3-PC was removed by serum, whereas much of the ^{125}I-N_3-PS remained. This is because the noncovalently bound material could not be removed by serum pretreatment (Figure 5) and is probably due to the fact that nitrenes generated by azido groups inefficiently label saturated fatty acids (Gupta et al., 1979; Brunner & Richards, 1980). There were also marked differences in the distribution of the probes on polyacrylamide gels. Particularly notable was the apparent preferential labeling of a M_r ~30000 peptide by ^{125}I-N_3-PS, which appeared to comigrate with integral membrane protein band 7 (Steck, 1974). At the present time, however, we have no indication of whether the radiation is bound to that protein or is comigrating with an unidentified minor species.

In summary, we have described a general method for the preparation of iodinated, photoactivatable and transferable phospholipid analogues. Since these lipids are strong γ emitters and are unaffected by quenching phenomena commonly encountered with the use of other labels, they can easily be detected by nondestructive techniques. They readily transfer into cells without any apparent mass vesicle adsorption or fusion, and therefore they should prove to be useful in studies of intracellular lipid trafficking/sorting and possibly provide a tool for identification of those proteins that might be involved in specific lipid transport processes.

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