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Photogenerated Reagents for Membranes: Selective Labeling of Intrinsic Membrane Proteins in the Human Erythrocyte Membrane†

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ABSTRACT: 1-[^{3}H]Spiro[adamantane-4,4′-diazirine], a lipophilic, photoactivatable reagent designed to label those segments of intrinsic proteins that lie within the lipid bilayer of biological membranes, has been evaluated. The reagent labels the intrinsic proteins of human erythrocyte membranes far more heavily than it labels the extrinsic proteins. This result, together with a detailed analysis of the label distribution in several well-characterized membrane proteins [Goldman, D. W., Pober, J. S., White, J., & Bayley, H. (1979) *Nature (London) 280*, 841], demonstrates that labeling with adamantanediazirine is a convenient and rapid method both for distinguishing intrinsic from extrinsic membrane proteins and for locating within intrinsic proteins those amino acid residues that are in contact with the hydrocarbon core of the lipid bilayer.

Those segments of intrinsic membrane proteins that are in close association with the hydrocarbon core of a lipid bilayer may in principle be identified by using hydrophobic, photogenerated reagents [e.g., Klip & Gitler (1974), Bercovici et al. (1978), and Bayley & Knowles (1978a,b); for related approaches, see Chakrabarti & Khorana (1975), Radhakrishnan et al. (1980), and Wisnieski & Bramhall (1979)]. The chemically inert reagent binds to the hydrocarbon core of the bilayer where it is subsequently activated by irradiation. The highly reactive photogenerated intermediate then reacts with the membrane constituents that lie within the bilayer. Since the amino acid residues that are exposed to the hydrocarbon phase are predominantly hydrophobic and chemically inert [e.g., Tomita et al. (1978), Frank et al. (1978), and Gerber et al. (1979)], only the most reactive reagents will derivatize them. For this reason we have chosen to use carbene precursors rather than aryl nitrene precursors as lipophilic reagents. We have previously demonstrated that phenyl nitrene does not react efficiently with the saturated fatty acyl chains in phospholipid vesicles, whereas the isosteric, isoelectronic phenyl carbene does so react [Bayley & Knowles (1978a,b); see also Gupta et al. (1979)]. We report here the synthesis and characterization of a lipophilic carbene precursor, [^{3}H]adamantanediazirine, of high specific radioactivity and demonstrate that this reagent can be used to label those segments of intrinsic membrane proteins that are in contact with the hydrocarbon core of the lipid bilayer.

Experimental Section

Chemical Synthesis

Adamantanone was from Aldrich. HNO_3 (100%) was prepared by distilling 90% HNO_3 from an equal volume of H_2SO_4. Hydroxylamine-*O*-sulfonic acid was prepared by the method of Matsuguma & Audrieth (1957), stored under vacuum over P_2O_5, and titrated before use. Palladium (10%) on charcoal was from Alfa. Na^{125}I was from New England Nuclear and [*ring*-G-^{3}H]aniline was from the Amersham Corp.

Thin-layer chromatography (TLC) was performed on silica gel 60 plates from Merck. Aluminium-backed plates were used for radiochemical analyses. The chromatogram was cut into small pieces which were counted in scintillation fluid (7 mL) made up from toluene (1 gal) and Liquifluor (New England Nuclear; 160 mL). Ethanol (1 mL) was added to each vial. Absolute radioactivity (dpm) based on a [^{3}H]hexadecane standard was measured in the same fluid without the addition of ethanol. The efficiency was 35.7%. A Beckman LS-233 scintillation counter was used for all radioactivity measurements. TLC systems were (A) petroleum ether (bp 40–60 °C)–ethanol (4:3 v/v), (B) petroleum ether–diethyl ether (3:1 v/v), and (C) hexane. Gas chromatography (GLC) was performed on a Varian Aerograph Series 1400 instrument, fitted with a flame ionization detector, using N_2 as the carrier gas. Glass columns were packed with 3% OV1 on Anakrom (Analabs). Proton magnetic resonance (^{1}H NMR) spectra were recorded on a Varian HFT 80 instrument in $CDCl_3$. Chemical shifts are reported as δ values relative to internal $(CH_3)_4Si$. Infrared (IR) spectra were recorded on a Perkin-Elmer 457A spectrometer, ultraviolet–visible absorption

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spectra were recorded on a Perkin-Elmer 575 instrument, and mass spectra were recorded on an AEI MS9 spectrometer. Microanalyses were carried out at Galbraith Laboratories, Knoxville, TN.

The synthetic route to 1-[^{3}H]spiro[adamantane-4,4′-diazirine] (VIII*) is shown in Figure 1.

1-Hydroxyadamantan-4-one (III). Adamantanone (I) was converted to the hydroxy ketone III according to Geluk (1972).

1-Bromoadamantan-4-one (IV) was prepared from III as described by Geluk & Schlatmann (1968). The crude bromo ketone was sublimed at 80–100 °C, at aspirator pressure, to give colorless crystals: GLC t_R(118 °C) 5.4 min, t_R(140 °C) 2.55 min; TLC (system A) R_f 0.7.

1-Bromoadamantan-4-one Ethylene Ketal (V). The bromo ketone IV (3.06 g) was dissolved in benzene (100 mL) containing ethylene glycol (0.95 mL) and toluene-*O*-sulfonic acid (monohydrate; ~1 mg). The mixture was refluxed for 5 h, and more ethylene glycol (0.25 mL) was then added. After being refluxed for a further 9 h, the solution was cooled and then washed with 5% Na_2CO_3 (2 × 60 mL) and water (2 × 60 mL). The organic layer was dried over K_2CO_3 and the solvent was removed by evaporation. The product was taken up in benzene, which was removed by evaporation, to yield a clear oil which crystallized on standing (3.48 g; 95% yield): GLC t_R(118 °C) 17.4 min, t_R(140 °C) 7.45 min; ^{1}H NMR δ 1.7–2.7 (br m), 3.93 (sp s, ketal protons).

Adamantanone Ethylene Ketal (VI). To a mixture of 10% Pd/C (220 mg) and anhydrous K_2CO_3 (1.06 g) under N_2 was added ethanol (30 mL), followed by the bromo ketal V (208 mg) in ethanol (5 mL). The flask was then evacuated and filled with H_2. The atmosphere of H_2 was maintained with a hydrogen-filled balloon and the reaction mixture was vigorously stirred. After 20 h at room temperature the reaction was complete, and the catalyst was removed by filtration under N_2. The solvent was carefully evaporated and the residue was extracted into benzene. The undissolved solid (KBr) was removed by filtration. Evaporation of the filtrate gave colorless crystals of the ketal (yield 88–94%): GLC t_R(118 °C) 4.6 min. The ^{1}H NMR spectrum was identical with that of ketal prepared directly from adamantanone: δ 1.55–2.22 (complex absorption, maximum at 1.74, protons on the adamantane nucleus), 3.93 (s, ketal protons). Deprotection (see below) yielded the ketone in 96% yield: GLC t_R(118 °C) 1.9 min; ^{1}H NMR δ 1.96 (br m, 12 H), 2.46 (br s, 2 H).

Spiro[adamantane-2,2′-diaziridine] (VII). The procedure of Schmitz & Ohme (1961) was followed [cf. Isaev et al. (1973)]. Adamantanone (6.3 g) was stirred with NH_3 (25% w/v) in CH_3OH for 45 min at −10 °C. A freshly prepared solution of hydroxylamine-*O*-sulfonic acid (5.7 g; 85% purity) in CH_3OH (50 mL) was added dropwise. The mixture was stirred for 18 h at 4 °C and the solvent was then removed by evaporation. The solid residue was triturated with CH_2Cl_2 (2 × 100 mL). The combined CH_2Cl_2 extracts were then extracted with 2 N H_2SO_4 at 4 °C (150 and 100 mL). The aqueous extracts were made alkaline with 2 N NaOH at 4 °C, and the product was extracted back into CH_2Cl_2. The combined extracts were dried over K_2CO_3, filtered, and evaporated to give the diaziridine (5.6 g; 81%): ^{1}H NMR δ 1.24 (br s), 1.88 (br m), 2.48 (br s), 3.51 (br s). On the addition of CF_3COOH, the absorption at 1.24 disappears and the following spectrum is obtained: δ 1.6–2.4 (br m, ~12 H), 2.93 (br s, 1 H), 3.52 (br s, 1 H).

Spiro[adamantane-2,2′-diazirine] (VIII). A modification of method b of Isaev et al. (1973) was used. All operations were done under dim light. Diaziridine VII (5.15 g) was partly dissolved in acetone (180 mL). A solution of CrO_3 (4.71 g) in 2 N H_2SO_4 (52.3 mL) was added dropwise at 0 °C with vigorous stirring over 20 min. The solution was then stirred at room temperature for 40 min. The mixture was poured onto ice (2 kg), and the precipitated product was extracted into pentane. The combined pentane extracts (375 mL) were washed sequentially with water (100 mL), 5% Na_2CO_3 (100 mL), and water (100 mL) and then dried over Na_2SO_4. Evaporation of the solvent (with care to avoid sublimation of the product) yielded the diazirine in 83–97% yield in different experiments. The product was further purified on a short column of silica gel, eluted with pentane: ^{1}H NMR δ 0.64 (br s, 2 H), 1.69, 1.84, 2.02, 2.16 (br m, ~12 H). The UV absorption spectrum showed major peaks at 372 (ϵ_{max} 245 ± 10), 359, 353.5, 348, 342, and 337 nm: IR ($CHCl_3$) ν_{CH} 2910, 2855, 1450 cm^{-1}, ν_{NN} 1540 cm^{-1} (with shoulders). Anal. Calcd for $C_{10}H_{14}N_2$: C, 74.03; H, 8.70; N, 17.27. Found: C, 73.84; H, 8.67; N, 17.02.

1-[^{3}H]Adamantan-4-one Ethylene Ketal (VI).* (This step was performed by the Amersham Corp. as their service TR3.) The bromo ketal V (20 mg) was exposed to $^3H_2$ gas (5 Ci) under conditions similar to those described above for the $^1H_2$ hydrogenolysis. The yield after removal of the catalyst and inorganic solids was 0.240 Ci (11% based on carrier-free $^3H_2$). TLC (system B, R_f 0.52) showed that the material was of 94% radiochemical purity and contained 1.5% of the corresponding ketone. It was stored in ethanol (8 mCi mL^{-1}) at −20 °C.

1-[^{3}H]Adamantan-4-one (I).* Unlabeled ketal VI (11.8 mg; made from the ketone by a method similar to that used for the bromo ketal V) was added to labeled ketal (55.9 mCi), and the ethanol was carefully removed by distillation. The residue was taken up in dioxane (1.04 mL), and the following were added: H_2O (250 μL), H_2SO_4 (25 μL), and KIO_4 (25 mg). The mixture was stirred for 6 h at room temperature and then poured into 5% Na_2CO_3 (5 mL). The product was extracted into pentane (3 × 3 mL). The radiochemical purity was 97% and the radiochemical yield (two preparations) was 84 and 94% (TLC: system B, R_f 0.38).

1-[^{3}H]Spiro[adamantane-4,4′-diaziridine] (VII).* The pentane solution of the ketone (46.0 mCi; dried over Na_2SO_4) was filtered through glass wool into a glass tube (4-mm diameter), and the solvent was evaporated in a slow stream of N_2. A small Teflon flea was placed in the tube which was then sealed with a septum. A solution of NH_3 (25% w/v) in CH_3OH (150 μL) was introduced through a cannula from a calibrated tube at 0 °C. After being stirred at 0 °C for 30 min, the solution was clear, and hydroxylamine-*O*-sulfonic acid (1 equiv by titration) in methanol (115 μL) was added in portions of 10 μL. The mixture was stirred at 10 °C for 16 h, and the solvent was then evaporated in a gentle stream of N_2. The residue was extracted with CH_2Cl_2 (15 × 200 μL). The CH_2Cl_2 solution was then extracted with 2 N H_2SO_4 (2 and 1 mL) and H_2O (1 mL) at 0 °C. The combined aqueous extracts were made basic with 2 N NaOH at 0 °C and the product was extracted into CH_2Cl_2 (3 × 4 mL). The final CH_2Cl_2 extracts were combined and stored at −20 °C. The radiochemical yield was 59 and 68% (two preparations).

1-[^{3}H]Spiro[adamantane-4,4′-diazirine] (VIII).* The preparation was carried out in dim light. In a 4-mm diameter tube, the diaziridine solution (30.2 mCi) was evaporated to dryness in a stream of N_2, and acetone (200 μL) was added. At room temperature, a solution of CrO_3 in 2 N H_2SO_4 (0.912 M, 65 μL) was added in portions (5 μL) by syringe over 10 min while the mixture was vigorously stirred. After a further 20 min, the mixture was injected into ice–water (4 g). The

precipitated product was extracted into pentane (1.5 mL), and this solution was washed sequentially with water, 5% Na_2CO_3, and water (each 2 mL). The washings were back-extracted with pentane (1.5 mL). The combined pentane extracts contained 80–89% of the initial radioactivity, 83% of which was diazirine (TLC: system B, R_f 0.62; system C, R_f 0.31; the remainder of the radioactivity stayed at the origin).

The pentane extracts were reduced to 0.5 mL in a stream of N_2, and the concentrate was applied to a silica column (2 cm × 1.2 cm^2). The column was eluted with pentane under slight pressure. A single fraction (0.75 mL) contained 55% of the initial diaziridine activity. The diazirine was pure by TLC (systems B and C) and had a UV spectrum identical with that of the unlabeled material (Figure 2). The measured specific radioactivity (from the dpm of a sample of known absorbance at 372 nm) was 837 mCi $mmol^{-1}$; that calculated for the starting ketal was 848 mCi $mmol^{-1}$. The overall yield from VI* was 30% (15.8 mCi). The purity of the labeled diazirine (1 mCi mL^{-1}) was estimated after storage for 4 months in various solvents (Sheppard, 1972; Evans, 1976) with the following results: ethanol (−20 °C), >99.9% purity (TLC systems B and C); pentane (−20 °C), 99.1%; benzene (+25 °C), 98.2%. Clearly, ethanol at −20 °C is preferred but this solvent could not be removed without large losses of material. Where very high concentrations of labeled reagent are required, storage in pentane, followed by repurification on silica gel, evaporation of the solvent, and transfer to the membrane preparation in a small portion of ethanol, would be necessary.

1-[^{125}I]Iodo-5-azidonaphthalene. Unlabeled material was synthesized by routes 2 and 3 of Bercovici et al. (1978). Material prepared by route 2 was always slightly contaminated with 1,5-diiodonaphthalene [m/z 380 (M^+), 253 ($M^+ - 127$)]. Because of this, radiolabeled material was prepared by route 3.

1-Nitro-5-aminonaphthalene was made in low yield by the methods of Hodgson et al. (Hodgson & Walker, 1933; Hodgson & Davey, 1939; Hodgson & Turner, 1943) or purchased from ICN Ltd. Conversion to 1-nitro-5-azidonaphthalene was by the method of Forster & Fierz (1907) (50% yield). Treatment of the product with $Na_2S_2O_4$–ethanol at room temperature produced 1-amino-5-azidonaphthalene, in 42% yield, which was converted to 5-azidonaphthyldiazonium hydrogen sulfate by the method of Bercovici et al. (1978). The diazonium salt was stored in 10% H_2SO_4 at −20 °C. A portion (0.6 μmol) was converted, as originally described, to 1-[^{125}I]iodo-5-azidonaphthalene with NaI (0.6 μmol) containing ^{125}I (1.0 mCi). The yield was 50% based on the amine. The reagent which after purification contained no detectable radiochemical impurities (TLC: system C) was stored at −20 °C in ethanol (0.2 mCi mL^{-1}), handled under a brown safelight, and tested for purity (TLC; autoradiogram) immediately before use. Unlabeled material prepared by route 3 gave the following peaks in the mass spectrum: m/z 295 (M^+, 38), 267 ($M^+ - N_2$, 98), 140 ($M^+ - N_2 - I$, 100). The peaks at 217 and 104 observed by Bercovici et al. (1978) were not detected.

[G-^{3}H]Phenyl Azide. [^{3}H]Phenyl azide of the highest possible specific activity was prepared as follows. Undiluted [*ring*-G-^{3}H]aniline (3.1 mg, 150 mCi $mmol^{-1}$) was dissolved in ethanol (100 μL) and transferred to one bulb of a small bulb-to-bulb distillation apparatus. Concentrated HCl (1.1 equiv) was added and, while the solution was stirred at 4 °C, neat isopentyl nitrite (1.1 equiv) was introduced all at once. After 10 min, NaN_3 (1.1 equiv; 8 μL of a concentrated aqueous solution) was added, and the mixture was stirred for 5 min at 4 °C and then for 45 min at 25 °C. Solid urea (~5 mg) was then added, followed by solid $NaHCO_3$ (~5 mg), and the mixture was stirred. The product was then codistilled with the solvent into the receiver bulb. The solution of [^{3}H]phenyl azide so obtained had an ultraviolet spectrum identical with that of the unlabeled material. The specific radioactivity was 97.2 mCi $mmol^{-1}$.

Labeling Experiments

Chymotrypsin (from bovine pancreas), aldolase (from rabbit muscle, Grade I), and wheat germ lectin–Sepharose 6MB conjugate were purchased from Sigma Chemical Co. Trypsin (TPCK treated) and lysozyme were from Worthington. The following buffers were used: A, 5 mM sodium phosphate, pH 8.0; B, 5 mM sodium phosphate, pH 8.0, containing EDTA (1 mM); C, same as B, containing BSA (10 mg mL^{-1}).

Phospholipid vesicles were prepared in 20 mM Tricine–HCl, pH 8.15, containing NaCl (0.1 M) as previously described (Bayley & Knowles, 1978a).

Unsealed human erythrocyte ghosts were prepared by the method of Steck & Kant (1974) from fresh or recently outdated blood. Common blood groups, usually O^+, were used, all of which appeared normal in protein content by electrophoresis. The ghosts were washed at least 3 times with buffer B. The final pellets, containing 3–4 mg mL^{-1} of protein (see below), were stored in portions (1 mL) at −20 °C for no longer than 1 week. Thawed ghosts were not refrozen. Chymotrypsin or trypsin treatment of intact erythrocytes before the preparation of ghosts was performed as described by Drickamer (1976). The method of Lowry, calibrated with BSA and modified by the addition of deoxycholate (Dunn & Maddy, 1976), was used to determine protein concentration. The results are uncorrected and probably underestimate the amount of membrane protein (Maddy et al., 1972).

Tritium in membrane samples and gel slices was determined by counting in scintillation fluid (10 mL) containing toluene (850 mL), Bio-Solve (Beckman; 100 mL), and Liquifluor (New England Nuclear; 50 mL). All samples were counted twice. The problem of quenching was overcome either by comparing samples of the same composition or by determining the absolute counting efficiency for a given sample by using [^{3}H]hexadecane.

Polyacrylamide gel electrophoresis was done in cylindrical tubes according to Fairbanks et al. (1971). The modifications of Steck & Yu (1973) were used for staining with Coomassie Blue or with Schiff's reagent (PAS).

Gels to be fractionated were run in duplicate or triplicate. Immediately after electrophoresis, one gel was sliced in sodium dodecyl sulfate (2% w/v) with a Gilson Aliquogel fractionator, and the others were stained. The recovery of radioactivity from the gels was virtually quantitative. Samples containing the tracking dye were not detectably quenched.

Reagent Binding to Phospholipid Vesicles. The partitioning of adamantanediazirine into single-bilayer vesicles was measured by equilibrium dialysis (Bayley & Knowles, 1978a). Because the diazirine is insoluble in buffer, the vesicles were first equilibrated with reagent by stirring for 20 min at 25 °C. A portion (800 μL) of the diazirine-containing vesicle solution was then dialyzed against buffer (5 mL).

Reagent Binding to Erythrocyte Ghosts. Packed ghosts (in buffer B) were diluted fivefold with the same buffer to ~0.6–1.0 mg mL^{-1}, and samples were taken for the exact determination of protein concentration. To each of a number of portions (1.0 mL) were added unlabeled diazirine (in 5 μL of ethanol) and labeled diazirine (5 μCi; in 5 μL of ethanol)

by syringe while the samples were vortexed vigorously. The samples were incubated for 15 min at room temperature and then for 45 min on ice. After the determination of the radioactivity in each suspension, the samples were centrifuged at 4 °C. The radioactivity in the supernatant and in the resuspended pellets was then measured. No radioactivity was lost during the centrifugation. The binding of iodoazidonaphthalene and phenyl azide were measured similarly. The binding of [^{3}H]phenyl azide to erythrocyte ghosts was also measured by the dialysis method used to investigate phenyl azide binding to phospholipid vesicles (Bayley & Knowles, 1978a). Preliminary equilibration at 4 °C was for 16–18 h. Final equilibration at 25 °C was for 5 h.

Time Course for the Covalent Incorporation of ^3H into Erythrocyte Ghosts Irradiated in the Presence of [^3H]-Adamantanediazirine. Ghosts (1 mL; containing 3.6 mg of protein in buffer B) in a septum-sealed glass tube were stirred magnetically under a slow stream of wet N_2 for 30 min. Thereafter a positive pressure of N_2 was maintained with a balloon. [^{3}H]Diazirine (10 μCi; in 10 μL of ethanol) was added while the suspension was vortexed. After incubation for 1 h at 4 °C, the well-stirred suspension was photolyzed at 3 cm from the center of a Rayonet RPR 3500-Å lamp which had been allowed to warm up for 1 h and which was not switched off during the course of the experiment. At each time point (see Figure 4) the tube was removed from the apparatus and duplicate samples were taken by syringe. Each sample was added to buffer C and vortexed. After incubation for 15–30 min, the membranes were recovered by centrifugation. The membranes were washed 3 more times in a similar manner and finally dissolved in sodium dodecyl sulfate (3.5% w/v) for counting.

Typical Small-Scale Procedure for Labeling Erythrocyte Ghosts with [^3H]Adamantanediazirine. Erythrocyte ghosts (1 mL; 3–4 mg of membrane protein in buffer A or B) were purged with wet N_2 for 30–60 min. [^{3}H]Diazirine ($\leq$15 μL of a 1 μCi μL^{-1} solution in ethanol) was added by syringe while the tube was vortexed. The suspension was stirred gently for 1 h at 4 °C. The membranes were then photolyzed for 15 min at 4 °C with thorough stirring, at 3 cm from the center of the RPR 3500-Å lamp. After photolysis, small portions were removed for counting. No radioactivity was lost during the photolysis. The membrane samples were diluted with at least 10 volumes of buffer C, thoroughly mixed, and incubated for 15 min at 4 °C. After centrifugation (12000g; 20 min) the pellet was resuspended in the same buffer and the procedure was repeated. After a total of four washes with the BSA solution, the membranes were washed four times with buffer B (without a 15 min incubation). Before the final centrifugation, the membranes were suspended to a known volume and small portions were removed for counting to determine the covalently bound radioactivity.

Ghosts Photolyzed with Unlabeled Adamantanediazirine before Photolysis with [^3H]Diazirine. Unlabeled adamantanediazirine (46.7 mM in ethanol; 4 μL) was mixed with a sample of N_2-purged ghosts (750 μL; 2.28 mg of protein). The suspension was incubated and photolyzed as before. After the suspension had been purged again with N_2, labeled diazirine (in 6 μL of ethanol) was added. After a further incubation, the sample was photolyzed once more and worked up as described previously. A parallel control differed only in the additions of pure ethanol rather than diazirine solution.

Treatment of Labeled Ghosts with NaOH. Washed, labeled ghosts (2.05 mg in 1 mL of buffer A) were diluted with ice-cold 100 mM NaOH (10 mL) and allowed to stand at 4 °C for 10 min. A control sample was similarly treated, but with buffer. After centrifugation (20000g; 1 h), the pellets were collected, washed 3 times with buffer B, and stored frozen before electrophoretic analysis.

Treatment of Labeled Ghosts with Chymotrypsin. Labeled ghosts (4.1 mg in 1.9 mL of buffer A) and chymotrypsin (100 μL of 2.5 mg mL^{-1} in buffer A) were stirred gently at 25 °C for 75 min.

Labeling of Membrane-Bound Lysozyme. Ghosts (1 mL; 2.36 mg of protein) in buffer B containing lysozyme (1.05 mg) were flushed with N_2, incubated with reagent, photolyzed, and washed as described above. A control contained no lysozyme. Half of the lysozyme-containing sample in buffer A (0.5 mL) was diluted with 100 mM NaOH (8.5 mL; 0 °C) and allowed to stand on ice for 15 min. This sample was centrifuged at 48000g for 30 min. The pellet was washed with buffer A.

Labeling of Membranes in the Presence of Aldolase. Ghosts were incubated for 20 min at 4 °C with 10 volumes of 5 mM sodium phosphate, pH 8.0, containing 0.15 M NaCl, and collected by centrifugation. This procedure removes band 6 (Kant & Steck, 1973). These ghosts, washed and resuspended in the appropriate buffers to a final concentration of $\sim$3 mg mL^{-1} protein, were used to prepare the following samples: (1) ghosts in 5 mM sodium phosphate, pH 7.0 (2.81 mg mL^{-1} protein); (2) same as (1) but containing aldolase (1.0 mg mL^{-1}); (3) ghosts in 5 mM sodium phosphate, pH 8.0, containing 0.12 M NaCl (2.65 mg mL^{-1}); (4) same as (3) but containing aldolase (1.0 mg mL^{-1}). After labeling as described, but before washing, portions (100 μL) were removed from the samples for direct electrophoretic analysis. All four samples were then centrifuged without dilution, and the supernatants were removed and frozen. The pellets were then washed as before.

Effect of Thiols on the Labeling Pattern of Erythrocyte Ghosts. Solutions of thiols in buffer A were freshly prepared and titrated to pH 8 under N_2 with aqueous NaOH. The thiols were present in the ghost samples from the start of the labeling procedure (that is, thiol was added before the N_2 purge). The rest of the labeling procedure was the same as described above. Glycophorin A was purified from labeled ghosts by affinity chromatography of membranes (solubilized in sodium dodecyl sulfate) on Sepharose 6B conjugated to wheat germ agglutinin (Kahane et al., 1976).

Erythrocyte Ghosts Labeled with [^{125}I]Iodoazidonaphthalene. The procedure was similar to that for [^{3}H]-adamantanediazirine. In all photolyses the ^{125}I level was 1–10 μCi mL^{-1}, the protein concentration was 2–4 mg mL^{-1}, and ethanol was kept below 1.5% (v/v). The time of photolysis (RPR 3500-Å lamp) was such that incorporation was at the peak level found in time-course experiments.

Erythrocyte Ghosts Labeled with [^3H]Phenyl Azide. The protocol used for photochemical labeling of erythrocyte ghosts with [^{3}H]phenyl azide was similar to that used with [^{3}H]-adamantanediazirine. Irradiation was at 254 nm, which meant that the exclusion of oxygen was particularly important. Irradiation of ghosts in air with the RPR 2540- and 3000-Å lamps leads to rapid cross-linking of the membrane proteins (data not shown). Incubation with the reagent was for 1 h at 4 °C. Photolysis was for 2.5 min 3 cm from the lamp with efficient stirring under a positive pressure of N_2.

Results

Synthesis and Chemical Characterization of 1-[^3H]Spiro[adamantane-4,4'-diazirine]. The synthesis of [^{3}H]-adamantanediazirine is outlined in Figure 1. The tritiated reagent can be synthesized from the readily accessible [^{3}H]-

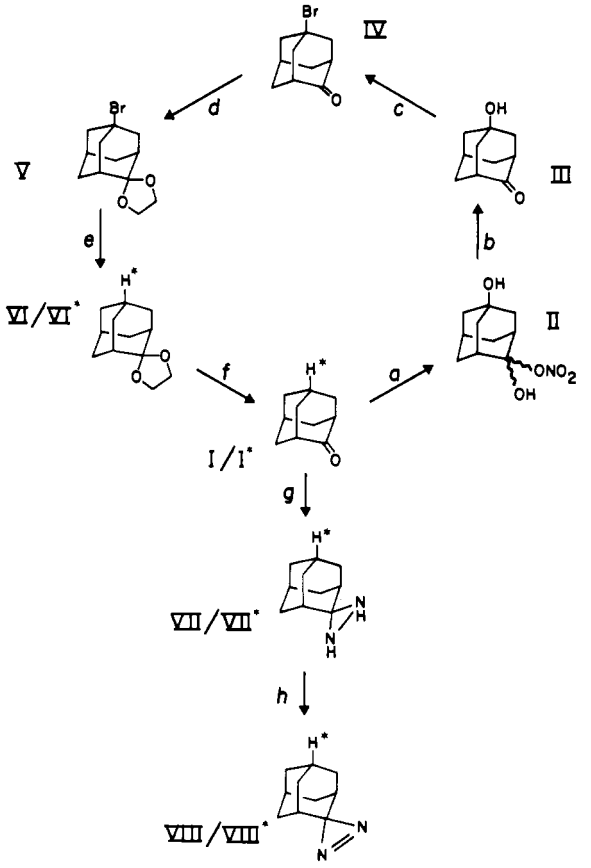

FIGURE 1: Synthesis of [^{3}H]adamantanediazirine. (a) 100% HNO_3, 25 °C, 69 h, and 60 °C, 2 h. (b) 25% H_2SO_4, 95 °C, 2 h. (c) 48% HBr, reflux for 7 h. (d) $HO(CH_2)_2OH$, TsOH, benzene, reflux for 14 h. (e) $^3H_2$, 10% Pd/C, K_2CO_3, ethanol, 25 °C, 20 h. (f) KIO_4, H_2SO_4, dioxane–water, 25 °C, 6 h. (g) NH_3/CH_3OH, NH_2OSO_3H, 10 °C, 16 h. (h) CrO_3–H_2SO_4, acetone, 20 °C, 30 min. The asterisk denotes a tritiated compound.

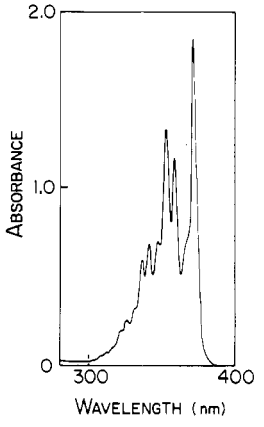

FIGURE 2: Ultraviolet absorbance spectrum of [^{3}H]adamantanediazirine in pentane. ϵ_{372nm} ~245.

adamantanone ethylene ketal and purified in a few days. Material of high specific radioactivity was obtained by replacement of the bridgehead bromine in V (Figure 1) by tritium, yielding the diazirine precursor VI* containing one atom of essentially carrier-free ^{3}H (26 000 mCi mmol^{-1}). In the present work, VI* was diluted with unlabeled material to ~1000 mCi mmol^{-1}. The near-ultraviolet spectrum of [^{3}H]adamantanediazirine is shown in Figure 2.

Binding of [^{3}H]Adamantanediazirine to Phospholipid Vesicles and to Erythrocyte Ghosts. Since the aim of this work is to label intrinsic membrane components, the concentration of the reagent precursor in the lipid bilayer should be far

Table I: Partitioning of Hydrophobic Reagents into Phospholipid Vesicles and Erythrocyte Ghosts[a]

ligand	$P_{DMPC}^{40°C}$	$P_{DOPC}^{25°}$	$P_{ghosts}^{4°C}$
adamantanediazirine	3700[b]	5050[b]	1 750[c]
phenyl azide	330[d]	370[d]	240[e]
iodoazidonaphthalene	n.d.[g]	n.d.	163 000[f]

[a] $P_{DMPC}^{40°C}$ is the partition coefficient for dimyristoylphosphatidylcholine vesicles at 40 °C and is defined as (ligand bound/milligram of lipid)/(free ligand/microliter of external solution). $P_{DOPC}^{25°C}$ is the partition coefficient for dioleoylphosphatidylcholine vesicles. $P_{ghosts}^{4°C}$ is the partition coefficient for erythrocyte ghosts at 4 °C and is defined as (ligand bound/milligram of membrane protein)/(free ligand/microliter of external solution). The values in the table were each determined several times and did not vary greatly with reagent and lipid concentrations. [b] Lipid, 10 mg mL^{-1}; ligand, 1–200 μM (concentration of ligand was averaged over the entire sample). [c] Protein, 0.85 mg mL^{-1}; ligand, 0.4–250 μM. [d] Bayley & Knowles (1978a). [e] Protein, 2.75–4.4 mg mL^{-1}; ligand, 0.46–4 mM; ethanol, 1–2% v/v. [f] Protein, 0.2 mg mL^{-1}; ligand, 65 nM; ethanol, 0.1% v/v. For further details, see Experimental Section. [g] n.d., not determined.

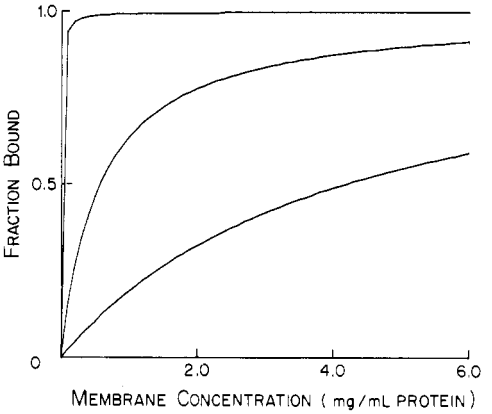

FIGURE 3: Partitioning of hydrophobic reagents into erythrocyte membranes in the dark. The curves are calculated (see the text) based on the measured values over a smaller concentration range (Table I). Upper curve, 1-iodo-5-azidonaphthalene; middle curve, adamantanediazirine; lower curve, phenyl azide.

greater than that in the surrounding buffer. That this was so for [^{3}H]adamantanediazirine was established by measuring the partitioning of the reagent into single-bilayer phosphatidylcholine vesicles and into human erythrocyte membranes. The results are summarized in Table I, which includes data for two other reagents: phenyl azide and 1-iodo-5-azidonaphthalene. Using the known lipid composition of erythrocyte ghosts (Steck & Dawson, 1974) and assuming that the density of the hydrocarbon core of the bilayer is 0.75 g cm^{-3}, the ratio of the concentration of the diazirine in the hydrocarbon core to that in the surrounding aqueous phase is ~1500. Similarly, the ratio of the diazirine concentration in the hydrocarbon core of the lipid vesicles to that in solution is 5000 for dimyristoylphosphatidylcholine and 6000 for dioleoylphosphatidylcholine.

If it is assumed that the binding of the hydrophobic compounds to erythrocyte ghosts is a simple partitioning, then $b/(b + f) = Pc/(1000 + Pc)$, where b = bound reagent per unit volume of solution, f = free reagent per unit volume of solution, c = concentration of protein (mg mL^{-1}), and P is defined in Table I. The data in Table I can then be extrapolated over a range of membrane concentrations (Figure 3).

Photochemical Incorporation of Label into Erythrocyte Ghosts. When [^{3}H]adamantanediazirine was allowed to bind to erythrocyte ghosts and subsequently irradiated at ~350 nm,

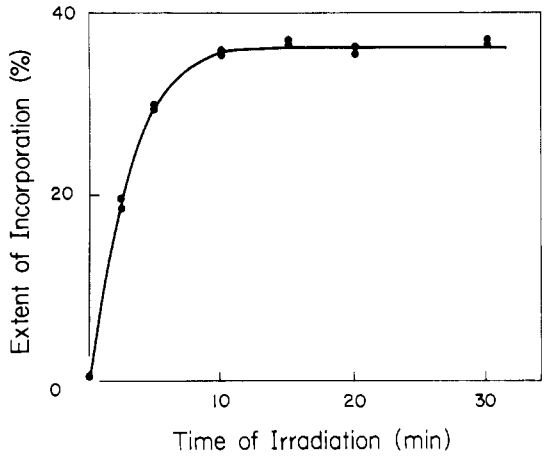

FIGURE 4: Time course for photochemical covalent attachment of [^{3}H]adamantanediazirine to erythrocyte membranes. Ordinate: the percentage of radiolabel originally present in the mixture that is covalently bound after irradiation. Abscissa: duration of irradiation. For details, see Experimental Section.

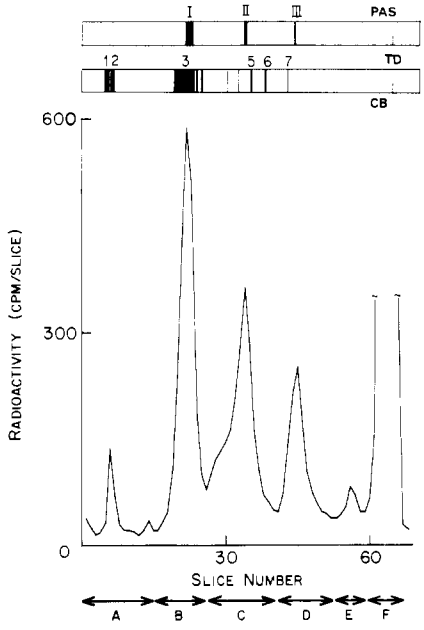

FIGURE 5: Electrophoretic profile of erythrocyte ghosts labeled with [^{3}H]adamantanediazirine. Numbers 1–7 indicate Coomassie Blue (CB) stained bands and roman numerals I–III indicate periodic acid–Schiff reagent (PAS) stained bands [Fairbanks et al. (1971) and Table II]. The position of the tracking dye is indicated by TD. Letters A–F indicate regions of the gel discussed in the text.

radiolabel became attached to the membranes and could not be removed by extensive washing with buffer containing bovine serum albumin (Figure 4). The attached label was not released on further irradiation. In contrast, label derived from [^{3}H]phenyl azide was slowly released on prolonged irradiation (data not shown) while that from [^{125}I]iodoazidonaphthalene was quite rapidly released under these conditions [data not shown; see also Bercovici et al. (1978)]. The distribution of label among the components of the membrane after labeling with [^{3}H]adamantanediazirine (see below) was unaltered after prolonged irradiation. The mean incorporation of label at the plateau level was 38% (±4 SD; n = 18) of the label present in the membrane suspension before irradiation.

Human Erythrocyte Membranes Labeled with [^{3}H]-Adamantanediazirine. To determine whether adamantanediazirine labels intrinsic membrane proteins more heavily than extrinsic proteins, we labeled erythrocyte ghosts with the [^{3}H]diazirine and analyzed the proteins electrophoretically (Figure 5).

Table II: Distribution of Label from [^{3}H]Adamantanediazirine among the Proteins of the Red Cell Membrane

region[a]	incorpd[b] label (%)	bands	% w/w[c]	monomers/cell[d]
A	5	1 (extrinsic)	15	200 000
		2 (extrinsic)	15	200 000
B	37	3 (extrinsic)	25	870 000
		PAS I (intrinsic)	1.6–2.0	450 000
C	34	PAS II (intrinsic)		
		5 (extrinsic)	5	300 000
		6 (extrinsic)	5	500 000
D	17	PAS III (intrinsic)		
E	7			

[a] See Figure 1. [b] Label in a given region as a percentage of the total label found in protein (regions A–E). Values quoted are the averages from six gels. [c] The weight percent of the total protein is given. Data are largely based on those of Steck (1974). The value for glycophorin A (PAS I and II) includes carbohydrate (Kahane et al., 1976). [d] Based on a value of 1.95 × 10^9 cells/mg of membrane protein (Lepke et al., 1976).

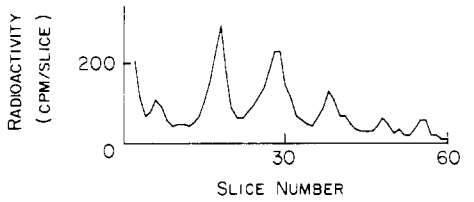

FIGURE 6: Electrophoretic profile of ghosts labeled with [^{3}H]-adamantanediazirine and then extracted with chloroform–methanol. Some aggregated material (630 cpm) was present in the first slice.

Four-fifths (mean = 79%) of the incorporated label ran close to the dye front (region F, Figure 5) in the gel. The radioactivity found in this region represents labeled lipid (Bayley & Knowles, 1978b) and perhaps noncovalently bound photolysis products (Bayley & Knowles, 1978a), although most of the label that is removed by the albumin washes runs in regions D–E. That the label in regions A–E is attached to protein is justified by the experiments described below.

In Table II are compiled the mean estimates of the label found in the various regions of the gel. Also shown are the major proteins in those regions, their properties, and their classification as intrinsic or extrinsic membrane proteins. It is clear that the amount of label in the regions of the gel containing known extrinsic proteins (bands 1, 2, 5, and 6) is low, while that in the regions containing known intrinsic proteins (band 3, PAS I–III, and the band 4.5 region) is relatively high. The small peaks at slice 14 (region A, Figure 5) and slices 56 to 57 (region E, Figure 5) were consistently observed but do not correspond to well-characterized proteins.

Variation of Labeling Conditions. The labeling pattern shown in Figure 5 was independent of the presence or absence of 1 mM EDTA or of 0.15 M NaCl, of changes in buffer (5 mM sodium phosphate, pH 7, and 10 mM Tris–acetate, pH 7.4), and of ethanol concentrations up to 3% (v/v). Label incorporation was proportional to the amount of diazirine bound before irradiation (Figure 3) over the range 0.3–3.0 mg of membrane protein mL^{-1}, and the distribution of radioactivity on electrophoresis was the same for all the samples. This suggests that nucleophiles at the membrane surface do not scavenge substantial amounts of photogenerated intermediates from the bulk phase.[1]

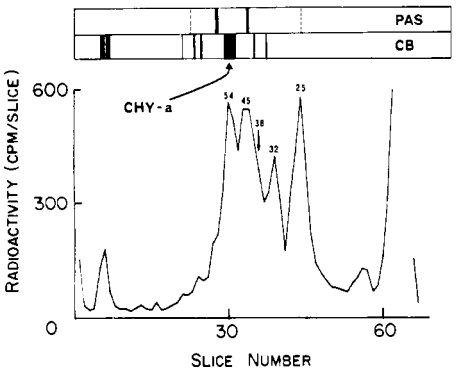

FIGURE 7: Electrophoretic profile of ghosts made from chymotrypsin-treated cells and labeled with [^{3}H]adamantanediazirine. Chy A designates the large chymotryptic fragment of band 3 (Drickamer, 1977).

To test the possibility that tight, efficiently labeled binding sites for adamantane diazirine might exist in the red cell membrane, we labeled membranes with nonradioactive diazirine at 25 times the concentration used in the radiolabeling experiments described here and *then*, without washing, relabeled them with radioactive diazirine. The distribution of radioactivity was not altered much in this experiment, but there was a significant (~10%) decrease of label in region B of the gel (Figure 5).

Labeled Bands Are Proteins. Experiments were carried out to demonstrate that the radioactivity in regions A–E indeed derives from labeled protein rather than noncovalently bound photolysis products, labeled lipids tightly bound to proteins [e.g., Shapiro & Marchesi (1977) and Armitage et al. (1977), but see, for instance, Oldfield et al. (1978) and Shukla et al. (1979)], or free labeled lipids running behind the dye front.

When labeled membranes were extracted with organic solvents to remove lipids, the radioactivity in region F of the gel was greatly diminished. The remainder of the pattern was largely unchanged (see Figure 6), although some aggregation of proteins occurred and radioactivity appeared at the top of the gel.

Membranes were subjected to proteolysis before or after labeling. When erythrocytes are treated with chymotrypsin, both glycophorin A and band 3 are degraded. The labeling pattern of ghosts from such a preparation shows no radioactivity in the regions corresponding to the intact proteins (Figure 7). A new peak of radioactivity (at 54 000 daltons) corresponds to the major chymotryptic fragment of band 3, Chy A (Drickamer, 1977). The label in region A (which corresponds to spectrin: bands 1 and 2) is unaltered as expected. In the case of ghosts from trypsin-treated cells, glycophorin A but not band 3 is degraded and this is reflected in the labeling profile (not shown). When ghosts are *first* labeled and then treated with chymotrypsin, no radioactivity appears in the first one-third of the gel. Of the numerous peaks of radioactivity with higher mobility, the most prominent corresponds to the 17 000-dalton membrane-bound fragment of band 3 (Steck et al., 1978).

Further proof that the radioactivity derives from labeled proteins is that on 9.6% acrylamide gels, band 3 and PAS I are resolved both by staining and in the radioactivity profile.

[1] If the extent of reaction with the membrane surface of the reagent in the aqueous phase were concentration dependent rather than a function of the total amount of external reagent, labeling of the membrane surface by unbound reagent would not be revealed by this experiment because the ratio of the concentration of bound and free reagent is unchanged by diluting the membranes.

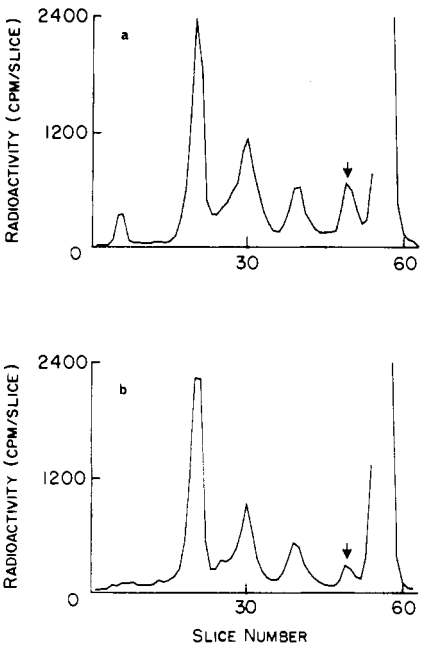

FIGURE 8: Electrophoretic profile of membranes containing bound lysozyme labeled with [^{3}H]adamantanediazirine. (a) Sample containing lysozyme (~30% w/w of the total protein). (b) Same as (a) but lysozyme (and other extrinsic proteins) was removed by a high pH wash before electrophoresis. The arrow shows the position to which lysozyme migrates.

Indeed, band 3 (Goldman, 1980) and glycophorin A (Goldman et al., 1979) can be purified to homogeneity from labeled membranes with no apparent loss of radiolabel.

Labeling of Membranes with Altered Extrinsic Protein Content. Except for spectrin, none of the endogenous extrinsic proteins are present in sufficient quantity to allow an unambiguous determination of their extent of labeling by adamantanediazirine. Two methods were used to manipulate the extrinsic proteins of the erythrocyte ghost and thus confirm that the extent of labeling of such proteins is low. In the first, these proteins were removed before or after labeling with the diazirine. In the second, exogenous proteins that bind tightly to the membranes were added to the membranes before labeling.

When labeled, washed membranes were treated with cold 100 mM NaOH to remove extrinsic proteins, more than 98% of the radioactivity was recovered in the 48000*g* pellet. Electrophoresis of this pellet showed only a single major band in the position of band 3 (from Coomassie Blue staining). The label in the spectrin region was eliminated, but otherwise the radioactivity profile was similar to that of a control sample (results not shown). To investigate the origin of the label in spectrin, we incubated ghosts at 37 °C in 0.3 mM sodium phosphate buffer, pH 8.0, which releases bands 1 and 2. The suspension containing ghosts and free spectrin was then labeled and the membranes were recovered by ultracentrifugation. Gel electrophoresis revealed that band 1 and most of bands 2 and 5 had been released. Fractionation and scintillation counting of the gels showed that free spectrin was labeled to approximately the same extent as bound spectrin. Ghosts washed with saline had the same labeling pattern as untreated ghosts. This demonstrates that band 6, which is removed by salt, is not heavily labeled.

We found that egg white lysozyme [pI = 11.4 (Wetter & Deutsch, 1951)] binds tightly to erythrocyte ghosts in buffers of low ionic strength and is not released from the membranes by our washing procedure [similar observations have been made previously [e.g., Gulik-Krzywicki et al. (1969)]]. When

lysozyme comprised ~30% by weight of the total membrane protein, the radioactivity in region E of the gel from a labeling experiment (Figure 8a) was approximately twice that observed in the absence of lysozyme (Figure 5). After treatment with cold 100 mM NaOH, lysozyme was removed (with the other extrinsic proteins) and the increment of radioactivity in region E of the gel was no longer seen (Figure 8b). Only 2.5% of the total covalently bound radioactivity was present in the 48000*g* supernatant after base treatment.

The extrinsic protein on the membrane was also varied by allowing aldolase to adhere to its binding site on band 3 (Strapazon & Steck, 1976). Aldolase is released at high ionic strength, and labeling experiments were done with either free or bound aldolase. Both free and bound aldolase were labeled, but to very low extents. In Figure 9 are shown the results of an experiment in which bound aldolase comprised 25% by weight of the membrane protein.

The specific radioactivities of lysozyme and aldolase labeled when bound to membranes was too low for precise quantitation. The values were approximately equal to that of the other lightly labeled extrinsic protein, spectrin.

Effect of Thiols on the Labeling Profile. Thiols can be used as scavengers for intermediates generated photochemically from diazirines (Bayley & Knowles, 1978b; D. N. Standring, unpublished work). Ghosts were labeled in the presence of water-soluble thiols to determine the extent to which labeling occurred through intermediates that are free in the aqueous phase. The *overall* extent of label incorporation was not measurably reduced in the presence of thiols (50 mM glutathione, 2-mercaptoethanol or thioglycolate, or 25 mM dithiothreitol), but there was a reproducible reduction in the labeling of glycophorin A and no reduction in the labeling of the other proteins. Glycophorin A isolated by affinity chromatography on Sepharose conjugated with wheat germ agglutinin contained ~6% of the total protein-bound label when membranes were irradiated with [^{3}H]adamantanediazirine in the absence of thiols and only ~3–4% of the label when labeling was performed in the presence of 50 mM glutathione.

Labeling with Phenyl Azide and Iodoazidonaphthalene. Erythrocyte ghosts were labeled with [^{3}H]phenyl azide to maximal incorporation. The distribution of radioactivity was, however, quite different from that observed with the diazirine. For example, spectrin and exogenous lysozyme were heavily labeled. In the presence of glutathione (16.5 mM), a fivefold decrease in overall label incorporation was observed. In the case of [^{125}I]iodoazidonaphthalene, we obtained similar results to those of Bercovici et al. (1978). Spectrin was not heavily labeled and neither was exogenous lysozyme, and the extent of labeling was not decreased by the presence of glutathione (17 mM). There was not, however, much label in the region of PAS I and band 3. [Glycophorin A (PAS I and II) *is* labeled, however (Kahane & Gitler, 1978), and it is possible that the bound label alters the electrophoretic properties of the glycoprotein [cf. Silverberg et al. (1976)].]

Discussion

The results presented demonstrate that photochemical labeling with the lipophilic molecule adamantanediazirine can be used to distinguish between intrinsic and extrinsic proteins. We have further shown (Goldman et al., 1979) that only the membrane-embedded segments of the intrinsic proteins are labeled. The labeling experiment is convenient, rapid, and direct, and it has some advantages over alternative methods for detecting hydrophobic peptides (such as amino acid analysis of isolated fragments, peptide sequencing, detergent binding studies, charge-shift electrophoresis, or inference from surface labeling and proteolysis experiments).

The reagent was selected on the basis of its low polarity, chemical stability, reasonable size, ease of synthesis, and ultraviolet absorption properties. The absorption between 300 and 400 nm (Figure 2) allows rapid photolysis of the reagent at wavelengths that are generally harmless to biological systems (Knowles, 1972; Bayley & Knowles, 1977). The photogenerated carbene is known to be highly reactive (Isaev et al., 1974; Bayley & Knowles, 1978b) and is expected to be of low polarity, like its precursor.

We have previously demonstrated that adamantylidene generated photochemically in phospholipid vesicles can insert into the carbon–hydrogen bonds of the bilayer lipids (Bayley & Knowles, 1978b). The synthesis of [^{3}H]adamantanediazirine of high specific radioactivity has now allowed us to examine the labeling pattern of biological membranes using low concentrations of this reagent. We chose the human red blood cell membrane for detailed investigation because it is a moderately complex yet well-characterized system.

Binding studies revealed that the concentration of the diazirine in the hydrocarbon core of the erythrocyte membrane before irradiation is 1500 times that in the surrounding solution. This concentration difference is evidently sufficient to give useful selectivity of labeling toward intrinsic proteins even though the extrinsic proteins and those parts of the intrinsic proteins that lie outside the bilayer are chemically more reactive.

On irradiation, almost 40% of the reagent becomes covalently attached to the membrane; 8% is incorporated into proteins and 32% is incorporated into lipid. This is surprising, since even with the relatively reactive unsaturated lipid, dioleoylphosphatidylcholine, only 13% covalent attachment to fatty acids is observed when the reagent is irradiated in the presence of single-bilayer vesicles (results not shown).[2] The lipid-to-protein labeling ratio (~4) is far higher with the carbene reagent used here than has been observed for nitrenes (H. Bayley, unpublished experiments; Bercovici et al., 1978; Karlish et al., 1977), and this presumably arises from the higher reactivity of carbenes. The stability of the incorporated label to further irradiation means that one can measure the extent to which proteins are labeled under different conditions, and we have recently used this property to detect a conformational change in Na,K-ATPase (Farley et al., 1980).

The results of Figure 5 show that intrinsic proteins of erythrocyte ghosts are labeled far more strongly by the diazirine than are extrinsic proteins. These data should be compared with those from general surface labeling studies of erythrocyte ghosts [e.g., Phillips & Morrison (1971), in which extrinsic proteins and the exposed parts of intrinsic proteins are labeled] and with studies using surface labels specific for carbohydrate [e.g., Steck & Dawson (1974), in which the exposed parts of intrinsic proteins are labeled]. On a weight for weight basis, glycophorin A and band 3 are labeled ~8 and ~18 times more heavily than spectrin. Since only 10% of the glycophorin A molecule lies within the bilayer, and the label appears to be confined to this segment (Goldman et al., 1979), adamantylidene reacts with the membrane-associated region of glycophorin ~80 times more strongly than it reacts with an extrinsic protein (e.g., spectrin). The exogenous extrinsic proteins, lysozyme and aldolase, show approximately

[2] The extent of incorporation of label into phospholipid vesicles varies inversely with the concentration of the reagent in the mixture. Presumably there are competing dimerizations. The result quoted here is for reagent at 7 μM and lipid at 10 mg mL^{-1} [compare Bayley & Knowles (1978b)].

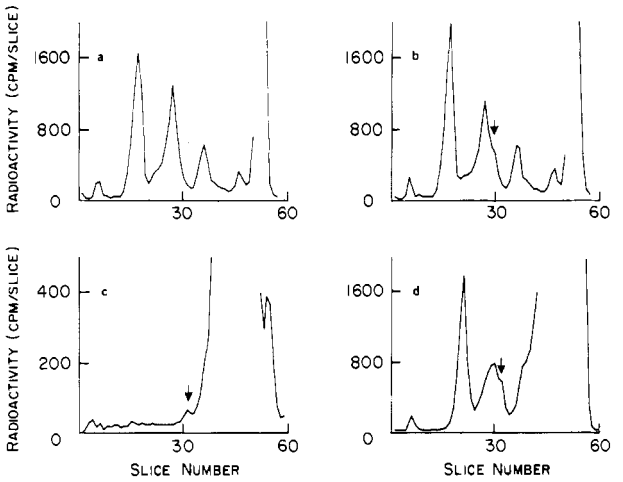

FIGURE 9: Electrophoretic profiles of erythrocyte ghosts containing bound rabbit muscle aldolase, labeled with [^{3}H]adamantanediazirine. Photolysis was in 5 mM sodium phosphate buffer, pH 7.0. (a) Control; no aldolase present. Ghosts were washed before electrophoresis. (b) Membranes contained 25% w/w aldolase, the larger part of which was tightly bound. Ghosts were washed before electrophoresis. (c) Supernatant from a portion of (b) directly after irradiation. A small amount of aldolase is present. (d) Same as (b) but membranes were directly subjected to electrophoresis after irradiation (no washing). The arrows indicate the position to which aldolase migrates. In a similar experiment conducted in the presence of 120 mM NaCl (see Experimental Section), the larger part of the aldolase was found to be free by centrifugation. This too was lightly labeled (data not shown).

the same reactivity as spectrin on a weight-for-weight basis. Spectrin and aldolase were labeled whether they were attached to the membrane or free in solution. This implies that the low labeling of extrinsic proteins occurs from the aqueous phase and that this background might be eliminated if a yet more hydrophobic reagent were used.

Various control experiments have been performed to confirm the validity of our conclusions. It was possible that the intrinsic membrane proteins were surrounded by tightly bound annular lipid [see Lee (1977) but compare, e.g., Oldfield et al. (1978)] and were inaccessible to the carbene. The peaks of radioactivity we observed on electrophoresis might have been labeled lipid bound to protein [but this should have been removed in sodium dodecyl sulfate; see, e.g., Shapiro & Marchesi (1977)] or even free lipid that did not travel near the dye front. The results of organic solvent extraction, proteolysis experiments, and the purification of labeled proteins, appear to rule out these possibilities. To eliminate the possibility of tight[3] efficiently labeled binding sites for the reagent (the external reagent concentration was ~ 1 μM), we first labeled the membranes with a high concentration of nonradioactive diazirine before labeling them with the radioactive reagent. Only small differences from the original labeling pattern were observed. To evaluate further the extent of labeling from the aqueous phase, we used the scavenger glutathione. The incorporation of label into glycophorin A (but not into the other membrane proteins) was somewhat lower in this experiment. Several points should be made. First, in the case of the carbene [but not for aryl nitrenes; see Results and Bayley & Knowles (1978a)], water and buffer components may be scavenging with maximal efficiency before the addition of glutathione. Second, a second reactive intermediate (in this case, 2-diazoadamantane; see below) may react more strongly with one membrane component (here glycophorin A) than the others, and this intermediate might be scavenged more efficiently by virtue of more rapid exchange into the medium and/or greater reactivity toward the scavenger. Third, the added thiol may produce a conformational change in the bilayer and the reorganized membrane could acquire a different distribution of label. These and other possibilities indicate that subtle changes in labeling patterns brought about by scavengers should be interpreted with caution. We have shown that the label distribution in glycophorin A (Goldman et al., 1979) and in Na,K-ATPase (Farley et al., 1980) is the same whether glutathione is present or not, even though the *extent* of labeling of both of these proteins is reduced in the presence of high concentrations of the scavenger. In contrast, observations such as the drastic reduction in labeling of all the proteins in erythrocyte ghosts by [^{3}H]phenyl azide in the presence of glutathione are of obvious consequence.

To obtain a direct comparison of adamantanediazirine with two aryl azide reagents that are in current use, we have labeled erythrocyte ghosts with those reagents. Our results with phenyl azide (Abu-Salah & Findlay, 1977; Bayley & Knowles, 1978a) indicate that this reagent is unsuited to its purpose. The combination of a rather poor partitioning into the bilayer and the relatively low reactivity of nitrenes [or other intermediates photogenerated from the azide (Chapman & Le Roux, 1978; Nielsen et al., 1978)] leads to extensive labeling of *extrinsic* proteins. The putative labeling species, phenyl nitrene, is presumably rather polar, and this property may contribute to its rapid exit from the bilayer and capture by external scavengers. On the other hand, iodoazidonaphthalene (Bercovici et al., 1978) does not label extrinsic proteins and is not scavenged by glutathione (Bayley, 1979). The major asset of this reagent is its high hydrophobicity (Table I and Figure 3). Nevertheless, not all intrinsic membrane components are labeled, and band 3 of erythrocyte ghosts, for instance, incorporated little if any label in our hands (Bayley, 1979). The conclusion that aryl azides that are bound only moderately tightly by membranes are unsuitable as hydrophobic reagents and that those of greater hydrophobicity (Gitler & Bercovici, 1980) do behave in the desired manner is confirmed by the results of Wells & Findlay (1979a,b).

Adamantanediazirine, though useful, is clearly not the "perfect" hydrophobic reagent. Carbenes exhibit some selectivity toward different functional groups, and we have not yet shown which groups within the bilayer are labeled. Indeed, it is possible that a hydrophobic reagent could label just outside the bilayer (Bayley & Knowles, 1978a,b; Goldman et al., 1979). For clarification of this point, sequence analysis of labeled peptides must be performed. The selectivity of the carbene presents a further problem. It would be tempting to use the specific radioactivities of the labeled intrinsic proteins to obtain an estimate of how much of each protein is buried in the bilayer. For instance, the data in this paper could be interpreted to suggest that band 3 is only partly buried in the bilayer, since on a weight-for-weight basis it is labeled only twice as strongly as glycophorin A. While this is in keeping with recent ideas on the structure of band 3 (Guidotti, 1977), such a result could arise if glycophorin A exhibits relatively high reactivity toward the carbene. Such an interpretation would be further compromised by the fact that 2-diazoadamantane (Bayley & Knowles, 1978b) may well be responsible for some of the observed labeling, and this species although very reactive is much more selective than the carbene. In short, we cannot assume that all hydrophobic peptides are equally reactive toward the reagent, and any differences in reactivity might be more evident in such a labeling experiment

[3] There appear to be few documented cases of soluble proteins binding hydrophobic fluorescent probes with values of $K_{association} > 10^6$ M^{-1} [see Edelman & McClure (1968) and references cited therein].

than, say, variations in detergent binding among different hydrophobic peptides (Clarke, 1977).

Despite these possible problems, our results indicate that adamantanediazirine can be used for the rapid characterization of membrane proteins. Since this work was completed, the reagent has been applied in structural studies of the red cell band 3 protein and Na,K-ATPase from dog kidney (Goldman, 1980; Farley et al., 1980).

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