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# A $^{125}$I-Radiolabeled Photoactivable Reagent: Iododiazofluorene for Labeling Membrane Hydrophobic Core

P. S. R. ANJANEYULU, D. PRADHAN, AND ANIL K. LALA[1]

*Biosciences and Engineering Group, Department of Chemistry, Indian Institute of Technology, Bombay, Powai, Bombay 400 076, India*

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2-Iododiazofluorene and 2,7-diiododiazofluorene have been developed as new reagents for labeling the membrane hydrophobic core. These compounds strongly absorb beyond 300 nm and rapidly insert into C-H bonds on photolysis in cyclohexane. 2-[$^{125}$I]Iododiazofluorene has been prepared and it easily partitions into egg phosphatidylcholine vesicles. Photolysis and product analysis indicated that 13.9% of the label inserted into the phosphatidylcholine. Further, the insertion was found to be exclusively associated with the fatty acyl chains and not the glycerophosphorylcholine part of phosphatidylcholine. The easy synthesis and purification procedure for 2-[$^{125}$I]iododiazofluorene and exclusive labeling of the hydrophobic core indicate that this reagent will be useful for studying artificial and natural membranes. 

## INTRODUCTION

The complex structure of membranes and the biomolecular interactions involved therein have been investigated using both chemical and photoactivable reagents (*1, 2*). Photoactivable reagents have an advantage over conventional chemical reagents as they are a more reactive species, which is desirable for labeling the hydrophobic core of membranes (*3, 4*). Iodonaphthylazide, a nitrene precursor, was among the first photoactivable reagents reported which could easily partition into the membrane interior and label hydrophobic domains of membrane-spanning proteins like Glycophorin (*5*). Bayley and Knowles later reported the use of the carbene precursors phenyldiazirine and adamantyldiazirine for labeling the membrane hydrophobic core (*6*). Based on these studies it was concluded that carbene precursors are preferred over nitrene precursors such as phenyl azide. Subsequently trifluoromethyl phenyldiazirine, another carbene precursor, was reported (*7*) and has been used to study various hydrophobic systems (*8, 9*).

We have reported the use of diazofluorene (DAF)[2] as a fluorescent photoactivable reagent for labeling the membrane hydrophobic core (*10, 11*). However, it

[1] To whom correspondence should be addressed.

[2] Abbreviations used: DAF, diazofluorene; 2-I-DAF, 2-iododiazofluorene; 2,7-I-DAF, 2,7-diiododiazofluorene; 2-[$^{125}$I]DAF, 2-[$^{125}$I]iododiazofluorene; PC, phosphatidylcholine; TLC, thin-layer chromatography.



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was difficult to quantitate the insertion of the probe into proteins. This was because the emission maxima of the inserted product and that resulting from the intrinsic protein fluorescence both appear in the 310- to 330-nm range. Thus it was decided to synthesize radioactive analogs of diazofluorene to provide a more sensitive and convenient method for monitoring insertion in lipids and proteins. Toward this end we have reported the synthesis and use of [2-$^3$H]diazofluorene for labeling erythrocyte membranes (*12*). This reagent labeled transmembrane components of both lipids and proteins in erythrocyte membranes, the anion transporter Band 3 being the major protein labeled. We report here the synthesis, characterization, and insertion properties of 2-iododiazofluorene (2-I-DAF) and 2,7-diiododiazofluorene (2,7-I-DAF). The use of iodine allows for a simple method of incorporating radioactivity. These probes can be easily prepared of desired specific activity and give rise to a high insertion yield of 13.9% in artificial membranes.

## MATERIALS AND METHODS

All chemicals and solvents used were of reagent grade. Fluorenone was purchased from Fluka and [$^{125}$I]sodium iodide from BARC, Bombay. Yellow HgO from E. Merck was purified by dissolving it in cold perchloric acid; it was reprecipitated with 20% cold NaOH solution, filtered, and washed with water and dried in a vacuum oven at 120°C for 2 h (*13*). Ultraviolet-visible spectra were recorded on a Shimadzu UV-260 and ir spectra on a Perkin–Elmer 681 spectrometer. NMR spectra were recorded in $CDCl_3$ on a Hitachi R-600 or Varian XL-100 A spectrometer. Mass spectra were recorded on a Varian MAT-112 S spectrometer. Accurate mass data were obtained on a VG 70-250 mass spectrometer. All photolyses were carried out using either a medium pressure mercury lamp (400 W) in an Annular photoreactor (Applied photophysics) or a Rayonet minireactor, RMR-500 (Southern New England) with four 3000 Å lamps. Photolyses were carried out in Pyrex tubes; degassing and removal of samples were carried out through septum ports. Samples were kept at a distance of 5 cm from the lamps. Deoxygenation of samples by evacuation wherever possible or by passing nitrogen or argon on the surface of the solution to be photolysed was found to be critical to obtaining higher insertion yields. Sonication was carried out using a Branson tip type sonicator. All preparations leading to the diazo compound, its purification, and subsequent reactions were carried out in a dark room.

*2-Iododiazofluorene.* 2-Iodofluorenenone (*14*), (2.8 g, 9.28 mmol in 15 ml ethanol), was refluxed with 100% hydrazine hydrate (0.7 ml, 13.92 mmol) for 1 h and concentrated. The resultant solid was crystallized from ethanol to give 2-iodofluorenone hydrazone (2.3 g) m.p. 141–42°C (decomposes), lit. (15) m.p. 141.5–43°C, in 77% yield. The hydrazone (2.39 g, 7.19 mmol in 140 ml dry ether) was treated with KOH (0.2 g) and water (0.1 ml). Yellow HgO (2.33 g, 10.78 mmol) was added in portions over a period of 1 h and stirring was continued for 2 h; it was filtered, dried over $Na_2SO_4$, and filtered again, and the ether was distilled off, the last traces being removed with nitrogen. The resulting red solid was

crystallized from hexane to give 2-iododiazofluorene m.p. 145–46°C, lit. (15) m.p. 145.5–46.5°C in 82% yield. Ultraviolet (methanol): 219 nm (ε 27,710), 241 (59,740), 288 (24,090), 299 (30,110), 336 (19,840), 351 (11,560) and characteristic diazo absorption in the visible region 455 nm (ε 72). Infrared spectrum clearly indicated the diazo peak at 2060 $cm^{-1}$. Mass spectral and chemical ionization mass spectral analysis gave the molecular ion at $m/z$ 318 and $MH^{+\cdot}$ at 319, respectively. Mass spectral fragments corresponding to loss of nitrogen $(M-28)^{+\cdot}$ and iodine $(M-I)^{+\cdot}$ at 290, 191, and 163 were also observed. Diazo compounds lose nitrogen readily; thus the base peak was observed in the mass spectrum at 290.

*2,7-Diiododiazofluorene.* To a mixture of fluorenone (1 g, 5.56 mmol in 12 ml glacial acetic acid), $CCl_4$ (5 ml), freshly sublimed iodine (2.82 g, 11.12 mmol) and concentrated $H_2SO_4$ (1.2 ml), concentrated $HNO_3$ (0.5 ml) were added. The mixture was stirred at 120°C for 4 h. The crude reaction mixture was then poured in water and extracted with chloroform. The chloroform layer was washed with water, dried over $Na_2SO_4$, and distilled off under reduced pressure. The residue was crystallized from ethanol to give 2,7-diiodofluorenone (2.02 g) in 84% yield, m.p. 200–201°C, lit. (14) m.p. 201–202°C.

2,7-Diiodofluorenone (1 g, 2.32 mmol in 30 ml ethanol) was refluxed with 100% hydrazine hydrate (0.17 ml, 3.47 mmol) for 1.5 h. The resulting solid obtained on cooling was filtered and crystallized from ethanol to give 2,7-diiodofluorenone hydrazone (0.85 g, 83%), m.p. 194–95°C (decomposes). The hydrazone (0.75 g, 1.68 mmol in 60 ml dry ether) was treated with KOH (30 mg), water (0.1 ml), and yellow HgO (0.55 g, 2.52 mmol) and stirred for 4 h. This suspension was filtered and dried over $Na_2SO_4$ and ether was distilled off, the last traces being removed with nitrogen. The resulting red solid was crystallized from hexane to give 2,7-diiododiazofluorene (0.49 g) m.p. 159–60°C in 66% yield. Ultraviolet (methanol): 227 nm (ε 23,300), 244 (38,290), 248 (42,740), 275 (25,540), 300 (27,750), 311 (32,750), 342 (8,326), and 354 (7,769). The ir spectrum gave a characteristic diazo peak at 2050 $cm^{-1}$. The mass spectrum indicated the molecular ion at $m/z$ 444. Fragments corresponding to loss of nitrogen and iodine, i.e. $(M-N_2)^{+\cdot}$ and $(M-N_2-I)^{+\cdot}$, were also observed at 416, 289, and 162, respectively.

Molecular ion calculates for $C_{13}H_6N_2I_2$: $m/z$ 443.8621. Found: 443.8623.

*2-[$^{125}I$]Diazofluorene.* Fluorenone (4 mg, 22 μmol) was taken in glacial acetic acid (1 ml). [$^{125}$I]Sodium iodide (3 mCi in 0.1 mM NaOH) was then added along with cold NaI (3.25 mg, 22 μmol). Concentrated sulfuric acid (100 μl) and concentrated nitric acid (100 μl) were then added to this and the mixture was stirred at 50°C for 3 h. It was then poured into water and extracted with $CHCl_3$. The extract was exhaustively washed with 5% sodium thiosulfate and finally dried over anhydrous $Na_2SO_4$. The iodofluorenone so obtained was then taken in alcohol (1 ml). Hydrazine hydrate (50 μl of a 99–100% solution) was added and the mixture was refluxed for 2.5 h on a water bath. Alcohol was then evaporated using nitrogen and the residue was dried under vacuum. The oxidation step was carried out in the dark. The hydrazone was taken in ether (5 ml) and KOH (3 mg), water (100 μl) and purified yellow mercuric oxide (6 mg) were added. The mixture was stirred for 2.5 h in the dark and filtered and dried over $Na_2SO_4$. The ether was evaporated and the residue was taken in 500 μl hexane. This was purified by

column chromatography using neutral alumina as a support (10 × 70 mm; 3 g alumina). Elution was carried out with increasing amounts of benzene in hexane. 2-[$^{125}$I]DAF was eluted with 50% benzene in hexane. The purity of this fraction and concentration was determined by uv spectroscopy. The overall yield of the reaction was 31.8%. The specific activity was found to be 14.6 mCi/mmol.

*Photolysis of 2-[$^{125}$I]DAF in phosphatidylcholine vesicles.* Phosphatidylcholine (PC) was purified from hen eggs by Singleton's procedure (*16*). The PC so obtained was made free of any oxidized impurities by washing with water and then precipitating with acetone (*17*). The concentration of purified PC obtained was determined by phosphate assay (*18*). PC vesicles were prepared by first drying a film of PC under vacuum for 8 h and then sonicating it for 5 min in buffer (20 mM Tris–HCl in 100 mM NaCl, pH 8.2). The vesicles were then centrifuged at 10,000 *g* to remove any titanium particles shed from the sonifier during sonication. A 3.3 mM PC vesicle preparation in buffer was incubated with an aliquot of a 9.4 mM solution of 2-[$^{125}$I]DAF solution in alcohol for 1 h in the dark at 25°C. The final 2-[$^{125}$I]DAF concentration was 94 $\mu$M and alcohol concentration 1% (v/v), thus bringing the PC : probe molar ratio to 35 : 1. After incubation the vesicle solution was photolyzed using a Rayonet minireactor for 5 min and concurrently a control sample was kept in the dark for 5 min. Both samples were extracted with $CHCl_3$ : $CH_3OH$ (2 : 1 v/v), the solvent was evaporated, and the residue was analyzed spectrophotometrically. The control sample indicated that 2-[$^{125}$I]DAF had remained intact, whereas the photolyzed sample showed complete disappearance of absorption bands beyond 300 nm. The residue was then analyzed by TLC on silica gel plates using $CHCl_3$ : $CH_3OH$ : $H_2O$ (65 : 25 : 4 v/v) as the solvent system. A number of bands were then scraped and counted on a gamma counter.

*Transesterification of labeled PC.* The band corresponding to PC in TLC of labeled product was extracted with $CHCl_3$ : $CH_3OH$ (2 : 1 v/v) and the solvent was removed under nitrogen. The residue was dissolved in 9 ml of 2.5% methanolic HCl and refluxed for 2 h. Water was then added and the transesterified product was extracted with hexane, evaporated, and counted. The aqueous phase was also counted.

## RESULTS

*2-Iodo-9-diazofluorene and 2,7-diiodo-9-diazofluorene.* 2-I-DAF and 2,7-I-DAF were prepared from fluorenone in an overall yield of 42 and 46%, respectively, and fully characterized. 2-[$^{125}$I]DAF has been prepared in an overall yield of 31.8% from fluorenone without isolating any of the intermediates given in the scheme for synthesis of 2-I-DAF (Fig. 1). Even though we have prepared 2-[$^{125}$I]DAF with specific activity of only 14.6 mCi/mmol, much higher specific activity material can be prepared based on the experimental requirement.

Both 2-I-DAF and 2,7-I-DAF are quite stable in the solid state in the dark at room temperature. Alcohol solubility of 2-I-DAF and 2,7-I-DAF is 3 and 1.5 mg/ml, respectively. The solutions are stable in the dark at 4°C, but should preferably be preserved at −20°C. More concentrated solutions of the probes can

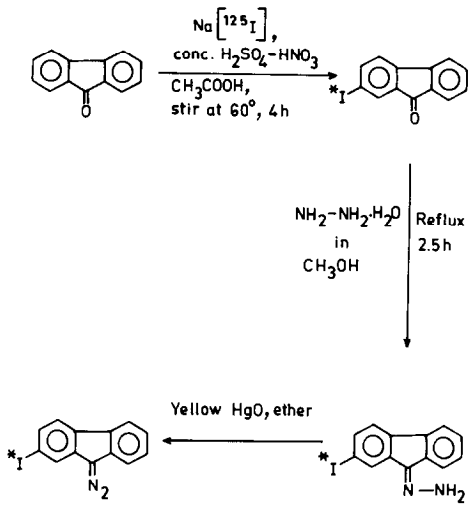


Fig. 1. Scheme used for synthesis of 2-[$^{125}$I]iododiazofluorene.

be prepared in tetrahydrofuran or dioxan in case a high probe to membrane lipid or protein ratio is desired, keeping the solvent concentration to less than 1% (v/v).

The uv-visible spectrum of DAF, 2-I-DAF, and 2,7-I-DAF is shown in Fig. 2. Interestingly the 237 and 291 nm absorptions of DAF shift to 242 and 299 nm in 2-I-DAF and to 248 and 311 nm in 2,7-I-DAF. The extinction coefficient of the 291 nm (17,740) absorption in DAF is 33% of its absorption at $\lambda_{max}$ 237 nm (53,850). In 2-I-DAF the intensity of 299 nm (30,120) absorption is 50% of its absorption at

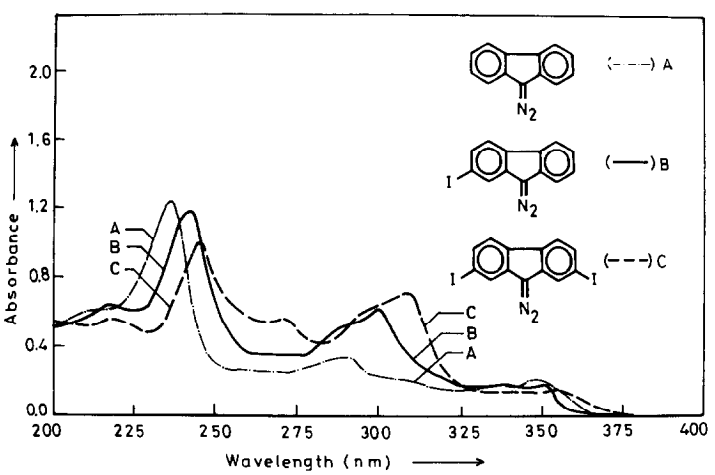


FIG. 2. Ultraviolet-visible spectra of diazofluorene (A), 2-iododiazofluorene (B), and 2,7-diiododiazofluorene (C) in methanol.

$\lambda_{max}$ 242 nm (59,740). Similarly in 2,7-I-DAF the corresponding absorption of 311 nm (37,250) is 77% of its absorption at $\lambda_{max}$ 248 nm (42,735). Thus addition of iodo groups to DAF results in an increase in absorption wavelength and intensity, beyond 300 nm.

*Photolysis of 2-I-DAF and 2,7-I-DAF in cyclohexane.* It is conventional to carry out the photolysis of a proposed membrane hydrophobic core photolabeling reagent in a nonpolar solvent-like cyclohexane to ensure its C-H insertion ability (*7*). Thus a 17 mM solution of 2-I-DAF was photolyzed in cyclohexane using a medium pressure mercury lamp in an annular photoreactor and 2-iodo-9-cyclohexylfluorene m.p. 120–121°C was isolated in 24% yield. The uv and ir spectra showed disappearance of diazo absorptions and the NMR spectrum clearly indicated the fluorene $C_9$-H as a doublet ($J$ = 3 Hz) at 3.90 ppm and the cyclohexyl protons as a multiplet between 0.8–1.8 ppm. The mass spectrum of the compound gave the molecular ion at $m/z$ 374 as expected and a fragmentation pattern corresponding to 2-iodo-9-cyclohexylfluorene. In a similar way, photolysis of 2,7-I-DAF in cyclohexane under identical conditions as in the case of 2-I-DAF gave 2,7-diiodo-9-cyclohexylfluorene m.p. 195–96°C which could be isolated in 25% yield. The uv and ir spectra showed the disappearance of diazo absorptions and the NMR spectrum indicated the $C_9$-H as a doublet ($J$ = 3 Hz) at 3.85 ppm, cyclohexyl protons appearing in the 1–2.1 ppm range. The aromatic proton region fully corroborated the structure. The mass spectrum gave the molecular ion at $m/z$ 500 and other fragments corresponding to loss of iodine and cyclohexyl units confirming the structure as 2,7-diiodo-9-cyclohexylfluorene.

The time course of 2-I-DAF and 2,7-I-DAF photolysis in cyclohexane was carried out in a Rayonet minireactor using four 3000 Å lamps. The photolysis was followed by observing the disappearance of the 2050 $cm^{-1}$ diazo absorption in the ir spectrum. Marked deviations from first-order kinetics were observed specially at a higher concentration, i.e., 50 mM. At lower concentrations near-linear plots were obtained. Thus for a 5.2 mM solution of DAF, 2-I-DAF, and 2,7-I-DAF, $t_{1/2}$ values of 37, 19, and 8 min were obtained. The values are derived from first-order

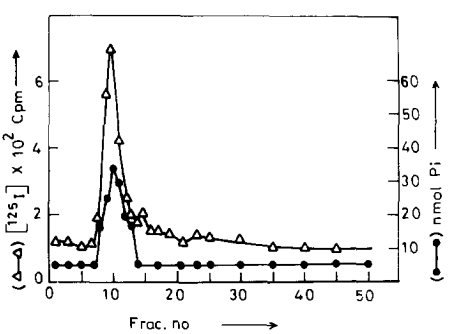


Fig. 3. Partitioning of 2-[$^{125}$I]iododiazofluorene in PC vesicles. 2-[$^{125}$I]DAF (0.18 μmol) was incubated with PC vesicles (8.3 μmol) in 20 mM Tris–HCl, 100 mM NaCl, pH 8.2, (2 ml) in the dark. The mixture was then passed over a Sephadex G-50 column preequilibrated with the same buffer. Fractions of 1 ml were collected and each fraction was analyzed for phosphate (●) and $^{125}$I (Δ).

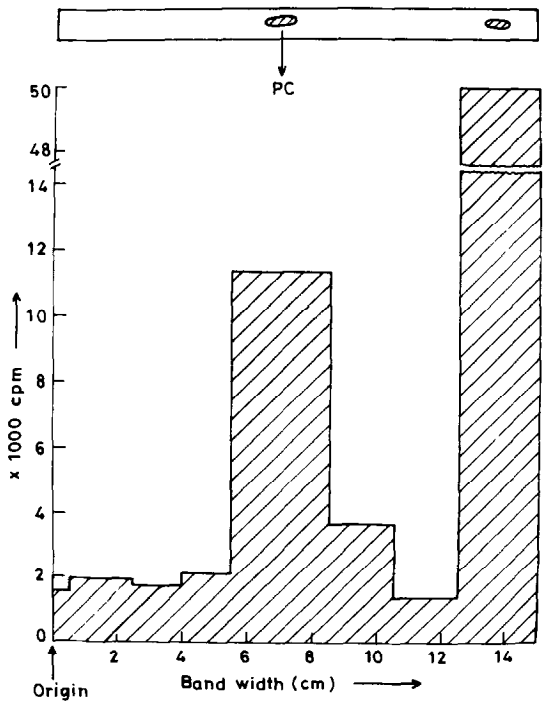


Fig. 4. Radio TLC of photolyzed products obtained after labeling of PC vesicles with 2-[$^{125}$I]DAF (s/s $CHCl_3$ : $CH_3OH$ : $H_2O$, 65 : 25 : 4 v/v).

approximations and should at best be taken as indicators of relative photolysis rates at the specified concentration.

*Partitioning and photolysis of 2-[$^{125}$I]DAF in phosphatidylcholine vesicles.* PC vesicles were incubated with 2-[$^{125}$I]DAF in the dark for 1 h and passed over a Sephadex G-50 column. The PC : probe molar ratio used was 35 : 1. The PC vesicles appeared in the void volume (Fig. 3) indicating that 2-[$^{125}$I]DAF partitions into the PC vesicles. The partition coefficient was determined by equilibrium dialysis essentially according to the procedure of Bayley and Knowles (*19*) in order to compare the data. A value of 15,000 was obtained by this method for partitioning of 2-I-DAF in PC vesicles.

The photolysis product of PC vesicles incubated with 2-[$^{125}$I]DAF was analyzed by TLC (Fig. 4). Of the applied radioactivity 13.9% was associated with the PC band ($R_f$ 0.45) and 70% with the solvent front ($R_f$ 0.83–1.0), corresponding to the photodecomposition products of I-DAF. These are usually the corresponding bifluorenyl, bifluorenylidene, and fluorenone derivatives, which have been identified in the case of DAF (*20*). Further analysis of labeled PC involved its extraction from the silica gel band corresponding to PC and transesterification of the extract. More than 99% of the radioactivity was found to be associated with the fatty acid methyl ester fraction and less than 1% with glycerophosphorylcholine fraction.

## DISCUSSION

Both 2-I-DAF and 2,7-I-DAF absorb above 300 nm with molar extinction coefficients greater than 7000. On the other hand, other carbene-based photoactivable reagents which absorb above 300 nm and are used in studying membranes have very low molar extinction coefficients, e.g., phenyldiazirine (382 nm, $\varepsilon$ 299), adamantyldiazirine (372 nm, $\varepsilon$ 245) (*6*), and 3-trifluoromethyl-3-(*m*-iodophenyl)-diazirine (353 nm, $\varepsilon$ 266) (*7*). Successive iodo substitution in DAF shifts the absorption to longer wavelength with an increased molar extinction coefficients (Fig. 2) leading to shorter duration of photolysis as seen in the relative rate of photolysis in cyclohexane. The formation and characterization of the cyclohexane insertion products further establishes the ability of these molecules to give C-H insertion products.

2-[$^{125}$I]DAF can be easily prepared from readily available fluorenone and [$^{125}$I] sodium iodide in an overall yield of over 30%. The final purification only involves alumina column chromatography. Further, 2-[$^{125}$I]DAF easily partitions into PC vesicles and has a higher partition coefficient (15,000) compared to phenyl azide (420) (*19*) and adamantyldiazirine (4700) (*6*). The ease of partitioning of I-DAF in PC vesicles and near exclusive (>99%) labeling of the fatty acyl portion demonstrates its ability to label the hydrophobic core of membranes. The 13.9% labeling of egg PC observed with 2-[$^{125}$I]DAF is better than the insertion reported with probes such as phenyldiazirine and adamantyldiazirine which result in 5.3 and 2.5% insertion in dimyristoyl lecithin vesicles and 9.8 and 5.0% insertion in dioleoyl vesicles, respectively (*6*). The specificity and the high insertion yields into the relatively nonpolar hydrophobic membrane core indicate that 2-[$^{125}$I]DAF would be a useful probe for studying membrane structure. Our studies with these probes in natural membranes are in progress.

In conclusion the availability of simple photoactivable reagents capable of labeling membrane hydrophobic core provides a convenient method for identifying a membrane-bound protein as integral or peripheral and can provide useful information on the membrane-spanning protein fragment. The convenient method reported here for the synthesis and purification of 2-[$^{125}$I]DAF will thus be useful in studying the structure of integral membrane bound proteins.

## ACKNOWLEDGMENT

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*Note added in proof.* Subsequent to the submission of this manuscript we noticed that Ray and Schuster (1987, *Photochem. Photobiol.* **45,** 439–443) have reported nonradioactive 2,7-diiodo-9-diozofluorene as a photoactivable reagent, though surprisingly they make no mention of our earlier preliminary report on this reagent in 1984 (see Ref. (*11*)).

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