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Photogenerated Reagents for Membrane Labeling. 1. Phenylnitrene Formed within the Lipid Bilayer†

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ABSTRACT: Phenylnitrene generated photochemically from phenyl azide that is bound to artificial phospholipid vesicles labels the fatty acid chains of the lipids in low yield. The labeling yield varies from approximately 3.3% with soybean lecithin (which is highly unsaturated) to approximately 0.25% with dimyristoyllecithin (which is completely saturated). Labeling is largely eliminated by reduced glutathione in the aqueous phase. Nitrenes are evidently unsatisfactory reagents for the labeling either of lipids or by analogy of the hydrophobic portions of membrane proteins. This is mainly because the long lifetimes and electrophilic character of nitrenes will lead to the preferential labeling of extrinsic membrane components. Phenyl azide itself is further compromised as a lipophilic reagent by its rather low partition coefficient into lipid bilayers, as measured by equilibrium dialysis.

During the past 10 years or so, a vague but nevertheless useful model for the structure of biological membranes has become accepted, in which proteins and glycoproteins are considered to be associated in various ways with a more or less fluid bilayer of lipid. The proteins and the lipids are vectorially arranged with respect to the two faces of the bilayer (the inside and the outside), and, of late, considerable effort has been devoted to the definition of this vectorial organization. Surface labeling of exposed membrane components using a variety of chemical reagents (Bretscher, 1971) continues to provide the most important information in this area (Hubbard & Cohn, 1976; Rothman & Lenard, 1977). Recently, photochemical surface labeling has been used in an attempt to overcome the reactivity limitations of ordinary chemical reagents (Staros & Richards, 1974).

The identification and characterization of membrane components situated *within* the bilayer have, however, been much less thorough. Such components may be whole molecules buried in the bilayer [e.g., small proteolipids (MacLennan et al., 1972)] or they may be parts of molecules [e.g., the hydrophobic tail of cytochrome b_5 (Spatz & Strittmatter, 1971), the hydrophobic peptide of glycophorin that is believed to cross the membrane (Marchesi et al., 1976), or multiply spanning segments as proposed for band 3 protein from human red blood cell membranes (Jenkins & Tanner, 1975)]. The location of such lipophilic components has been indirectly inferred from

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peptide sequence data, from the solubility characteristics of derived peptides, from surface-labeling experiments, or from the results of in situ proteolytic digestion. In one instance, that of the purple membrane protein from *Halobacterium halobium*, direct evidence for a transmembrane location has been obtained by high-resolution electron microscopy (Henderson & Unwin, 1975). There is, however, a dearth of general methods that would complement the surface-labeling reagents by labeling those parts of membrane proteins that are embedded in the lipid bilayer. It is obvious that if labeling of superficial components is to be avoided, the reagent must be generated within the membrane. This fact, together with the lack of chemical reactivity of intramembrane components (whether these be the lipids themselves or hydrophobic parts of membrane proteins), makes a lipophilic or amphipathic photogenerated reagent (Knowles, 1972; Bayley & Knowles, 1977) the most appropriate choice.

Amphipathic reagents have been developed (Bayley, 1974; Chakrabarti et al., 1974; Stoffel et al., 1976), but as yet only one rather unsatisfactory application to a biological system has appeared (Stoffel et al., 1977).

The use of lipophilic photogenerated reagents for membrane labeling was first reported by Klip and Gitler (1974). In this and in later work (Klip et al., 1976) the extent of protein labeling was low and the site of labeling within the protein was not well characterized. Lipid labeling appeared to be high but the labeled lipids were not identified. Mohiuddin et al. (1976) have labeled high-density lipoproteins from plasma with phenyl azide, and Nieva-Gomez & Gennis (1977) have used pyrene azide to label *E. coli* membranes. Both these papers report only minimal characterization of the products. Abu-Salah and Findlay (1977) have labeled egg-yolk lecithin and myelin with phenyl azide, and this work is discussed in the present paper. Recently, a careful study (Bercovici & Gitler, 1978) has appeared in which membrane proteins have been labeled in high yield with 5-iodonaphthyl 1-azide. The authors present evidence that only intrinsic proteins are labeled. However, when this reagent was used to label purified (Na^+, K^+)ATPase and the site of labeling was partly defined by proteolytic digestion of the labeled membranes, not all of the membrane-bound peptides were labeled (Karlish et al., 1977).

It is apparent that no clear-cut conclusions about the usefulness of such photogenerated reagents in the study of uncharacterized membrane systems can be drawn until detailed studies of the appropriate model systems have been completed. It is to this end that the present work is directed. Phenylnitrene has been generated photochemically within several artificial phospholipid bilayers and the nature and extent of lipid labeling has been evaluated. The implications for the design of both hydrophobic and amphipathic photolabile reagents are discussed. In the following paper of this issue, we compare these results with those obtained using analogous carbene reagents.

Materials and Methods

All chemicals and buffer components were commercial grades of high purity. [^{14}C]Aniline and [^{3}H]aniline were obtained from Amersham-Searle. Lipids and glutathione were obtained from Sigma. The synthetic lipids were at least 98% pure in the fatty acid side chains (as determined by GLC).

Phenyl Azide. Aniline (10 mL, 110 mmol, distilled under N_2 from Zn dust) was dissolved in aqueous HCl (6 M, 100 mL) and cooled to 0 °C. Dropwise addition of $NaNO_2$ (7.15 g, 104 mmol, in 20 mL of cold water) was followed by NaN_3 (7.97 g, 123 mmol, in 30 mL of cold water). After stirring for 1 h at room temperature, the oil was separated, washed with 0.1 N K_2CO_3 (50 mL), and filtered through anhydrous Na_2SO_4. Distillation in vacuo from molecular sieves was carried out on a 1-g scale, with the distillation flask held below 40 °C at all times to prevent explosion. Phenyl azide was obtained as a pale-yellow liquid, of density 1.106 ± 1.5% at 22 °C, and λ_{max} 250 nm, ϵ 10 200 ± 3% (in ethanol). Literature values for these properties vary considerably. Shoulders were visible at 277 and 286 nm. All samples of phenyl azide were stored at −20 °C in the dark.

[^3H]*Phenyl azide* was prepared from [G-^{3}H]aniline (5 mCi) as described above, but the product was not distilled. The labeled material had an ultraviolet spectrum identical to that of the unlabeled compound and showed only one diffuse radioactive component by thin-layer chromatography. The specific radioactivity (1.4 mCi/mmol) was determined spectrophotometrically. For most labeling experiments, a solution (0.44 mM) in ethanol was used.

[^{14}C]*Phenyl azide* was prepared from [U-^{14}C]aniline (50 μCi) as described above. The product was extracted with pentane and isolated by careful removal of the solvent by distillation. The specific activity was 2.2 mCi/mmol.

Photochemical reactions were followed using a Pye-Unicam SP1800 spectrophotometer. All other spectral measurements were made using a Perkin-Elmer 575 instrument. Thin-layer chromatography was performed on Merck silica gel 60 plates of 0.25-mm thickness. A Beckman LS-233 scintillation counter was used for all radioactivity measurements. The scintillation fluid contained toluene (2460 mL), ethanol (1120 mL), naphthalene (210 g), PPO[1] (10.5 g), and POPOP (0.42 g) and was used unless otherwise stated. Other fluids contained PPO (0.5%, w/v), naphthalene (10%, w/v) in dioxane (Abu-Salah & Findlay, 1977), or toluene (1240 mL), Triton X-100 (500 mL), and Liquifluor (60 mL; from New England Nuclear).

Preparation of Phospholipid Vesicles. Lipid (100 mg) from which solvent had been removed by exhaustive pumping was vortexed with buffer [10 mL of 20 mM Tricine-HCl, pH 8.15, containing NaCl (100 mM)] until a homogeneous opaque suspension was obtained. This suspension was ultrasonically irradiated (Heat Systems W-350 sonifier, large probe tip, power setting 2.5, 0.5-s pulse, 0.5-s delay) for approximately 15 min under nitrogen. The vessel was surrounded by ice water. The resulting clear suspension was centrifuged at 27 000*g* for 30 min at 25 °C. Phosphorus assay (Veerkamp & Broekhuyse, 1976) showed that the phospholipid concentration in the supernatant was close to 10 mg/mL. Chromatography of the supernatant on Sepharose 4B showed the presence of small vesicles. No lipid decomposition could be detected by thin-layer chromatography. Vesicles from soybean lecithin, egg-yolk lecithin, and dioleoyllecithin were stored at 4 °C and discarded after 1 week. Vesicles from dimyristoyllecithin were used within 24 h.

Vesicle Labeling. Vesicles (1 mL of 10 mg/mL) were transferred by syringe into a small quartz tube containing a magnetic stirring bar and fitted with a septum. Before this transfer the tube was flushed with N_2. The vesicles were stirred under a gentle stream of wet N_2 for at least 1 h. A portion (10 μL) of [^{3}H]phenyl azide in ethanol was then added, and stirring was continued in the dark for 1 h under an N_2 atmosphere maintained with a gas-filled balloon (a stream of N_2 rapidly removes the volatile phenyl azide). For experiments in air, the

[1] Abbreviations used are: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

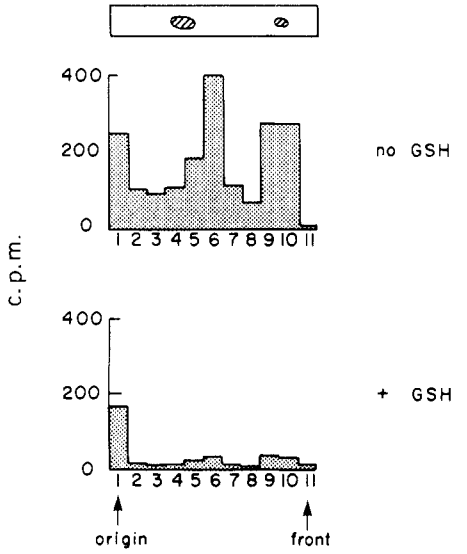

FIGURE 1: Chromatography of dialyzed products from the labeling of egg-yolk lecithin by phenylnitrene. Egg-yolk lecithin (10 mg/mL) in the Tricine-NaCl buffer, pH 8.15, was incubated with [^{3}H]phenyl azide (3.0 mM) for 105 min at 25 °C. The mixture was then photolyzed for 150 s at 254 nm. Reactions were done in the absence (upper) and presence (lower) of reduced glutathione (15 mM). The glutathione was added 5 min before photolysis. After exhaustive dialysis and freeze-drying (see Materials and Methods), a portion (90 μg of lipid) was subjected to thin-layer chromatography on silica gel using chloroform–methanol–NH_4OH (7 M) (90:55:11, v/v) as the eluent. The recovery of radioactivity from the TLC plate was approximately 60%. The hatched areas in the diagram of the TLC plate represent the I_2 vapor staining pattern.

N_2 purging was omitted. For experiments at 40 °C, the preincubation was for 30 min only, to avoid losses of phenyl azide. Portions (5 or 10 μL) were then removed for scintillation counting, and the mixture was irradiated for 4 half-lives of the reagent (150 to 210 s, 3 cm from the center of a single RPR 2537-Å lamp in a Rayonet minireactor; alternatively for 90 min, 3 cm from a RPR 3000-Å lamp). Vigorous stirring was maintained during the photolysis, and the disappearance of phenyl azide was monitored spectrophotometrically.

After the photolysis, portions were removed for scintillation counting, and the mixture was then dialyzed exhaustively against 2 mM sodium phosphate buffer, pH 7.0, containing 0.5% ethanol at 4 °C for 2 days. Any volume change was recorded, and the mixture was freeze-dried in portions of 200 μL. These were stored over P_2O_5 at 4 °C until analyzed (within 24 h).

Analysis of Labeled Lipid. A freeze-dried portion (above) was redissolved in chloroform (200 μL) and two samples (10 μL of this solution) were subjected to thin-layer chromatography on aluminum-backed silica gel plates, eluting with chloroform–methanol–NH_4OH (7 M) (90:55:11, v/v). One lane was stained with iodine vapor, and the other lane was cut into 11 pieces (1 cm^2), which were soaked in their scintillation fluid for 2 days before counting.

To analyze the fatty acids, a freeze-dried portion (200 μL) of labeled lipid was incubated with a solution (50 μL) of sodium methoxide (1 M) in benzene–methanol (2:3, v/v) [prepared from purified sodium and dry solvents according to Glass (1971)]. After 8 min at room temperature, the product was partitioned in the two-phase system of chloroform–butanol–water (4:2:3, v/v; Abu-Salah & Findlay, 1977). Each phase was subjected to TLC on silica gel plates, eluting with methanol–chloroform (3:1, v/v) or chloroform–methanol–water (13:5:1, v/v), and the plates were cut and counted as described above.

TABLE I: Yields of Labeling of Different Lipids by Phenylnitrene.[a]

Lipid	Av yield[b]	Ratio[c]	Deg of unsaturation[d,g]	Doubly allylic methylenes[e,g]
Soybean lecithin	3.3 ± 1.2	1.0	2.9	1.3
Egg-yolk lecithin	2.6 ± 0.7	0.79	1.5	0.55
L-α-Dioleoyllecithin	0.75 ± 0.25	0.23	2.0	0
L-α-Dimyristoyllecithin	0.25 ± 0.15	~0.08[f]	0	0

[a] All experiments were done at room temperature, except those with dimyristoyllecithin which were done at 40 °C. Dioleoyllecithin gives similar yields at both 25 and 40 °C. [b] The percentage of phenyl azide present before photolysis that inserted into phospholipid. Mean of at least three experiments. [c] Relative to soybean lecithin. [d] The average number of carbon–carbon double bonds per phospholipid molecule. [e] The average number of doubly allylic methylene groups per phospholipid molecule. [f] This low value is less precise than the others. [g] Data for *d* and *e* are from Debuch (1957) and from Hanahan et al. (1960).

Solubility Measurements. Phenyl azide (50 μL) was shaken with buffer solution (3 mL) at 25 °C in a sealed vessel for 18 h. The mixture was allowed to stand for 1 h and the supernatant was then centrifuged at 25 °C. A portion of the supernatant after centrifugation was diluted for spectrophotometric determination of phenyl azide. In quadruplicate samples the measured solubility deviated by $\lesssim$2.5% from the mean.

Equilibrium Dialysis. A portion of vesicles (10 mg of lipid in 1 mL) in a small dialysis bag was immersed in buffer solution (5 mL) in a stoppered tube. Phenyl azide solution in ethanol was added to the buffer [final ethanol concentration, 1% (v/v); final phenyl azide concentration averaged over the whole sample, ~0.3 mM]. The system was allowed to equilibrate at 4 °C, the buffer solution being stirred magnetically. For measurements above 25 °C, equilibrium was first established at 4 °C before final equilibration at the higher temperature. This procedure minimized lipid hydrolysis. Phenyl azide concentrations inside and outside the dialysis tube were determined spectrophotometrically. Volume changes were found to be negligible.

Results

Photochemical Labeling of Lipid Vesicles with Phenyl Azide. The results from a typical labeling experiment with egg-yolk lecithin vesicles are shown in Figure 1. After exhaustive dialysis of the irradiated sample, 11.7% of the original radioactivity remained associated with the vesicles. After freeze-drying, followed by dissolution in chloroform, 10.0% of the radioactivity remained. Figure 1 shows the distribution of the label on TLC. The radioactive peak from fractions 5–7, which runs just ahead of the free lipid peak that is stained by iodine, contained 26% of the radioactivity applied to the plate assuming that the recovery of each component from the silica was the same.[2] The estimated percentage of counts incorporated into the lipid fraction is therefore approximately 2.6%. When similar experiments were performed with a number of different lipid types, the results summarized in Table I were obtained. While the absolute labeling levels were somewhat

[2] Extraction of the labeled components from a preparative chromatogram by a method which quantitatively recovers lipid, followed by scintillation counting of each fraction, gave essentially the same yield. The yield was also independent of the scintillation solvent used (see Materials and Methods).

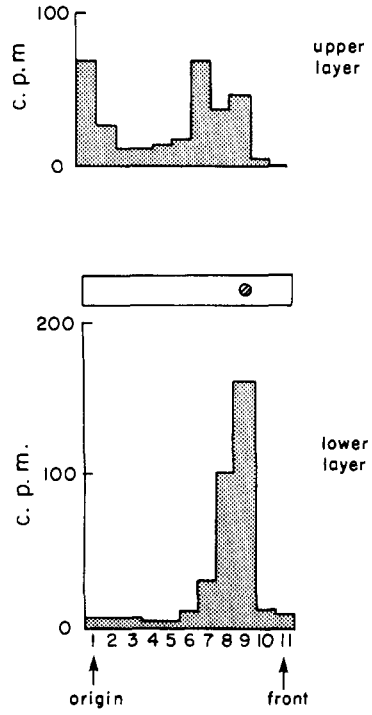

FIGURE 2: Chromatography of transesterified products from the labeling of egg-yolk lecithin by phenylnitrene. The sample of labeled lipid was subjected to methanolysis, and then partitioned in the two-phase system chloroform–butanol–water (4:2:3, v/v) according to Abu-Salah and Findlay (1977). A quantitative recovery of the fatty acid methyl esters was obtained in the lower phase which was analyzed chromatographically as described under Materials and Methods. The hatched area in the diagram of the TLC plate represents the I_2 vapor staining pattern for the lower phase.

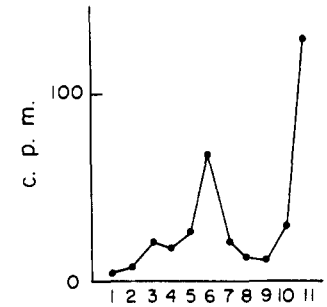

FIGURE 3: The nature of the radioactive materials that run as "fatty acid methyl ester" in the chromatographic analysis of Figure 2 (lower, fractions 8–9). Fractions from a preparative lipid separation (analogous to the analytical separation shown in Figure 1) were subjected to transesterification and then analyzed (as shown in Figure 2, lower) for radioactivity that *runs as* fatty acid methyl ester. Ordinate: cpm in fractions 8–9 of an analytical chromatographic separation (as Figure 2, lower). Abscissa: fraction number from the preparative lipid separation done prior to transesterification and analysis. For further details, see the text.

variable, the ratios of yields for different lipids in parallel experiments were always close to the values shown in Table I. Labeling yields (from egg-yolk lecithin vesicles) were found to be lower if the dialysis following photolysis was performed at 25 rather than at 4 °C. This evidently derives from some instability of the link between the label and the lipid, since the lipid itself was recovered intact after such treatment.

The yield of labeled product was unaffected by lowering the concentration of ethanol, by performing the photolysis under nitrogen, by prolonging the incubation time before photolysis to 4 h, or by irradiating the preparation at 300 rather than at 254 nm. Using egg-yolk lecithin, the extent of labeling correlated well with the extent of photolysis of the phenyl azide measured spectrophotometrically. Other control experiments demonstrated that *all* the radioactivity was lost in the dialysis step if the photolysis step was omitted. When [^{3}H]phenyl azide in buffer was photolyzed in the absence of lipid vesicles and subjected to the analytical procedure, virtually all the radioactivity was lost on dialysis and freeze-drying. The small amount that remained (0.1–0.3%) chromatographed solely at the position of fractions 9 and 10 (Figure 1). Analysis of a mixture made by mixing *prephotolyzed* phenyl azide and vesicles revealed radioactivity near the origin and near the solvent front but none in fractions 5–7. The peaks near the origin and the front of the chromatogram (Figure 1) evidently arise from the photodecomposition product(s) of phenyl azide which binds tightly but noncovalently to the liposomes. The radioactivity in fractions 5–7 derives from the labeling of the phospholipid. To check on the possibility that tritium from [^{3}H]phenyl azide was being lost photochemically, experiments with egg-yolk lecithin and dioleoyllecithin were also carried out with [^{14}C]phenyl azide. Closely similar results were obtained.

Effect of Glutathione on Labeling by Phenylnitrene. The effect of reduced glutathione on the labeling of egg yolk lecithin by [^{3}H]phenylnitrene is illustrated in Figure 1. Phenyl azide undergoes negligible reaction with glutathione in the dark at pH 8 (Staros et al., 1978) and the absorption of glutathione does not affect the photolysis of phenyl azide at 254 nm under the conditions of the experiment. The presence of reduced glutathione in the aqueous phase reduces the extent of lipid labeling approximately eightfold. Experiments with soybean lecithin gave similar results even when the glutathione concentration was as low as 6 mM.

Analysis of the Fatty Acid Methyl Esters Derived from Labeled Lipid. When the fatty acid methyl esters prepared by methanolysis of labeled lipid were analyzed by thin-layer chromatography, the apparent yield of radiolabeled product was invariably higher than that based upon the direct lipid analysis described above. For example, when the transesterification products from an egg-yolk lecithin experiment were analyzed by the methods used by Abu-Salah and Findlay (1977), there were about twice as many counts in the fatty acid methyl ester region (see Figure 2) as had been seen in the whole lipid region when the lipids were analyzed directly (Figure 1). The origin of this radioactivity was investigated as follows. When photolabeled lipid (*not* esterified) was analyzed in the TLC system used for fatty acid methyl esters, the lipid was found to run at low R_f, and a large peak of radioactivity was observed at the R_f of fatty acid methyl ester. It appears, therefore, that a photodecomposition product of phenyl azide cochromatographs with labeled fatty acid methyl ester in this system. To check this, labeled lipid (1 mg) was subjected to preparative TLC under the conditions of Figure 1. Each fraction from this separation was then eluted from the silica gel with chloroform–methanol–water (10:5:1, v/v) and the solvent removed. Transesterification of the residues with sodium methoxide was followed by the partitioning step to isolate the "fatty acid methyl esters" which were then analyzed [Figure 2 (lower)]. In Figure 3 is plotted the amount of radioactivity in each fraction that runs as "fatty acid methyl ester" after transesterification. It is clear from this experiment that photodecomposition products of phenyl azide (which run well ahead of labeled lipid in lipid analysis such as that shown in Figure 1) must be removed before any transesterification step is performed, otherwise these products may be erroneously designated as photolabeled fatty acid.

Solubility and Partitioning of Phenyl Azide. To establish the location of the phenyl azide before the photolysis, the solubility of the reagent and its binding to vesicles were measured. Under the conditions of our experiments, the solubility of phenyl azide in water is 4.2 mM, and in 50 mM sodium phosphate buffer, pH 8.0, it is 3.8 mM. The presence of 1% (v/v) ethanol has a negligible effect on these values.

The binding of phenyl azide to the lipid vesicles was investigated, and the following binding constants (K) were obtained [K = (ligand bound/mg of lipid)/(free ligand/μL of external solution)]. For egg yolk lecithin, $K_{4°C}$ = 415, $K_{25°C}$ = 420; for L-α-dioleoyllecithin, $K_{4°C}$ = 450, $K_{25°C}$ = 370; and for L-α-dimyristoyllecithin, $K_{4°C}$ = 95, $K_{40°C}$ = 330. The phase-transition temperatures for these three lipids are −15 (broad), −22, and +23 °C, respectively (Chapman, 1975).

Discussion

The results described above show that the phospholipid molecules in single bilayer vesicles are subject to photolabeling in low yield with the photosensitive lipophilic compound phenyl azide. The extent of labeling roughly correlates with the degree of unsaturation and the number of doubly allylic methylene groups in the fatty acid side chains (see Table I), which suggests that most of the labeling occurs in this part of the lipid. Autoradiography of a chromatogram of dioleoyllecithin labeled with [^{14}C]phenyl azide reveals two barely separated components that run just ahead of the unlabeled lipid. It is possible that these products arise either from nitrene insertion into the two differently situated fatty acid side chains, from the two product aziridine diastereoisomers, or from partial ring-opening of the first-formed aziridine during workup. The labeled products from lipids containing heterogeneous fatty acid side chains are presumably more complex.

As is evident from Table I, the yields of labeled lipid are not high. Since the initial concentration of phenyl azide in the bilayer is large (approximately 0.25 M), the low labeling of lipid demonstrates the limitations of phenylnitrene, much of which must be dimerizing or reacting with water and buffer components at the bilayer surface. This view is quite consistent with the relatively long lifetime (and comparatively low reactivity) of arylnitrenes. To confirm this, we have performed labeling experiments in the presence of reduced glutathione, which was predicted to act as a scavenger of nitrene and of other reactive species derived from it, *in the aqueous phase* of the vesicle suspension. Figure 1 shows that glutathione indeed reduces the labeling of lipid, which suggests that phenylnitrene (or a reactive intermediate derived from it) generated within the bilayer has a half-life long enough to diffuse to the bilayer surface where it may be scavenged efficiently.

The second possible disadvantage of nitrenes as lipid reagents is their well-known predilection for electron-rich bonds. That is, nitrenes are electrophilic. This means that a nitrene may preferentially react with such nucleophilic groups as may be available to it, such as water, buffer components, the surface residues of extrinsic proteins, and the extramembrane portions of intrinsic proteins. This view is supported by the very low labeling of the saturated hydrocarbon chains of dimyristoyllecithin (Table I) even in the absence of an added scavenger. It seems that very little insertion into unactivated carbon–hydrogen bonds occurs. Furthermore, when phenylnitrene is generated within the membrane of erythrocyte ghosts (Goldman & Bayley, unpublished work), most of the membrane proteins *as well as* exogenous proteins that can bind to the cell surface (such as lysozyme) are labeled. The protein labeling is largely eliminated by the addition of glutathione. The longevity and chemical selectivity of nitrenes may therefore lead to preferential labeling of membrane components that are exposed to solvent, *even though* the reagent may have been generated within the bilayer.

In agreement with this is the selectivity of *N*-(4-azido-2-nitrophenyl)taurine (a water-soluble nitrene precursor) in labeling the cell-surface components of human red blood cells, which cannot be entirely attributed to the degree of exposure of the various labeled proteins (Staros et al., 1975). Besides the possibility suggested by these authors that there are different local concentrations of reagent at various parts of the cell surface due to electrostatic interactions, functional-group selectivity of the nitrene may also account for their observations. On the other hand, the results of Matheson et al. (1977) show the almost random modification of amino acids including the hydrophobic residues of ribonuclease A on flash photolysis of the enzyme in a solution containing *N*-(4-azido-2-nitrophenyl)taurine. Only glycine, glutamate (which were relatively little affected), and arginine (which was heavily modified) deviated very significantly from the mean "labeling" level. It is, however, not clear that nonselective insertion by nitrene produced the observed modifications. The enormous concentration of free radicals produced after the flash by hydrogen atom transfer to the nitrenes, could give rise to intramolecular cross-linking and other modifications not observed in more gentle photolyses.

Another weakness of phenyl azide as a membrane reagent is its relatively low partition coefficient into lipid bilayers. The binding constants reported here show that in our experiments 75–80% of the azide was noncovalently bound to the vesicles before photolysis, at a high lipid concentration (10 mg/mL). The partition coefficient of phenyl azide into red blood cell ghosts is considerably lower (unpublished results).

The danger of misinterpreting labeling patterns obtained with arylnitrenes is not necessarily alleviated by the use of more hydrophobic precursors, since the site of nitrene generation may bear little or no relation to the ultimate site(s) of reaction. It would be of considerable interest to know exactly which residues are labeled by the reagent used by Bercovici and Gitler (1978) (5-iodonaphthyl 1-azide) which appears to label only intrinsic proteins. That only the externally situated parts of these proteins or the few nucleophilic groups present within the bilayer have been selectively modified cannot be ruled out. Indeed, these authors observed that the ratio of protein to (presumed) lipid labeling decreases with increasing reagent concentration, which suggests the presence of saturatable, easily labeled residues on the proteins in question or an increase in label dimerization that was mistaken for lipid labeling. Furthermore, when the reagent was applied to (Na^+,K^+)-ATPase (Karlish et al., 1977) the large subunit (100 000 daltons) was selectively labeled. When the membranes containing the labeled enzyme were treated with trypsin, less than 10% of the label was released into the supernatant, although 50–55% of the protein was released, but most of the remaining membrane-bound peptides were *not* labeled. On gel electrophoresis of the digest, all the membrane-bound radioactivity was found in a broad band at approximately 12 000 daltons, implying that selective labeling of only a portion of the largely hydrophobic segments occurred. On the basis of the present work, we should expect that surface residues of nucleophilic character have been attacked from the membrane interior by the lipophilic nitrene.

Even photolabile groups bound to amphipathic molecules such as lipids (Chakrabarti & Khorana, 1975) may present similar difficulties. Here, the photolabile substituent may loop back to the membrane surface as has been suggested for

spin-labeled lipids (Cadenhead et al., 1975), though this need not be a permanent configuration. The lipophilic photoactivated cross-linking reagents of Mikkelson and Wallach (1976) and of Huang and Richards (1977) may also need reevaluation with the problem of nitrene electrophilicity in mind.

While this work was in progress, Abu-Salah and Findlay (1977) reported the labeling of egg-yolk lecithin by phenylnitrene. We have been unable to confirm the report of these workers that a labeling yield of around 12% can be achieved with this system. Indeed, our results suggest why the estimate of Abu-Salah and Findlay (1977) may be erroneously high. First, if prephotolyzed phenyl azide is mixed with vesicles, we have shown that it is impossible to remove more than approximately 90% of the radioactivity by exhaustive dialysis. (This is in contrast to the effective removal of all *un*photolyzed phenyl azide by this method.) This means that the residual radioactivity after dialysis of photolabeled vesicles (which those authors used as a measure of labeling efficiency) is largely a measure of *noncovalently bound* photolysis products of phenyl azide. Only by thin-layer chromatography of the whole lipid can these products be separated from bona fide labeled lipid (see Figure 1). Secondly, we have shown (Figure 3) that a large fraction of the radioactivity that cochromatographs with the fatty acid methyl esters (after lipid transesterification) derives from the photolysis products of phenyl azide that are seen even in the absence of lipid. Since Abu-Salah and Findlay (1977) chromatographed the methyl esters but not the lipids themselves, their estimate of the amount of label attached to fatty acid was too high. [In control experiments, we have shown that use of the unusual Tris–lysine buffer employed by Abu-Salah and Findlay (1977) does not increase the labeling yield.] Thirdly, these authors suggest that some 2% of label is incorporated into the head groups of the phospholipids. Yet the fact that <0.25% of label is incorporated into dimyristoyllecithin (Table I) shows that this cannot be generally true. Finally, our findings that not all the nondialyzable radioactivity is associated with lipid and that the radioactivity that *is* associated with lipid does not cochromatograph exactly with the unlabeled lipid (Figure 1) casts doubt upon the claim that there is an even distribution of photolabel amongst the various lipid classes comprising the myelin bilayer.

In summary, we have shown that phenylnitrene generated within a lipid bilayer will insert in rather low yield into unsaturated fatty acid chains and in even lower yield into saturated lipid. Most of the internally generated phenylnitrene can be scavenged by a thiol in the aqueous phase. We must conclude that nitrenes are unsatisfactory reagents for the labeling not only of lipids but also of the hydrophobic amino acid side chains of membrane proteins that are contiguous with lipid. In the following paper of this issue, the superiority of carbenes for these purposes is demonstrated.

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