Photogenerated Reagents for Membrane Labeling. 2. Phenylcarbene and Adamantylidene Formed within the Lipid Bilayer†

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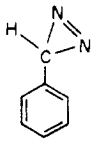
ABSTRACT: Phenylcarbene and adamantylidene have been generated photochemically from the corresponding diazirines within lipid bilayers. Reasonable yields of labeled fatty acid side chains have been observed. The products have been characterized by gas chromatography–mass spectrometry and derive both from the insertion of the carbene into carbon–hydrogen bonds of saturated fatty acids and from the addition of the carbene to the carbon–carbon double bonds of unsaturated fatty acids. In contrast to the results found using phenylnitrene, the lipid labeling by carbene is not reduced by the water-soluble scavenger glutathione. Carbenes generated from diazirines are evidently superior reagents for the photolabeling of lipids and should be useful for identifying the intrinsic hydrophobic sections of membrane proteins.

In the preceding paper of this issue (Bayley and Knowles, 1978), we have presented a study of the efficacy of the nitrene derived from phenyl azide as a labeling reagent for lipids and for the hydrophobic portions of intrinsic membrane components. Phenylnitrene was found to be a poor lipid labeling reagent largely because of its longevity and its electrophilicity. In the present work, we report on the utility of carbenes derived photochemically from diazirines as lipophilic labeling reagents. In general, carbenes are much more reactive than nitrenes (Jones and Moss, 1973; Moss and Jones, 1975), the diazirine precursors can be photolyzed at longer wavelengths than can azides (Smith and Knowles, 1975), and the products of the expected carbene insertion reactions are chemically more stable than those derived from nitrenes. Results using phenyldiazirine (I) and adamantanediazirine (II) as precursors of carbenes are reported.



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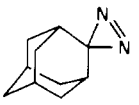

II

Materials and Methods

Unless otherwise stated, the materials and methods used here were as described earlier (Bayley and Knowles, 1978).

3-Phenyl-3H-diazirine was prepared by the second method of Smith and Knowles (1975), involving the treatment of 1,3,5-triphenyl-2,4,6-triazabicyclo[3.1.0]hexane (Schmitz, 1962), with *tert*-butyl hypochlorite in methanol. The final extraction was done with *n*-pentane. The product was purified by preparative thin-layer chromatography on silica gel, eluting with hexane–chloroform (3:1, v/v) at 4 °C. The product (R_f 0.45) was extracted into pentane, and the solvent was then carefully removed by evaporation at 10 mm and 0 °C to leave the pale yellow oil of phenyldiazirine. This had an identical ultraviolet spectrum to that described by Smith and Knowles. The concentration of stock solutions was determined spectrophotometrically using an extinction coefficient of 299 at 362 nm.

Spiro[adamantane-2,2'-diazirine] was prepared by the method of Isaev et al. (1973). After purification by chromatography on silica gel using hexane as eluent, a white crystalline solid was obtained which was a single spot in various TLC systems. The ultraviolet spectrum had λ_{max} 372.5, 359, 354, 342.5, and 337 nm, and ϵ (372.5 nm) 167.

Photolyses were carried out in a glass tube or glass jacketed cell tightly sealed with a septum. The solution was vigorously stirred. Irradiation was with an RPR 3500-Å lamp at a distance of 3 cm for 8 min. The half-life of the diazirines under these conditions is approximately 2 min. Further details can be found in Tables I and II. After photolysis, the solutions were divided into 200-μL portions which were freeze-dried. Transesterification was done with sodium methoxide (Glass, 1971). When the reaction was complete, the methyl esters were extracted by partitioning the products between hexane and water after acidification with methanolic HCl. The organic layer was dried over Na_2SO_4. After evaporation of the solvent with a stream of N_2, the residue was dissolved in methanol. This solution was used for gas chromatography and gas chromatography–mass spectrometry.

Analytical gas chromatography 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). Peak area measurements were made by planimeter. The yield (the percentage of diazirine in the incubation that inserted into the fatty acid side chains) was determined assuming that the signal observed was proportional to the mass of the product. Integration with respect to the major peak (the unmodified fatty acid) or with respect to an added standard (an *n*-alkane) gave similar results.

The products were identified by gas chromatography–mass spectrometry on an AEI 1073 instrument equipped with a Pye Unicam Series 104 chromatograph and a Data General minicomputer.

Results

Products from the Photochemical Labeling of Phospholipid Vesicles. In Table I are summarized the results of the gas chromatographic analysis of labeled fatty acids from saturated (dimyristoyllecithin) and unsaturated (dioleoyllecithin)

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TABLE I: Gas Chromatographic Analysis of Fatty Acid Methyl Esters from Labeled Lipid Vesicles.

Lecithin	Label	Mean t_R of product esters	(min)	Col Temp (°C)
L-α-Dimyristoyl	Phenylcarbene	Myristic acid	1.05	240
		1	5.4	
L-α-Dioleoyl	Phenylcarbene	Oleic acid	1.2	275
		2	4.25[a]	
		3	4.7[a]	
		4	5.4[a]	
L-α-Dimyristoyl	Adamantylidene	Myristic acid	0.83	260
		5	6.9	
L-α-Dioleoyl	Adamantylidene	Oleic acid	1.2	275
		6	9.8[b]	
		7	10.6[b]	

[a] Ratio of **2**/**3**/**4**, 1:1.9:2.1. [b] Ratio of **6**/**7**, 1:5.4.

phospholipid vesicles. With dimyristoyllecithin, new methyl esters were detected on labeling either with phenylcarbene or with adamantylidene (Table I, **1** and **5**, respectively). In both cases, gas chromatography–mass spectrometry demonstrated that carbene insertion into saturated carbon–hydrogen bonds of the lipid had occurred. Peak **1** (Table I) contains the benzylmyristic acid methyl esters and gave *m/e* 332 (M^+, 6.6%), 241 ($M^+ - C_7H_7$, 34%), 209 ($M^+ - C_7H_7 - CH_3OH$, 7%), 91 ($C_7H_7^+$, 100%). Peak **5** (Table I) contained the adamantylmyristic acid methyl esters and had *m/e* 209 ($M^+ - C_{10}H_{15} - CH_3OH$, 2%) and 135 ($C_{10}H_{15}^+$, 100%).

When adamantylidene was generated within the bilayer of vesicles made from the unsaturated lipid dioleoyllecithin and the derived fatty acid methyl esters were subjected to gas chromatography–mass spectrometry, two new products (or sets of products) were observed (Table I, **6** and **7**). The mass spectrum of **6** is dominated by *m/e* 135 ($C_{10}H_{15}^+$), and this peak contains the family of products from the carbon–hydrogen insertion reaction of the carbene. Product **6** from oleic acid is analogous to **5** from myristic acid. In contrast, the mass spectrum of peak **7** (shown in Figure 1) is clearly the double bond insertion product of adamantylidene. The parent ion at *m/e* 430 fragments with cleavage of the C_{18} chain on either side of the spirocyclopropane, giving strong ion intensities at *m/e* 273 and 317. The ion at *m/e* 161 is formed when both these cleavages occur, and the base peak (*m/e* 135) derives from the adamantylcarbonium ion itself. Peak **7** showed a slight shoulder which may represent a small degree of nonstereoselective addition to the cis double bond. Adamantylidene therefore undergoes both insertion and addition reactions with oleic acid in a ratio of about 1:5.4 (Table I).

In the case of the reaction of phenylcarbene with dioleoyllecithin vesicles, three new products (or sets of products) were seen (Table I; **2**–**4**). Peaks **3** and **4** each had a small shoulder (see below). On the basis of the results with adamantylidene, peaks **2**–**4** are expected to be the set of benzyloleic acid methyl esters derived from carbon–hydrogen insertion, and two of the four possible stereoisomers (Figure 2) from phenylcarbene insertion into the double bond of oleic acid. [The major products are expected to be A and B (Figure 2), since phenylcarbene is known to add to olefins with high stereospecificity (Closs and Moss, 1964). The roughly equal ratio of **3** and **4** (**3**/**4** is 0.9, see Table I) is not unexpected: the sterically more hindered product from the addition to olefins of phenylcarbene generated by the photolysis of phenyldiazomethane (the "syn" product) is usually formed in slightly greater amounts than the less hindered ("anti") product (Closs and Moss, 1964). The

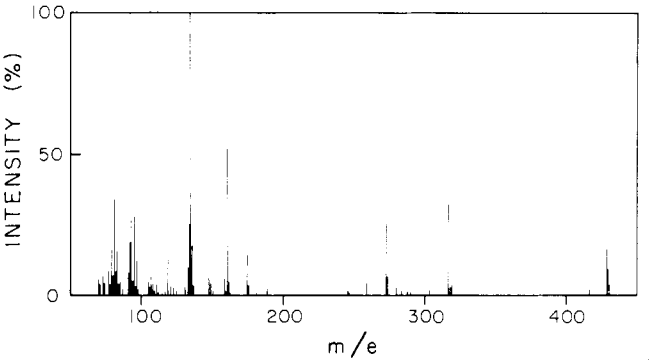

FIGURE 1: Mass spectrum of the double-bond addition product of adamantylidene to dioleoyllecithin. The spectrum is derived from peak 7 (Table I). For the assignments, see the text.

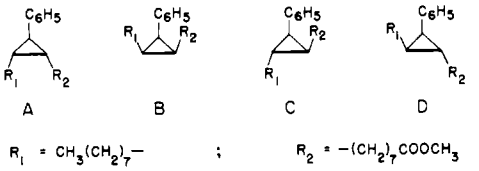

FIGURE 2: Possible stereoisomers deriving from phenylcarbene addition to oleic acid.

products C and D (Figure 2) may well be the small shoulders observed on peaks **3** and **4**. Small amounts of the nonstereoselective addition products have been observed on photolysis of phenyldiazomethane with olefins (Gutsche et al., 1962; Closs and Moss, 1964).] By gas chromatography–mass spectrometric analysis, peak **2** clearly contains benzyloleic acid methyl esters. [The upper end of the mass spectrum is dominated by *m/e* 295 ($M^+ - C_7H_7$) and 263 ($M^+ - C_7H_7 - CH_3OH$).] While peak **3** did not give an unequivocal mass spectrum, the spectrum of peak **4** identifies this product as deriving from double-bond insertion by phenylcarbene. Peak **4** has a very much lower ion intensity at *m/e* 295, the dominant ion above *m/e* 150 being 229, which derives from cleavage between C-8 and C-9 of the carbene addition product. The molecular ion (*m/e* 386) and the ion from cleavage between C-10 and C-11 (*m/e* 273) were also relatively strong. For peak **3** the ions *m/e* 229 and 273 were weaker, and the remainder of the spectrum did not indisputably demonstrate double-bond addition. However, the gas chromatographic results and the known selectivity of this carbene for carbon–carbon double bonds over carbon–hydrogen single bonds (which we recognize might be different in the liquid-crystalline environment of the bilayer) leads us to assign **3** and **4** as double-bond addition products. This gives a ratio (not statistically corrected) of approximately 1:4.0 (Table I) for carbon–hydrogen insertion to double-bond insertion.

Labeling Yields. In Table II the results from representative quantitative experiments are compiled. In these and in other experiments, the addition of glutathione appeared to have little effect on the outcome, nor was the labeling yield significantly changed when the experiment was performed under nitrogen. To prevent losses of the volatile phenyldiazirine the preincubation time was kept to a minimum. Thus, when an extended preincubation of phenyldiazirine with dimyristoyllecithin was carried out at 40 °C (Table II), the yield of labeled product was lowered significantly. The labeling was totally dependent on photolysis. In summary, the labeling yields from phenylcarbene with dimyristoyl- and dioleoyllecithins were approximately 5 and 10%, respectively, and the yield from adamantylidene with the same lipids approximately 3 and 5%.

TABLE II: Representative Labeling Yields from the Photolysis of Phenyldiazirine and Adamantanediazirine in Phospholipid Vesicles.[a]

	Phenyldiazirine[b]				Adamantanediazirine[c]	
Lecithin	Preincubat time (min)	Glutathione concn (mM)	Extent of labeling[d] (%)	Atm	Glutathione concn (mM)	Extent of labeling[d] (%)
1-α-Dimyristoyl[e]	~0.5	0	5.3	Air	0	2.5
	1	0	4.9	N_2	0	3.1
	10	0	3.2	N_2	17.6[f]	3.5
	10	0	<0.1[g]			
	~0.5	17.6[h]	5.1			
1-α-Dioleoyl[i]	5	0	9.8	Air	0	5.0
	5	17.6[h]	10.7	N_2	0	5.5
				N_2	17.6[f]	5.6

[a] Lipid concentration, 9.1 mg/mL. The solution contained 0.9% (v/v) ethanol and was photolyzed at 350 nm. [b] Photolysis in air. Phenyldiazirine concentration, 5.6 mM. [c] Adamantanediazirine concentration, 4.6 mM. Preincubation time, 15 min for dimyristoyllecithin, 30 min for dioleoyllecithin. [d] Percentage of reagent attached to phospholipid after photolysis. Based on the initial diazirine concentration at the start of the incubation (See Materials and Methods). These values are not precise to better than ±10%. [e] 40 °C. [f] Added 5 min before photolysis. [g] No photolysis. [h] Added just before diazirine. [i] 25 °C. [j] Glutathione present during incubation.

Properties of the Adamantanediazirine. Heating the adamantanediazirine in the dark in ethanol at 60°C for 2 h produced no significant decomposition. On photolysis in hexane, the diazirine absorptions smoothly disappeared and a new strong band at 234.5 nm appeared which decayed at room temperature in the dark. This band was very rapidly destroyed by acetic acid. By analogy with the work of Smith and Knowles (1975), the unstable photolysis product was tentatively identified as 2-diazoadamantane.

Discussion

Phenyldiazirine (I) and the adamantanediazirine (II) lose nitrogen on irradiation, yielding highly reactive carbenes (Smith and Knowles, 1975; Isaev et al., 1973). Unlike most alkylcarbenes, these species cannot rearrange to olefins by 1,2-hydrogen migration. Adamantylidene can undergo an *intra*molecular insertion reaction to give dehydroadamantane (Udding et al., 1966) and this reaction partly competes with intermolecular reactions (Isaev et al., 1974).

In accordance with the greater reactivity of carbenes compared with nitrenes (Jones and Moss, 1973; Moss and Jones, 1975), the results presented in Table II show that phenylcarbene is a more efficient reagent than phenylnitrene (see the previous paper in this issue) for the labeling of membrane lipids. The carbene is about 20 times more efficient in labeling a saturated lipid such as dimyristoyllecithin and about 10 times more efficient for an unsaturated lipid such as dioleoyllecithin. Since 75–80% of the phenyl azide is in the lipid bilayer under our conditions, the difference in labeling yields is a minimum estimate of the higher reactivity of the carbene. Adamantylidene also labels these lipids in reasonable yield (Table II).

In contrast to the results obtained with phenylnitrene, it is evident from Table II that glutathione cannot scavenge the carbenes. This supports the interpretation that the relatively long-lived and electrophilic nitrene diffuses to the bilayer surface where it can react with glutathione, whereas the more reactive carbene reacts with the lipid or is destroyed (by coupling, rearrangement, etc.) before a significant fraction of it can react at the bilayer surface. It therefore appears that diazirines are superior reagents for the photochemical labeling of lipids, and, by analogy, of the hydrophobic segments of intrinsic membrane proteins that are believed to be rich in amino acids with hydrocarbon side chains (Marchesi et al., 1976).

Diazirines have further advantages over azides. First, the diazirine functional group absorbs near ultraviolet radiation directly with a maximum in the region 340–380 nm. Phenyl azide has an absorption which tails to about 300 nm. Azides that absorb light of longer wavelength are likely to be more polar (e.g., nitrophenyl azides) or more bulky (e.g., naphthyl azides). Furthermore, irradiation at longer wavelengths is inefficient for such azides (Mattheson et al., 1977). Secondly, the carbene insertion products are likely to be more stable than those derived from nitrenes and this can be an important factor where harsh methods of protein degradation are used.

The major disadvantages of the diazirines that we have explored so far is that photolysis generates, as well as the carbene, the rearranged linear diazo compound. This has been observed for phenyldiazirine (Smith and Knowles, 1975) and, in this work, for adamantanediazirine. Very recently, a detailed study of spiro[cycloalkanediazirines] (of ring sizes 5–8) has appeared (Bradley et al., 1977). It appears that approximately 40% of phenyldiazirine and about 60% of spiro[cyclohexyldiazirine] decompose on photolysis via the diazo compound. Although the diazo compounds are in turn photolyzed at suitable wavelengths to give the carbene, they are also powerful electrophiles and may be attacked by nucleophiles at the membrane surface. To evaluate this problem, we are investigating the label distribution in the well-characterized membrane-protein glycophorin.

The competing intramolecular insertion reaction of adamantylidene may be the reason why the yields with this reagent are lower than those with phenylcarbene, but this cannot be established until binding constants to the membrane have been measured. The comparative ease of synthesis and greater stability of this alkyldiazirine may more than compensate for its lower labeling efficiency.

Besides the diazirines, there are other possibilities for photoactivatable lipophilic reagents. These include carbonyl compounds (Galardy et al., 1973) and the particularly stable α-fluorodiazo compounds (see, e.g., Chowdhury et al., 1976). Our own preliminary experiments with diazocyclopentadiene have also been encouraging.

In conclusion, we have found that the properties of diazirines are such that they should replace the presently used nitrene precursors for photochemically labeling the interior of membranes. Besides simply labeling the membrane interior, our results show that a lipid-soluble photochemical cross-linking reagent is a viable prospect and that the "photochemical depth charge", a photosensitive group attached by a hydrocarbon tail of defined length to a polar head group (Bayley, 1974; Chak-

rabarti et al., 1974), may prove a success if based on carbene rather than on nitrene chemistry.

Acknowledgments

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References

Bayley, H. (1974), Part II Thesis, Oxford University.
Bayley, H., and Knowles, J. R. (1978), *Biochemistry 17* (preceding paper in this issue).
Bradley, G. F., Evans, W. B. L., and Stevens, I. D. R. (1977), *J. Chem. Soc., Perkin Trans. 2,* 1214.
Chakrabarti, P., Paisley, K., and Khorana, H. G. (1974), Abstracts, 168th National Meeting of the American Chemical Society, Atlantic City, N.J., No. BIOL-150.
Chowdhury, V., Vaughan, R., and Westheimer, F. H. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1406.
Closs, R. L., and Moss, R. A. (1964), *J. Am. Chem. Soc. 86,* 4042.
Galardy, R. E., Craig, L. C., and Printz, M. P. (1973), *Nature (London), New Biol. 242,* 127.
Glass, R. L. (1971), *Lipids 6,* 919.
Gutsche, C. D., Bachman, G. L., and Coffey, R. S. (1962), *Tetrahedron 18,* 617.
Isaev, S. D., Yurchenko, A. G., Stepanov, F. N., Kolyada, G. G., Novikov, S. S., and Karpenko, N. F. (1973), *Zh. Org. Chim. 9,* 724.
Isaev, S. D., Yurchenko, A. G., Murzinova, Z. N., Stepanov, F. N., Kolyada, G. G., and Novikov, S. S. (1974), *Zh. Org. Khim. 10,* 1338.
Jones, M., Jr., and Moss, R. A. (1973), Carbenes, Volume I, New York, N.Y., Wiley-Interscience.
Marchesi, V. T., Furthmayr, H., and Tomita, M. (1976), *Annu. Rev. Biochem. 45,* 667.
Mattheson, R. R., Van Wart, H. E., Burgess, A. W., Weinstein, L. I., and Scheraga, H. A. (1977), *Biochemistry 16,* 396.
Moss, R. A., and Jones, M., Jr. (1975), Carbenes, Volume II, New York, N.Y., Wiley-Interscience.
Schmitz, E. (1962), *Chem. Ber. 95,* 688.
Smith, R. A. G., and Knowles, J. R. (1975), *J. Chem. Soc., Perkin Trans. 2,* 686.
Udding, A. C., Strating, J., Wynberg, H., and Schlatmann, J. L. M. A. (1966), *J. Chem. Soc., Chem. Commun.,* 657.

Structural Mapping of Aspartate Transcarbamoylase by Fluorescence Energy-Transfer Measurements: Determination of the Distance between Catalytic Sites of Different Subunits†

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ABSTRACT: Fluorescence energy transfer measurements have been used to determine the distances between active sites on different catalytic subunits of aspartate transcarbamoylase and between active sites and sulfhydryl groups (which are adjacent to active sites) on different catalytic subunits. Catalytic subunits were covalently labeled with pyridoxamine phosphate, which binds at the active sites, and reconstituted with regulatory subunits and with either unmodified catalytic subunits or catalytic subunits with sulfhydryl groups modified with 2-mercuri-4-nitrophenol. The unmodified subunit was subsequently modified with pyridoxal phosphate, which binds at the catalytic site, and fluorescence energy transfer was measured between pyridoxamine phosphate and pyridoxal phosphate by steady state fluorescence (quantum yield) and fluorescence lifetime measurements. The energy transfer between pyridoxamine phosphate and 2-mercuri-4-nitrophenol also was determined by steady-state fluorescence measurements. In both cases significant energy transfer was measured, and control experiments indicated conformational changes were not causing the observed changes in fluorescent properties. No significant changes in fluorescent properties or in energy transfer were detected in the presence of the allosteric effectors cytidine 5′-triphosphate or adenosine 5′-triphosphate or in the presence of the substrate carbamoyl phosphate alone or with the substrate analogue succinate. If the active sites of each catalytic subunit are assumed to define an equilateral triangle, with the two planes of the triangles being parallel, the distance between pyridoxamine phosphate and the closest pyridoxal phosphate is approximately 33 Å. The distance between pyridoxamine phosphate and the closest mercurinitrophenol is 28 Å. Thus the distance between catalytic sites on different subunits is concluded to be about 30 Å.

Aspartate transcarbamoylase catalyzes the formation of carbamoyl-L-aspartate and is the first enzyme in the biosynthetic pathway leading to the formation of pyrimidine nucleotides (Jacobson & Stark, 1973a; Gerhart, 1970). The enzyme is subject to feedback regulation by nucleotides (Gerhart & Pardee, 1962). Reaction with mercurials results in dissociation of the enzyme into two distinct kinds of subunits: the catalytic subunit, which is more active than the native enzyme, and the regulatory subunit, which binds nucleotides (Gerhart & Holoubek, 1967). The catalytic subunit has a molecular weight of 100 000 and is a trimer. The regulatory subunit has a molecular weight of 34 000 and is a dimer (Rosenbusch & Weber, 1971a). The intact enzyme has a molecular weight of 300 000 (Rosenbusch & Weber, 1971b) and is composed of two catalytic trimers and three regulatory dimers. The struc-

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