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Design and synthesis of new fluorescent photoaffinity labels to study membrane structure

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Abstract. Photoaffinity labelling has been used as a technique to study membrane structure. This technique necessitates design and synthesis of suitable carbene and nitrene precursors referred to as photoaffinity (PA) labels. The PA labels should preferably be hydrophobic in nature, photolyse with light of wavelength greater than 300 nm to give reactive intermediates *i.e.*, carbenes which should undergo intermolecular insertion exclusively. The latter reaction, on incorporation of the PA labels in membranes, gives rise to crosslinked products, the analyses of which give useful information on the nature of bio-molecular interaction in membranes.

We have prepared diazofluorene and quantitatively studied the products formed on photolysis in polar and nonpolar organic solvents. Products from both singlet and triplet carbenes were observed. Various other analogues like iodo, carboxy and alkyl substituted diazofluorenes have been prepared to get greater extinction coefficients and absorptions above 300 nm. These extrinsic PA probes have been incorporated in artificial membranes and photolysed. The covalently-linked fluorescent products so formed have been analysed.

Fatty acids containing the diazofluorene unit have also been synthesized and linked to phospholipids. Studies with these intrinsic photolabelled phospholipids as well as erythrocytes are in progress.

Keywords. Photoaffinity labelling; carbenes; diazofluorene; fluorescence; artificial membranes.

1. Introduction

During the last few years, a photochemical approach has been developed to label and study membrane structure (Creed 1974; Bayley and Knowles 1977; Khorana 1980). It involves the use of reagents which on photoactivation give rise to highly reactive carbenes or nitrenes (figure 1). These reactive intermediates in turn insert into the neighbouring molecules. Carbenes have been preferred over nitrenes as probes (Bayley and Knowles 1977, 1980) and this article will deal only with carbene precursors.

A useful photoactivable (PA) reagent must satisfy various criteria before it can be used as a probe. Thus a carbene precursor to be used as a PA reagent, on photolysis must undergo only intermolecular insertion reactions. A carbene undergoing intramolecular insertion, *e.g.*, formation of cyclohexene from diazocyclohexane, would not be useful as a PA probe as it would lose its capacity to insert into neighbouring molecules. Other essential criteria to be met with are that (a) the carbene precursor should be photolysable with light of wavelength greater than 300 nm, (b) it should not be very bulky as otherwise it would perturb the biomolecular system, (c) it should undergo indiscriminate insertion, (d) it should be easy to synthesize and covalently link to biomolecules, (e) it should be stable. All these constraints have posed a challenge to bio-organic chemists to design and synthesize photoactivable reagents. Phenyl diazirine (II)

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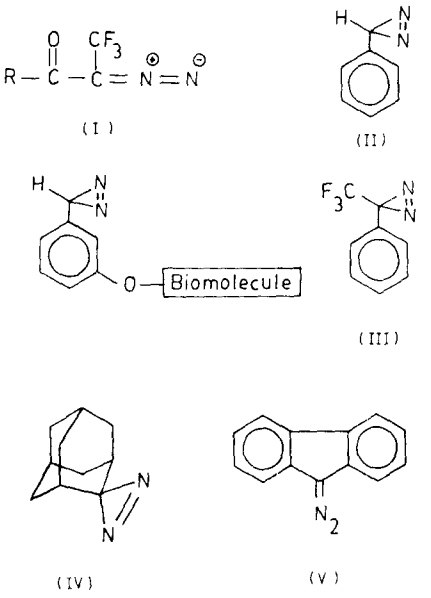

Figure 1. Photoactivable probes.

and adamantyl diazirine (IV) (Bayley and Knowles 1978, 1980), were one of the first carbene probes to be used in membranes. These two probes are extrinsic in nature and need to be incorporated into membranes prior to photolysis. Khorana's group has been involved in the synthesis of various intrinsic phospholipid PA probes for studies of biological membranes (Khorana 1980; Radhakrishnan *et al* 1980). Keto carbenes are not preferred as they easily undergo Wolf rearrangement. 2-diazo-3,3,3-trifluoropropionyl esters (I) have been used by Westheimer's group to overcome this problem (Choudhary *et al* 1976). Brunner *et al* (1980), Brunner and Richards (1980) and Brunner (1981) have recently introduced the use of 3-trifluoromethyl-3-phenyl diazirine (III) as a useful carbene precursor which can be prepared in fairly high yields. *p*-toluenesulphonyl-diazoacetates and (dansyl-diazomethyl) phosphinates have also been reported as photoaffinity labelling reagents (Choudhary and Westheimer 1978; Stackhouse and Westheimer 1981) though they are fairly polar in nature.

Depending on the location or disposition of an intrinsic PA probe in the membrane, it covalently labels the membrane components. The polarity of an extrinsic PA probe determines whether it will label the surface (Staros and Richards 1974; Dockter 1979) or the hydrophobic core of membranes (Brunner and Semenza 1981). Despite the useful information provided by PA probes, their insertion into membrane components is fairly low, *ca.* 5% (Bayley and Knowles 1977). Consequently, PA reagents with very high specific activities, *e.g.*, 1500 mCi/nmol have to be prepared. However, fluorescence can be used as an alternative sensitive method for monitoring the degree of insertion. Recently, Dockter (1979) reported the use of 3-azido-2,7-naphthalenedisulphonate (ANDS) for fluorescent photochemical labelling of the erythrocyte surface.

We report here the use of diazofluorene (DAF) (V) as a PA probe. It is easily prepared, extremely hydrophobic and strongly absorbs beyond 300 nm. Being lipophilic in

nature, it partitions easily into the membrane hydrophobic core. Here it has been used to label phosphatidyl choline (PC) vesicles. Even though DAF is not fluorescent by itself, the inserted products formed on photoactivation are highly fluorescent.

2. Chemistry of fluorenylidine

DAF strongly absorbs beyond 300 nm *i.e.* 302 nm (ε 12200), 332 nm (ε 9860) and 345 nm (ε 11970), though its λ_{max} appears at 237 nm (ε 3900). This makes it very easy to photolyse DAF with light above 300 nm—an important prerequisite for photoactivable reagents. Fluorenylidine formed on photolysis of DAF could be a singlet or triplet carbene depending on the solvent. Actually an equilibrium between singlet and triplet fluorenylidene has been suggested (Moss 1973). Zupanick and Schuster (1980) recently observed singlet and triplet fluorenylidene in acetonitrile at room temperature. Reactions of both singlet and triplet carbenes can be observed in acetonitrile, depending on the reactant.

We have carried out the photolysis of DAF in cyclohexane, initially reported by Krimse *et al* (1958), and completely analysed the products. All photolyses were carried out using a pyrex filter. Besides the expected 9-cyclohexylfluorene (67%), bifluorenyl (14%) and fluorenone (4%) are also formed (figure 2). Trace amounts of bifluorenylidine, azine and bicyclohexyl were also observed. The major product is an intermolecular C–H insertion product 9-cyclohexyl fluorene, thus making DAF a promising photoactivable reagent. It appears that products from both singlet and triplet fluorenylidine are formed. 9-cyclohexylfluorene could be formed from both singlet and triplet carbenes. On the other hand, the formation of bifluorenyl and bifluoridine could take place only from triplet (abstraction-recombination) and singlet (dimerisation) carbene respectively. The higher concentration of bifluorenyl in the crude reaction mixture suggests that the singlet-triplet inter-system crossing rate must be rapid compared to the reaction rate for singlet fluorenylidine with cyclohexane. DAF on photolysis in a mixture of cyclohexane and methanol (1 : 6 molar ratio) gives largely

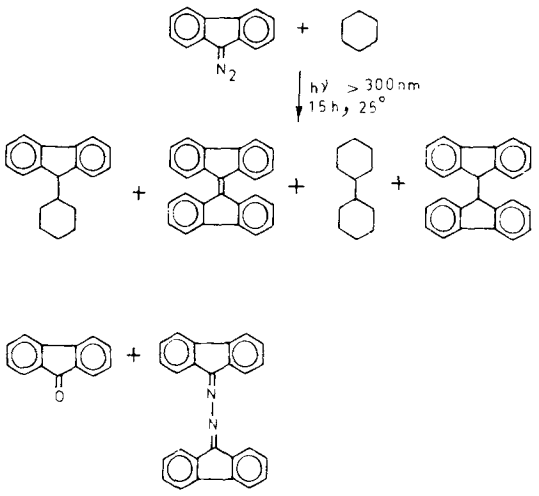

Figure 2. Insertion products of 9-diazofluorine in cyclohexane.

9-methoxy fluorene with trace amounts of 9-cyclohexyl fluorene suggesting a preference of O–H insertion over C–H insertion. This is quite expected of electron-deficient carbenes. But a carbene should be indiscriminate in order to act as a reporter molecule in membranes and a search for indiscriminate carbenes must continue. It is interesting that DAF on photolysis in cyclohexane: methanol gives rise to at least 5% 9-cyclohexyl fluorene. Since the amount of bound water in membranes is very limited relative to the hydrophobic milieu, DAF could probably act as a useful PA reagent for membranes. To further ascertain the potential of DAF as a PA probe, it was photolysed in cyclohexane: methyl oleate and cyclohexane: methyl palmitate mixtures. In either case, a number of C–H insertion products were obtained from the fatty acid fraction of the photolysed product. Gas chromatographic and mass spectral analyses indicated an indiscriminate C–H insertion along the fatty acyl chain. The fatty acid insertion products not only encouraged us to use DAF in membranes, but also proved useful for comparison with similar fatty acid insertion products obtained from photolysis of DAF in membranes.

3. Photolysis of DAF in phosphatidyl choline (PC) vesicles

PC vesicles were prepared by sonication and incubated with a 41·6 mM solution of DAF in alcohol. The final concentration of alcohol was 1% v/v. After incubation for 1 hr the solution was centrifuged. The supernatant so obtained indicated a molar ratio of 43:1

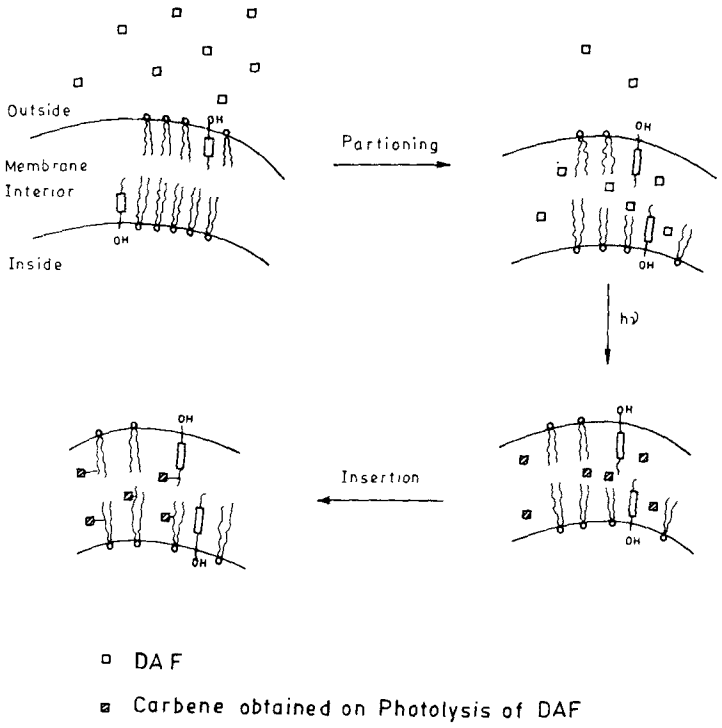

Figure 3. Partitioning and insertion of DAF in membranes.

(PC: DAF). The Sephadex G-50 run of the supernatant indicated that both PC vesicles and DAF appear in the void volume. This clearly indicated that DAF had partitioned into PC vesicles (figure 3). Photolysis of the supernatant fraction mentioned above was carried out in an annular photoreactor. After photolysis the whole solution was extracted with chloroform:methanol and separated on a silica gel plate. The PC fraction so obtained in its absorption spectrum gave bands beyond 280 nm where PC does not absorb at all, indicating clearly that fluorenylidine, formed on photolysis of DAF, had inserted into PC. Further, this PC fraction was also found to be highly fluorescent. In the control experiment involving unphotolysed samples, no fluorescence was observed in the PC fraction isolated as mentioned above. The insertion yield in PC was 3–5%. Further on trans-esterification of the PC fraction only the fatty acid portion was highly fluorescent giving a fluorescence spectrum very similar to that of 9-cyclohexyl fluorene (figure 4). The glycerophosphoryl choline fraction was nonfluorescent. Interestingly the fatty acid fraction obtained from DAF inserted PC gave a fluorescence spectrum very similar to that obtained on photolysis of DAF in methyl palmitate and cyclohexane.

It is thus evident that DAF partitions into the hydrophobic interior of vesicles and labels the fatty acyl chains. DAF would thus serve as a useful photoactivable reagent for labelling integral membrane proteins. Besides giving rise to fluorescent insertion products, its flat structure and hydrophobic nature makes it an attractive photo-

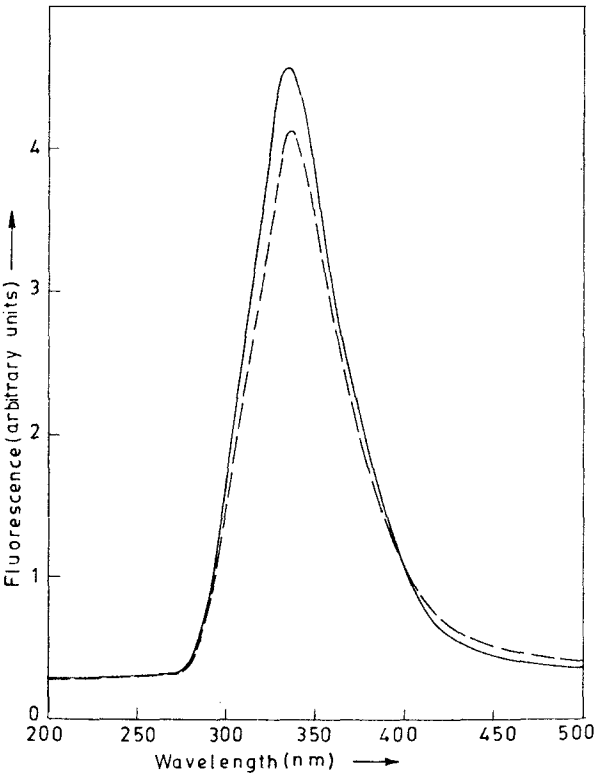

Figure 4. The fluorescence spectrum of the fatty acid fraction obtained from PC-DAF photolysed mixture (—) and 9-cyclohexyl fluorene(– – –) in methanol.

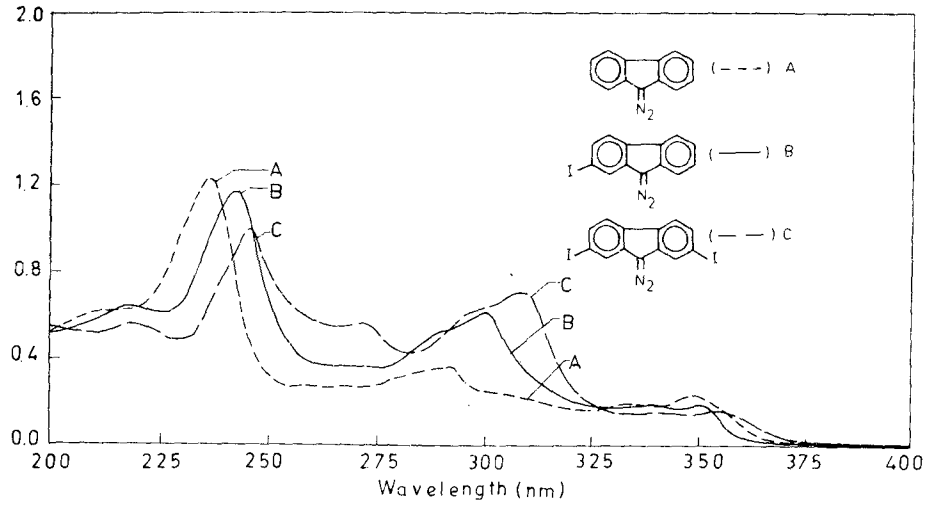

Figure 5. UV spectra of diazofluorene, 2-iododiazofluorene and 2,7-diiododiazofluorene in methanol.

activable reagent. In order to make DAF radioactive we have also prepared 2-ethyl diazofluorene wherein ^{14}C can be easily introduced in the ethyl group. Finally 2-iodo and 2,7-diiododiazofluorene were prepared. This provided us with a method of introducing radioactivity in DAF using iodine, which can be obtained carrier-free and thus permits one to prepare iodo-DAF of high specific activity. Thus in cases where insertion yields are very low one could use iodo DAF or ethyl DAF to follow the course of photolysis. Interestingly, introduction of iodo groups (which makes the molecule much less fluorescent, heavy isotope effect) not only made the molecule radioactive but also shifted the DAF 291 nm absorption beyond 300 nm (figure 5). This considerably helps in cutting the time for photolysis. Thus whereas a 5·2 mM solution of DAF in cyclohexane is photolysed (> 300 nm) in 1·5 hr, 2-iodo-DAF takes 1 hr and 2,7-diiodo DAF takes about 20 min. PA labelling studies with these compounds in PC vesicles also indicated that they insert into fatty acyl chains. We are continuing the synthesis of DAF based labels, which should be useful as surface labels and intrinsic membrane probes. Our studies with erythrocytes are in progress. The use of these reagents should provide interesting information on the supramolecular structure of membranes.

Acknowledgement

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