Labelling of Egg Phosphatidylcholine Vesicles and Myelin Membrane with a Photoreactive Lipophilic Reagent

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The preparation and isolation of [^3H]phenyl azide, a photosensitive non-polar probe, is reported. The reagent partitions into the lipid bilayer of egg phosphatidylcholine vesicles and bovine myelin membranes. On photoactivation to generate the nitrene grouping, as much as 90% of the covalently attached label is associated with the fatty acyl residues of the constituent phospholipid molecules. The remainder is found in the polar head groups. The cholesterol component of myelin membranes is also heavily labelled. These results suggest that such reagents may be used to probe the hydrophobic regions of natural membranes.

The ability of proteins to exist in the phospholipid bilayer of biological membranes and to bind lipid molecules is presumed to depend on the presence of hydrophobic amino acid sequences exposed at the reactive surface of the polypeptide molecule. Moreover, lipid binding (Segrest *et al.*, 1974) and reconstitution studies (Warren *et al.*, 1975) suggest that such regions may possess a high degree of specificity in their phospholipid–protein interactions. This ability may be a function of the hydrocarbon tail region as well as the polar head group of the lipid molecule. The structural nature of lipid-binding sites on the polypeptide chain is, however, almost completely unknown. To date, only one membrane-bound protein has been sequenced, but no evidence exists as to either the conformation of the lipid-soluble segment or to the exact identity of those amino acid residues exposed to the hydrophobic milieu of the membrane (Tomita & Marchesi, 1975).

One possible experimental approach to determining such exposed sequences may lie in the covalent attachment of a photosensitive hydrophobic label. Two groups (Klip & Gitler, 1974; Chakrabarti & Khorana, 1975; Greenberg *et al.*, 1976) have reported the synthesis and use of such reagents. In the present paper we report the synthesis and preliminary characterization of a similar material, phenyl azide. Two principal criteria have governed our choice of reagent. First, the probe must have pronounced hydrophobic properties. Secondly, since our aim is to utilize the reagent in specialized mammalian cell types, the probe must be small enough to partition into the pre-formed membrane in substantial quantities and to react non-specifically with the components of the bilayer. For these latter reasons, phospholipid derivatives were eventually excluded from this investigation. Where active reconstituted systems can be

prepared, however, such photoreactive lipid derivatives may be preferable, since they more closely represent the native environment for the protein molecule.

Materials and Methods

Materials

Phosphatidylcholine type III-E from egg yolk and phospholipase C from *Clostridium welchii* were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Anderman and Co., London S.E.1, U.K., supplied Kieselgel H. [^3H]Aniline was purchased from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Cationic-exchange resin AG 50W-X4 (analytical grade) was obtained from Bio-Rad Laboratories, Bromley, Kent, U.K.

All other chemicals were reagent grade or better. Bovine brains were obtained from a local abattoir.

Methods

Preparation of [^3H]phenyl azide. The preparation, purification and characterization of [^3H]phenyl azide were carried out essentially as described by Smith & Brown (1951). The synthesized material occurred as a yellowish oily liquid, which was immiscible with the final reaction mixture and hence could be recovered by centrifugation (5000g for 10min). The reagent may be further purified under dark conditions by ion-exchange chromatography on AG 50W-X4 with ethanol/water (2:1, v/v) adjusted to pH 3.0 with 1 M-HCl as the solvent (Fig. 1). T.l.c. on Kieselgel H with *n*-hexane and *n*-decane as solvents revealed only one radioactive spot with R_f 0.33 and 0.5 respectively. The values obtained for the partition coefficients

Fig. 1. Elution profile of [³H]phenyl azide after loading on a column (1.5 cm × 34 cm) of AG 50W-X4 equilibrated and eluted with ethanol/water (2:1, v/v), pH 3.0, then washed through with 0.1 M-NaOH. The fraction volume was 4.0 ml.

at 22°C of phenyl azide in the organic phases of the three biphasic systems of *n*-hexane, *n*-decane and olive oil with water were 60.0, 375.0 and 876.0 respectively, and are in accordance with the increasing hydrophobic nature of these organic solutions. The reagent was dissolved in ethanol and stored in sealed glass vessels at 4°C in the dark. The specific radioactivity was 1.5 mCi/mmol, a molar extinction coefficient for phenyl azide of 9940 litre · mol⁻¹ · cm⁻¹ at 248 nm and a density of 1.4 g/ml being used.

The u.v. spectrum of purified phenyl azide exhibited a maximum at 248 nm and two shoulders at 275 and 285 nm, all characteristic of this molecule (Lieber *et al.*, 1966). On photoactivation, all the characteristic peaks disappeared from the u.v. spectrum, to be replaced by a peak at 230 nm.

The i.r. spectrum contained two peaks, one at a wavenumber (2130 cm⁻¹) attributed to the asymmetric stretch in -N=N+=N- azide bonds and a second band at 1300 cm⁻¹ assigned to the symmetric stretch in the same bonds (Reiser & Wagner, 1971). All peaks were consistent with pure phenyl azide; no evidence was obtained of any contaminating aniline.

Preparation and labelling of egg phosphatidylcholine. Egg phosphatidylcholine was dried under a stream of N₂, ethanol was added and the mixture injected into water (Batzri & Korn, 1973). No sonication was performed. [³H]Phenyl azide, dissolved in ethanol, was added to this lipid preparation such that the concentration of ethanol never exceeded 1.3% (v/v). Alternatively, liposomes were prepared by sonication of a suspension of egg phosphatidylcholine in 0.02 M-Tris/0.1 M-lysine buffer, pH 7.3, in the absence of

ethanol. A molar ratio of phenyl azide to phospholipids of 1:1 was used. The mixture was incubated in a sealed glass vessel at 30°C for 3 h before photoactivation for 60 min with a Philips Ultraphil Health Lamp KL 2866. The vesicle preparation was applied to a column (2.2 cm × 56 cm) containing Sepharose 6B resin equilibrated and eluted with 1.3% (v/v) ethanol (see Fig. 2) or the Tris/lysine buffer, or dialysed against water adjusted to pH 7.4 with 0.1 M-NaOH. Where the liposomes were prepared in the absence of ethanol, this latter material was omitted from the column buffer. Phosphate was determined by the Bartlett method as described by Dittmer & Wells (1969).

Cleavage of phospholipids. To examine the distribution of label in the phosphatidylcholine after photoactivation, lipid was subjected to two cleavage systems. (A) Incubation in methanolic 0.25 M-KOH (Sheltawy, 1975) yielded the fatty acyl methyl esters and glycerol-3-phosphorylcholine. These products were separated by the biphasic system chloroform/butan-2-ol/water (4:2:3, by vol.). (B) Exhaustive digestion with phospholipase C yielded the phosphoryl head group and diacylglycerols. The reaction was carried out at 37°C for 3 h in 0.01 M-Tris/HCl buffer, pH 7.3, containing 5 mM-CaCl₂. In this case, the products were extracted with the chloroform/methanol/water (10:10:9, by vol.) biphasic system of Bligh & Dyer (1959). The constituents of both layers were subjected to t.l.c. with chloroform/methanol/water (65:25:4, by vol.) as solvent and detected with I₂ vapour. The silica-gel plate was divided up into sections, and the gel scraped from the glass support and counted for radioactivity (Tables 2 and 4) by using as scintillant 10% naphthalene and 0.5% 2,5-diphenyloxazole with respect to redistilled dioxan. The recoveries from the gel plates were carefully monitored.

Purification and labelling of myelin membranes. Bovine white matter was obtained from the brains of freshly slaughtered animals. Myelin was prepared from this white matter by published procedures (Rumsby *et al.*, 1970). Protein concentrations were determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as the standard. [³H]-Phenyl azide was added to purified myelin in water, pH 7.4 (20 mg of freeze-dried myelin/ml), at a molar ratio of reagent to phospholipid of 1:1. The mixture was sonicated in ice for 5 s and subsequently incubated for 3 h at 30°C. Photoactivation and dialysis were carried out as described above.

Isolation and chemical and enzymic cleavage of labelled myelin phospholipids. Total lipids from aqueous myelin suspensions were extracted by the procedure of Folch *et al.* (1957). Labelled myelin membrane (10 mg) was suspended in 1.5 ml of water pH 7.4, and 28.5 ml of chloroform/methanol (2:1, v/v) was added. All the components of myelin dis-

solved in this system, except for the acidic protein, which can be removed by centrifugation at 3000g for 30 min. KCl was added to a final concentration of 0.1 M, with respect to the original volume of myelin in water. A biphasic system was thereby generated and the basic protein formed a precipitate at the interface. The lower chloroform phase was retained, to which was added 4 vol. of diethyl ether. This mixture was incubated for 4 h at 4°C to precipitate proteolipid proteins, leaving the purified myelin lipids dissolved in the chloroform layer. The protein was removed by centrifugation at 2000g for 15 min. Total lipid classes and subclasses were separated and identified by t.l.c. on silica-gel H plates. The plates were developed and spots detected, and the radioactivity was determined as above (Table 4).

Total myelin lipids were subjected to the chemical and enzymic cleavage procedures and reaction products analysed as discussed above for the egg phosphatidylcholine preparations. Phosphate determinations were carried out on both lipid phases.

Results

Attachment to egg phosphatidylcholine vesicles

The association of radioactivity with the lipid peaks as indicated by the elution profile shown in Fig. 2 is clear indication of liposomal labelling by [³H]-phenyl azide. Unbound material appears as a peak at the column volume.

Further lipid preparations were divided into two equal fractions. The first was photoactivated as above, before exhaustive dialysis (3 days against 2×1 litre of 1.3% ethanol in water) at 4°C. The second fraction was not photoactivated, but was dialysed under the same conditions. Samples from the material contained in both dialysis sacs were counted for radioactivity after drying. The results show that [³H]phenyl azide is largely dialysed out unless covalently attached to the phospholipid molecules (Table 1). This finding is consistent with a continued partitioning of the reagent between the lipid and water phases during the course of the dialysis.

Fig. 2. Elution profile of labelled egg phosphatidylcholine from a column (2.2 cm × 56 cm) of Sepharose 6B ●, Radioactivity (d.p.m.) in each fraction; ▲, phosphate concentration (A_{830}) in each fraction; ○, light-scattering (A_{300}). Fraction volume was 3.5 ml. The column was eluted with 0.2 M-Tris/0.1 M-lysine, pH 7.3.

Table 1. Extent of labelling after exhaustive dialysis with and without photoactivation
In each case 3 mg of phosphatidylcholine and 30 mg of freeze-dried myelin were used.

Preparation used	Photoactivation	Radioactivity before dialysis (d.p.m.)	Radioactivity after dialysis (d.p.m.)
Egg phosphatidylcholine vesicles	—	717400	8960
	+	717400	332100
Myelin membrane	—	697728	1650
	+	697728	460000

To determine the optimum time at which maximal covalent labelling occurred, photoactivation for different time-intervals was performed, with the [^3H]phenyl azide/phospholipid ratio and the overall phenyl azide concentration kept constant. Samples were withdrawn, dialysed and counted for radioactivity after drying. The results have been corrected for the specific amount of phosphate in each fraction. Fig. 3 indicates that optimum attachment was obtained after photoactivation for 1 h. At that time the

Fig. 3. Time-course of photoactivation of egg phosphatidylcholine

A molar ratio of [^3H]phenyl azide/lipid of 1:1 was kept constant throughout the whole experiment.

amount of reagent bound corresponds to a ratio of 1 mol of [^3H]phenyl azide/8 mol of phospholipid.

Increasing the molar ratio of [^3H]phenyl azide/phosphatidylcholine above 1:1 resulted in increasing attachment of label. However, a greater proportion of the reagent became incorporated into the phospholipid head group rather than the hydrophobic tail region. This could be attributed to saturation of the bilayer, although no proof of this was obtained.

Site of labelling

To investigate the site of attachment of activated phenyl azide, the labelled phospholipid molecules were degraded and their constituent fragments isolated and identified as described in the Materials and Methods section (results in Table 2). The high percentage of radioactivity in fatty acyl chains and diacylglycerides, allied with the low percentage in the head groups in both systems, indicates that phenyl azide partitions preferentially into the hydrophobic core of egg phosphatidylcholine vesicles. After photoactivation, the tritiated reagent became covalently attached to the fatty acyl chains of this core. Phosphate determinations indicated that more than 95% of the phosphate, in the form of the lipid head group, was found in the aqueous layer.

A higher percentage of labelled material appeared in the aqueous phase than was recovered from the area on the t.l.c. plate corresponding to the position of the polar head group (see Table 2). This discrep-

Table 2. Chemical and enzymic cleavage of egg phosphatidylcholine

Recoveries from t.l.c. ranged between 50.0 and 60.0%. Each experiment was performed a minimum of three times. Results indicate the average of the values obtained, all of which lay within 5% of the values given.

Cleavage method	Reaction products	Percentage of radioactivity in each phase in the biphasic system	Percentage of P_i in each phase	Percentage of radioactivity in each reaction product after loading on t.l.c.
(A) Chemical	1. Methyl ester fatty acid	74.0	5.0	85.0
	2. Glycero-3-phosphorylcholine	26.0	95.0	15.0
(B) Enzymic	1. Diacylglycerol	76.5	4.0	85.5
	2. Phosphorylcholine	23.5	96.0	14.5

Table 3. Bovine myelin lipid classes and subclasses isolated after labelling the myelin membrane

Freeze-dried myelin (10mg) was labelled with [^3H]phenyl azide as described in the Materials and Methods section. The extracted lipids were separated by t.l.c.

Lipid classes of myelin	R_F	Reported values for molar percentages of lipid classes in myelin total lipids	Percentage of each lipid class after elution from t.l.c.
Cholesterol	1.00	38	43
Total glycolipids	0.83	22	28
Total phospholipids		40	29
(a) Phosphatidylethanolamine	0.63	18	13
(b) Phosphatidylcholine	0.30	15	10
(c) Phosphatidylserine and sphingomyelin	0.17	7	5

Table 4. *Chemical and enzymic cleavage of phospholipids obtained from labelled myelin membrane*
Recoveries from t.l.c. ranged between 50.0 and 60.0%. The experiments were performed a minimum of three times. Results indicate the average of the values obtained, all of which lay within 5% of the values given.

Cleavage method	Reaction products	Percentage of radioactivity in each phase in the biphasic system	Percentage of P ₁ in each phase	Percentage of radioactivity in each reaction product after loading on t.l.c.
(A) Chemical	1. Methyl ester fatty acid	69.0	4.5	78.0
	2. Glycero-3-phosphorylcholine	31.0	95.5	22.0
(B) Enzymic	1. Diacylglycerol	84.5	4.0	87.5
	2. Phosphorylcholine	15.5	96	12.5

ancy was resolved by the presence of radioactive material in this phase which migrated at rates inconsistent with the presence of polar species.

Labelling of myelin

The results shown in Table 3 indicate that [³H]-phenyl azide partitions readily into myelin membranes and, on photoactivation, binds covalently to all the major lipids found in membranes (Singh *et al.*, 1971).

Assuming similar recoveries for all the lipid classes, the percentage of radioactivity in cholesterol and glycolipids is slightly larger than its reported percentage in myelin membrane. The percentage in phospholipids is correspondingly smaller.

Chemical and enzymic cleavage of myelin lipids (Table 4) yields analogous results to experiments with egg phosphatidylcholine. More than 90% of the phosphate again appears in the aqueous layer, but only about 15% of the total radioactivity is associated with phosphate-containing head groups. The structure of cholesterol appears to be largely unaffected by these procedures as monitored by its behaviour on t.l.c.

Discussion

In this paper we report the synthesis and some of the necessary primary properties of a photosensitive chemical probe which readily partitions into the hydrophobic portion of the lipid bilayer. On photoactivation, up to 90% of the attached label is found in the fatty acyl chains of phosphatidylcholine vesicles. The nature of the chemical bond(s) has not been ascertained, but on the basis of earlier comprehensive studies (Knowles, 1972) it is presumed that direct insertion into carbon-hydrogen bonds or addition across any double bonds present are the most likely possibilities. A small proportion of the reagent is found in the head-group region of the phospholipid molecules. Such labelling may occur as a function of the orientation of the probe in the bilayer. Alternatively, incorporation may result from the presence of small amounts of the probe in the aqueous environ-

ment surrounding the bilayer. For, although the concentration of the probe in this hydrophilic phase is very low in comparison with its concentration in the bilayer, its overall amount is nevertheless relatively high on account of the very small volume occupied by the lipid molecules. If this labelling does occur from the aqueous phase, increasing the partition effect still further may decrease the binding.

The use of the probe with a natural membrane (myelin) is also reported here. This membrane system is considered to be among the most impervious of any natural bilayer. However, our results indicate that most if not all of the lipid components of myelin are capable of being labelled. In particular, cholesterol appears to have a high affinity for the reagent, a finding which attests even more strongly to the ease with which phenyl azide permeates into the hydrocarbon core of the bilayer. This situation is also seen with the erythrocyte membrane (E. Wells & J. B. C. Findlay, unpublished work).

The ultimate potential of this probe is its use as a covalent label for membrane proteins. Preliminary results indicate that the reagent becomes covalently attached to the intramembranous regions of most proteins in both the erythrocyte (E. Wells & J. B. C. Findlay, unpublished work) and myelin (K. M. Abu-Salah & J. B. C. Findlay, unpublished work) membrane systems. Moreover, the label is still present in the proteins after treatment with sodium dodecyl sulphate to ensure complete removal of all tightly bound phospholipid. Radioactive major sialoglycoprotein (glycophorin) has been isolated from human erythrocytes. Sequence studies on the molecule will allow us to determine the distribution of the covalently attached [³H]phenyl grouping along the polypeptide chain and the nature of the amino acid residues carrying the label. Such information is necessary for the proper evaluation of the potential of this probe as an indicator of intramembranous surface structure.

Labelling of intramembranous domains indicates that phenyl azide may be able to penetrate through the tightly bound boundary lipid supposedly associated with intrinsic membrane proteins and this

may represent an advantage over more native phospholipid derivatives. A very important point to note, moreover, is that such labelling does not appear to affect markedly the transport function of one of these labelled proteins (E. Wells & J. B. C. Findlay, unpublished work). This suggests that rearrangement of the polypeptide chain is not occurring on attachment of the phenyl grouping. We have no evidence, however, as to the effect that the introduction of the reagent has on the fluidity characteristics of natural membranes.

It is to be hoped, therefore, that further development of this type of probe, which can be used in native membrane systems, will help to resolve some of the questions associated with the integration of mammalian proteins into the lipid bilayer.

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