Use of Photosensitive Hydrophobic Probes to Label the Membrane of the Human Erythrocyte

By EDWARD WELLS and JOHN B. C. FINDLAY

Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.

(Received 29 June 1978)

Two photosensitive hydrophobic probes, azido[^3H]benzene and 1-azido-4-iodo[^3H]benzene, have been compared for their effectiveness in labelling, selectively, the intramembranous domains of lipid and proteins. Both partition preferentially into the lipid bilayer and, upon irradiation, covalently attach to both phospholipids and membrane proteins; the more extrinsic polypeptides have a significantly lower specific radioactivity than that of the intrinsic species. Proteolytic experiments also reveal higher labelling of intramembranous regions of the proteins. Consistently, the iodinated form of the probe showed the greater preference for the non-polar phase and a higher degree of selectivity for labelling hydrophobic regions. The results also suggest that penetration through the annulus of tightly bound lipid surrounding integral proteins occurs readily.

For an integral membrane protein to exist within the hydrophobic core of the phospholipid bilayer, it is conventionally assumed that sequences of non-polar amino acids must be exposed to the lipid hydrocarbon environment. It is also envisaged that the interaction between lipid and integral protein is of sufficient strength to immobilize an annulus of lipid around the intramembranous polypeptide chain (Jost *et al.*, 1973; Vanderkooi, 1974). Indeed, such interactions may not only be highly specific for both the head group and the hydrocarbon tail regions (Warren *et al.*, 1975) but also could play an important role in the modulation of protein activity (Warren *et al.*, 1975).

The method we have chosen to study the protein sequences exposed to the lipid environment makes use of lipophilic probes capable of generating reactive species within the bilayer when subjected to u.v. irradiation. Such probes must not only be very hydrophobic in nature but also partition into the bilayer in sufficient amounts, without disrupting the structural and functional integrity of the membrane. The synthesis and use of such reagents have been reported (Klip & Gitler, 1974; Abu-Salah & Findlay, 1977; Gupta *et al.*, 1977). In this report we compare two such probes, azido[^3H]benzene and 1-azido-4-iodo[^3H]benzene, to evaluate the theoretical soundness of this approach and their potential usefulness as labels for intramembranous peptides. The study has been carried out on the erythrocyte since its membrane contains protein species whose disposition in the membrane has already been characterized (Steck, 1974). In addition, several functional capacities identified with integral membrane proteins can readily be measured in order to monitor the effect of the probes on membrane activity.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials

All chemicals were obtained from British Drug Houses (Poole, Dorset, U.K.), unless listed below. AnalaR grade was used when available.

SdS, Proteinase (type VI from *Streptomyces griseus*) and Tris (7-9 or Trizma grade) were obtained from Sigma (Poole, Dorset, U.K.). Sepharose 4B was from Pharmacia (Uppsala, Sweden), Kieselgel H from Anderman and Co. (London, U.K.). All radiochemicals were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Human packed erythrocytes were supplied by the Regional Blood Transfusion Centre, Seacroft Hospital, Leeds, U.K.; these were stored at 4°C and used before the expiry date. Thiopentone was obtained from May and Baker, Dagenham, Essex, U.K.

Methods

Syntheses

Azido[^3H]benzene. The preparation of azido[^3H]benzene from [^3H]aniline was essentially as described by Smith & Brown (1951). The azide occurred as a yellow oily liquid, immiscible with the reaction mixture. It was recovered by centrifugation (5000g for 5 min) and used without further purification at a specific radioactivity of 0.93 Ci/mol.

1-Azido-4-iodo[^3H]benzene. 4-Iodo[^3H]aniline was prepared from [^3H]aniline as described by Vogel (1964). 1-Azido-4-iodo[^3H]benzene was then synthesized by the method of Smith & Brown (1951); it appeared as a brown precipitate, which, after washing with water, was used without further purification at a specific radioactivity of 0.74 Ci/mol.

Human erythrocyte membranes

Isolation and labelling. 'Ghosts' were prepared by hypo-osmotic lysis with Tris-based buffers by the method of Dodge *et al.* (1963). The membranes were washed with lysing buffer containing 0.1 mM-phenylmethanesulphonyl fluoride until free of visible haemoglobin. Membrane suspensions (5 mg/ml) in 15 mM-Tris/H₂SO₄/100 mM-lysine, pH 7.4, were incubated with 1.1 mM-1-azido-4-iodo[³H]benzene or 2 mM-azido[³H]benzene for 30 min at 24°C. The azides were added in ethanol from 1 M and 1.4 M stock solutions respectively. Irradiation for 60 min at 24°C was carried out on 100 ml batches of membranes in a 250 ml Pyrex Quickfit flask 10 cm from a 300 W Phillips Ultraphil lamp.

Removal of non-covalently bound by-products. When necessary, this could be done by extensive dialysis (4 × 250 vol. over 48 h at 4°C). Further removal of very hydrophobic by-products, which were not removed by dialysis, could be accomplished by incubating the membranes (5 mg of protein/ml) at 35°C for 10 min with 10 vol. of bovine serum albumin (10 mg/ml) in 15 mM-Tris, pH 7.4. The membranes were recovered by centrifugation (80 000 g for 15 min). This was repeated until no further radioactivity appeared in the protein-containing supernatant. Any residual bovine serum albumin was then removed by three similar washes in a suitable buffer.

Extraction of lipid from erythrocyte membranes. Lipid was extracted from the membrane suspension with 5 vol. of chloroform/methanol (2:1, v/v), with stirring for 2 h at 4°C. The lower, chloroform, layer was washed with 1.25 vol. then with 0.5 vol. of 0.4 M-trisodium citrate/methanol (3:2, v/v) and evaporated to dryness (Sheltawy, 1975). The lipid extracts were stored in chloroform under nitrogen at -20°C.

Thin-layer chromatography of membrane lipid extracts. The lipid extract was applied as a band to 0.25 mm layers of Keisegel H that had been activated at 110°C for 1 h just before use. The plates were developed in chloroform/methanol/water (65:35:4, by vol.) at 4°C. Lipid bands were detected under u.v. light after spraying with dichlorofluorescein (0.1% in ethanol). Phosphatidylserine and phosphatidylethanolamine were detected by using ninhydrin (0.25% in acetone). The plates were divided into 1 cm strips and the silica was scraped off into Pasteur pipettes. The lipid was eluted with 3 ml of chloroform/methanol (13:7, v/v) and then 3 ml of chloroform/methanol (1:1, v/v). The eluates were dried under nitrogen, taken up in 10 ml of scintillant [0.4% 2,5-diphenyloxazole in toluene/methoxyethanol (3:1, v/v)] and counted for radioactivity (Beckman LS 230 instrument).

Proteinase digestion of labelled 'ghosts'. Labelled 'ghosts', after extensive dialysis against 100 mM-

ammonium bicarbonate, were suspended to 2–2.5 mg of protein/ml. These were incubated with 1% proteinase (type VI from *Streptomyces griseus*) (1 mg/100 mg of protein) for 3 h at room temperature, and then centrifuged (80 000 g for 30 min). The supernatant was retained for the determination of specific radioactivity. The pellet was washed by centrifugation as described above, then extracted with 5 vol. of chloroform/methanol (2:1, v/v). The protein was precipitated at the interphase and, after washing with chloroform/methanol (1:1, v/v), methanol and then with water, was redissolved in 5% SDS in 25 mM-Tris/H₂SO₄, pH 8.0. A control tube, without proteinase, was treated similarly to ensure that there was no unbound material in the supernatant and to give a specific radioactivity for the total membrane protein.

Determinations

Acetylcholinesterase assay. The acetylcholinesterase activity associated with the membrane was assayed by the method of Steck & Kant (1974).

Protein assay. Protein, in the 10–80 µg range, was determined by the manual ninhydrin method described by Hirs (1967), with bovine serum albumin used as a standard.

Organic phosphate. This was determined by the method of Bartlett (1959) after prior digestion with 72% perchloric acid to liberate inorganic phosphate.

Phosphate transport

Erythrocytes, washed thoroughly in 0.3 M-triethanolamine/citrate, pH 6.8, were preincubated with thiopentone, azidobenzene or 1-azido-4-iodobenzene for 30 min at 24°C. Then 3 µCi (0.5 ml) of 100 mM-KH₂³²PO₄ (specific radioactivity 0.06 µCi/µmol of phosphate) was added to 4.5 ml of a 50% haematocrit of washed erythrocytes in 0.3 M-triethanolamine/citrate, pH 6.8. Samples (0.7 ml) were withdrawn at 0, 10, 20, 30, 40, 50 and 60 min intervals, the tubes centrifuged at 5000 g for 1 min and 0.1 ml of the supernatant was transferred into 0.4 ml of 5% trichloroacetic acid. Thorough mixing was again followed by centrifugation at 5000 g for 5 min and duplicate 0.1 ml samples were sampled for scintillation counting in 2 ml of scintillant (0.5% 2,5-diphenyloxazole/10% naphthalene in redistilled dioxan). Transport rates were calculated from the graph of log(*C_t/C₀*) against time, where *C_t* represents c.p.m. remaining in the supernatant at time *t*.

Results

Characterization of probes

The spectral data obtained for both probes were consistent with values in the literature (Reiser & Wagner, 1971; Klip & Gitler, 1974).

The u.v. spectrum of azido[^3H]benzene exhibited a peak at 248 nm with shoulders at 277 and 285 nm, and the spectrum of 1-azido-4-iodo[^3H]benzene exhibited a peak at 258 nm and a shoulder at 297 nm.

Two major peaks, characteristic of aromatic azides, were present in the i.r. spectra of both compounds, the pseudosymmetric stretch at 1297 cm^{-1} and the symmetric stretch at 2135 cm^{-1} (Reiser & Wagner, 1971).

Thin-layer chromatography of azido[^3H]benzene on Kieselgel H gave a single radioactive spot of R_F 0.34 when developed with n-hexane, and of R_F 0.78 with light petroleum (b.p. $40\text{--}60^\circ\text{C}$)/diethyl ether/acetic acid (80:20:1, by vol.). Upon irradiation with u.v. light, the compound could be detected on the plate as a brown spot coincident with the radioactivity.

Thin-layer chromatography of 1-azido-4-iodo[^3H]benzene in n-hexane gave a major spot, R_F 0.36–0.39, containing 94% of the radioactivity, with the remain-

ing 6% at the origin. When light petroleum (b.p. $40\text{--}60^\circ\text{C}$)/diethyl ether/acetic acid (80:20:1, by vol.) was used as the solvent, 94% of the radioactivity moved with R_F 0.78, 3.5% of the radioactivity co-ran with a 4-iodoaniline standard, R_F 0.15, and 2.5% of the radioactivity ran with R_F 0.39–0.43. In both systems the major radioactive spot was photosensitive, yielding a brown colour upon u.v. irradiation.

Partitioning into erythrocyte membranes

Azido[^3H]benzene and 1-azido-4-iodo[^3H]benzene were incubated with suspensions of human erythrocyte 'ghosts' (5 mg of protein/ml) at the concentrations shown in Table 1. The fraction of the probes partitioning into the bilayer was calculated as the percentage of the radioactivity co-pelleting with the 'ghosts'

Fig. 1. Effect on phosphate uptake by intact erythrocytes of azido[^3H]benzene (Δ — Δ), 1-azido-4-iodo[^3H]benzene ($\blacktriangle$ — $\blacktriangle$) and thiopentone ($\circ$ — $\circ$)

Fig. 2. Effect of irradiation time (a) on the acetylcholinesterase activity associated with the membrane and (b) on incorporation of azido[^3H]benzene

In (b) incorporation is expressed as the percentage of radioactivity counts not removed by extensive dialysis.

Table 1. Partitioning of azido[^3H]benzene and 1-azido-4-iodo[^3H]benzene into human erythrocyte membranes. Radioactivity in the membrane suspension (5 mg of protein/ml) is given as a ratio to the radioactivity remaining in the same volume of supernatant after centrifugation ($80000g$, 15 min) $\pm$ s.d. with the numbers of experiments in parentheses.

Azide	Concentration (mM)	^3H radioactivity	
		d.p.m. in suspension/d.p.m. in supernatant	In bilayer (%)
Azidobenzene	2	2.15 ± 0.05 (4)	53
1-Azido-4-iodobenzene	1	8.41 ± 1.2 (6)	88
	2	8.24 ± 0.91 (4)	88

upon centrifugation. It is evident that a significantly greater proportion of 1-azido-4-iodo[^3H]benzene partitions into the bilayer. For labelling experiments, therefore, 2 mM-azido[^3H]benzene and 1.1 mM-1-azido-4-iodo[^3H]benzene have been used; these concentrations give comparable incorporations of the probes into the bilayer.

Equilibrium is rapid and the percentage incor-

poration was found to be constant for incubation times ranging from 30 min (the minimum readily determined) up to 12 h. The percentage of label that dissolved in the membrane also remained constant, suggesting that the probes repartitioned upon dialysis, upon washing by centrifugation and upon incubation with the resin, Amberlite XAD-2, which adsorbs hydrophobic compounds.

Fig. 3. Protein, radioactivity and phosphate distribution obtained by gel filtration of azido[^3H]benzene-labelled membranes labelled in the presence of 100 mM-lysine (a) and in the absence of lysine (b)

Gel filtration of the labelled membranes (60 mg of protein), solubilized in 2.5% SDS/1% 2-mercaptoethanol, was on a Sepharose 4B column (105 cm × 2.4 cm) in 0.025 M-Tris sulphate/0.1% SDS, pH 8.0. Flow rate was 12 ml/h; fraction volume was 5.4 ml. Peak 1 contains spectrin components I and II; peak 2 contains III, IV-1 and IV-2; peak 3 contains V, VI and VII; peak 4 contains the mixed micelles of SDS with membrane lipid and very hydrophobic by-products; peak 5 contains unbound material. The sialoglycoprotein is distributed between peaks 2 and 3. ○, Protein concentration (A₂₈₀); ▲, radioactivity; △, organic phosphate.

When one considers these partition figures, it must be remembered that the membrane constitutes approximately 1% of the volume of the suspension (10 mg total weight/ml) and only half of this is lipid. Thus the concentration of the probe within the lipid matrix is up to 3 orders of magnitude greater than in the aqueous phase.

Effect on phosphate transport

As a measure of the perturbation caused by the partitioning of these lipophilic reagents into the lipid bilayer, the effect on phosphate uptake by the human erythrocyte was determined (Fig. 1). Compared with thiopentone, a lipid-soluble general anaesthetic that attains a maximal inhibitory effect at 0.5 mM, neither of the hydrophobic probes showed significant inhibition either at this concentration or at the concentrations used in these studies. Only when these materials are used at concentrations nearing 10 mM is any inhibition detectable.

Photoactivation and covalent attachment

The effect of irradiation time on the acetylcholinesterase activity associated with the membrane is shown in Fig. 2(a). The relationship between irradiation time and the incorporation of azido[^3H]-benzene, as non-diffusible radioactivity, assumed to be covalently bound, or very hydrophobic reaction by-products, is shown in Fig. 2(b). An irradiation time of 1 h was chosen; this gives a decrease in acetyl-

cholinesterase activity of 15%, and photoactivation of azido[^3H]-benzene is close to a maximum. After this period of irradiation, 10% and 40% of the radioactivity added as azido[^3H]-benzene and 1-azido-4-iodo[^3H]-benzene respectively could not be removed by exhaustive dialysis.

Despite the concentration of the probes outside the membrane being very much lower than within the bilayer, 100 mM-lysine was incorporated, as a precaution, into the reaction buffer to 'mop up' free nitrene formed in the aqueous phase. This is perhaps more important with azido[^3H]-benzene since there is more of this probe present in the aqueous phase than with 1-azido-4-iodo[^3H]-benzene.

If the labelled membrane is dissolved in 2.5% SDS and applied to Sepharose 4B, the profiles seen in Figs. 3 and 4 are obtained. The protein profile is identical with that obtained previously (Ho & Guidotti, 1975) with the positions of the various bands, as detected on SDS polyacrylamide gel disc electrophoresis, being indicated (Fairbanks *et al.*, 1971). The lipid fraction runs as a mixed micelle with SDS. Profiles of solubilized membranes labelled with azido[^3H]-benzene, in both the presence and the absence of 100 mM-lysine, are shown in Figs. 3(a) and 3(b). When lysine is present, the percentage of radioactivity counts running with the lipid is greatly increased. Labelling of the spectrin components, generally considered more extrinsic proteins, is much diminished compared with that of the other proteins, most of which have significant hydrophobic interaction with the bilayer. The profile of solubilized

Fig. 4. Gel filtration of 1-azido-4-iodo[^3H]-benzene-labelled membranes

Gel filtration of the labelled membranes (40 mg of protein) was on a Sepharose 4B column. Details are given as for Fig. 3, except that the fraction volume was 5.5 ml. $\circ$, Protein concentration (A_{280}); $\blacktriangle$, radioactivity.

membranes labelled with 1-azido-4-iodo[^3H]benzene illustrates a more pronounced version of this effect.

Upon labelling with azido[^3H]benzene and 1-azido-4-iodo[^3H]benzene, 51 % and 28 % respectively of the radioactivity present in the membranes after extensive dialysis is associated with the protein fraction precipitated during extraction with chloroform/methanol.

Labelling of the lipid fraction

Thin-layer chromatography of lipid extracted from 1-azido-4-iodo[^3H]benzene-labelled 'ghosts' is shown in Fig. 5(a). Much of the radioactivity runs ahead of the phospholipid bands in the region occupied by more hydrophobic species such as cholesterol.

In order to investigate the nature of this fast-running material the following control was devised: 200 mM-1-azido-4-iodo[^3H]benzene in ethanol, the approximate concentration in the membrane, was irradiated under normal irradiation conditions. The photolysis products were added to untreated 'ghosts' to give a final concentration of 1 mM. It was calculated that these conditions would approximate to those pertaining in the lipid matrix, although the procedure must result in a gross overestimate of by-product formation owing to the absence of any other component in the irradiation system (e.g. lipid and protein). Of these photolysis products 92 % became associated with the membrane (cf. 88 % in the presence of membrane) and, after exhaustive dialysis in the dark, the radioactivity in these 'control ghosts' was 30 % of that retained in 'ghosts' labelled normally. This indicates that some of the more hydrophobic by-products might remain associated with the membrane even after exhaustive dialysis.

An attempt was made to remove these hydrophobic species by washing the dialysed membranes with a 1 % solution of bovine serum albumin (Table 2); 37 % of the non-covalently bound by-products are eluted under these conditions from the control system. Although the control represents an extreme case, it does demonstrate some uncertainty about how much of the radioactivity associated with the lipid extract in the reaction system is not covalently bound to lipid after washing with bovine serum albumin. With dialysed labelled 'ghosts', washing with bovine serum albumin removes 31 % of the radioactivity. Thin-layer chromatography of lipid extracted from such washed membranes (Fig. 5b) shows that, although much of the high-mobility material has been removed, much of the remaining radioactivity does not co-run with the phospholipid bands. Because of the uncertainty as to whether we are observing unbound by-products or lipid species of mobility altered by binding of the probe, it is difficult to arrive at any meaningful estimate of the

Fig. 5. Thin-layer chromatography of total lipid extracts from 1-azido-4-iodo[^3H]benzene-labelled membranes (a) extracted after extensive dialysis and (b) extracted after dialysis and washing with bovine serum albumin.

For details see the Methods section. SM, Sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine.

total amount of iodinated probe bound by lipid, or its distribution among the lipid classes. Labelled lipid would constitute less than 5 % of the total and would not be detected by the fluorescent stain if the mobility was altered.

It is, however, important to note that, upon extraction of 'control ghosts' with chloroform/methanol,

Table 2. Removal of very hydrophobic by-products from labelled and control erythrocyte 'ghosts'

Values represent the radioactivity present before washing, that removed by each wash with bovine serum albumin and the total radioactivity remaining associated with the washed membranes. Control 'ghosts' are defined in the text.

	Labelled 'ghosts'		Control 'ghosts'	
Protein (mg)	... 3.2	3.5	3.8	3.6
Dialysed 'ghosts' (d.p.m.) washes in bovine serum albumin	337180	391710	129680	122743
Wash 1 (d.p.m.)	105000	105780	45510	43630
Wash 2 (d.p.m.)	10810	10900	8450	7560
Wash 3 (d.p.m.)	3400	3350	2480	2220
Washed 'ghosts' (d.p.m.)	227320	280380	78060	78060
d.p.m. removed (%)	33	29	38	36

whether after dialysis alone or after washing with bovine serum albumin, all the by-products partitioned into the chloroform phase and less than 3% of the radioactivity seen in the experimental system remained associated with the protein fraction.

Proteinase digestion of labelled 'ghosts'

Specific radioactivities were determined for the peptide material removed by proteolysis and for material remaining membrane-bound, as described in the Methods section. The ratio of these values (Table 3) is used as a rough indication of the degree of labelling occurring from within the lipid bilayer. With this criterion, it can be seen that both probes preferentially labelled intramembranous domains, and that 1-azido-4-iodo[³H]benzene is by far the more efficient of the two probes in this respect.

Similar experiments on control 'ghosts' indicate that few radioactivity counts were released from the membrane along with the proteinase-solubilized peptides. The protein remaining membrane-bound in this case had a specific radioactivity not more than 3% of that obtained after proteolysis of labelled

'ghosts'. No correction for unbound reaction by-products was therefore necessary.

Discussion

Those regions of integral membrane proteins exposed to the hydrophobic milieu of the membrane are of obvious importance to the function of the protein. In order to ascertain the nature of these regions and their location in the intact polypeptide, we have explored the possibility of labelling techniques that can be used on the intact membrane rather than on reconstituted systems. We regard this as a safer approach to the problem, bearing in mind the uncertainties that accompany reconstitution *de novo*. In this paper we report the usefulness of two such photosensitive hydrophobic probes. The results indicate, firstly, that covalent attachment to membrane components occurs readily and, secondly, that there is a preference for intramembranous regions (see also Abu-Salah & Findlay, 1977). At the concentrations used, neither probe affects anion transport in the intact erythrocyte. The proteolysis experiments indicate that the more hydrophobic the probe, the greater is its propensity to label membrane-bound constituents, suggesting that this binding is to the intramembranous regions of the polypeptide chain.

The results also show that there may be some labelling from the aqueous phase by azido[³H]benzene, even with the addition of lysine to the incubation buffer. With 1-azido-4-iodo[³H]benzene such surface labelling is much diminished. The addition of lysine to the buffer may mop up nitrene in, or available to, the aqueous phase, but the overall total labelling is not drastically decreased. Rather, the specificity of reagent-binding is increased. We would suggest, however, that this picture presents the worst possible situation, since it is likely that some hydrophobic sections are released from the membrane on proteolysis and that many hydrophilic peptides remain associated with the membrane. Both eventualities will affect the ratio of specific radioactivities to lessen the

Table 3. Proteinase treatment of labelled erythrocyte 'ghosts'

Values represent the ratio of the specific radioactivity of protein remaining membrane-bound after proteinase treatment (1 mg of proteinase/100 mg of membrane protein) to the specific radioactivity of the peptide material released into the supernatant, $\pm$ S.D., with the numbers of experiments given in parentheses.

Azide	Protein released (%)	Sp. radioactivity ratio bound protein/ released protein
2mm-Azido-benzene	49	1.75 ± 0.18 (6)
1.1mm-1-Azido-4-iodobenzene	51	7.14 ± 0.61 (5)

apparent preference for hydrophobic domains. Indeed, if the results are expressed in the form used by Klip & Gitler (1974), after labelling with 1-azido-4-iodo[³H]benzene, it can be calculated that less than 5% of the radioactivity is released in conjunction with 50% of the protein.

The usefulness of these probes is much more satisfactorily evaluated by exploring the binding pattern to an integral membrane protein of known molecular anatomy. This has now been achieved for the major sialoglycoprotein of the human erythrocyte. The results show that, with 1-azido-4-iodo[³H]benzene, up to 90% of the radioactivity bound to the protein is to be found in the 25-residue peptide that is thought to traverse the bilayer (Wells & Findlay, 1979).

The ability of the probe to bind covalently to integral membrane proteins implies that the molecule is able to penetrate through the annulus of tightly bound phospholipid, which is believed to be associated with intrinsic polypeptides. However, the concentrations of the probes used for labelling cause no alteration in activity of the anion-transport protein, in contrast with the effect seen with much lower concentrations of hydrophobic anaesthetics. The results for thiopentone are shown here, but other lipid-soluble general anaesthetics also inhibit, some more strongly than thiopentone (I. M. Mitchell & J. B. C. Findlay, unpublished work). We believe therefore that probes, such as these investigated here, have a high potential in the study of intramembranous protein sequences, membrane protein reconstitution, the interaction between membrane proteins within the non-polar milieu and in elucidation of the molecular anatomy of integral membrane proteins.

We are grateful to Dr. L. A. D. Tovey for supplying blood. E. W. is in receipt of an S.R.C. Studentship. This research was supported by S.R.C. grant B/RG82179 to J. B. C. F.

References

- Abu-Salah, K. M. & Findlay, J. B. C. (1977) *Biochem. J.* **161**, 223–228
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Gupta, C. M., Radhakrishnan, R. & Khorana, H. G. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4315–4319
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 326–329
- Ho, M. K. & Guidotti, G. (1975) *J. Biol. Chem.* **250**, 675–683
- Jost, P. C., Griffith, O. H., Capaldi, R. A. & Vanderkooi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 480–484
- Klip, A. & Gitler, C. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1155–1162
- Reiser, A. & Wagner, H. M. (1971) in *The Chemistry of the Azido Group* (Patai, S., ed.), pp. 441–501, John Wiley and Sons, London
- Sheltawy, A. (1975) *Res. Methods Neurochem.* **3**, 293–307
- Smith, P. A. S. & Brown, B. B. (1951) *J. Am. Chem. Soc.* **73**, 2438–2441
- Steck, T. L. (1974) *J. Cell Biol.* **62**, 1–19
- Steck, T. L. & Kant, J. A. (1974) *Methods Enzymol.* **31A**, 177
- Vanderkooi, G. (1974) *Biochim. Biophys. Acta* **334**, 307–345
- Vogel, A. (1964) *Practical Organic Chemistry*, p. 647, Longmans, Green, London
- Warren, G. B., Houslay M. F., Metcalfe, J. C. & Birdsall, M. J. M. (1975) *Nature (London)* **255**, 684–687
- Wells, E. & Findlay, J. B. C. (1979) *Biochem. J.* **179**, 265–272