basic protein molecule. The N-terminal portion of the polypeptide chain favours hydrophobic interactions with lipid (London et al., 1973; London & Vossenberg, 1973), whereas ionic interactions with lipid occur at the C-terminal region (Jones & Rumsby, 1977). These structural features are considered to be important in maintaining the cytoplasmic apposition in myelin. The basic protein interacts with both opposed surfaces (Fig. 1). The alternating arrangement for the basic protein shown in Fig. 1 arises as a result of the compaction process outlined in Fig. 2.

The proteolipid protein has the properties of an intrinsic membrane component. Some labelling of this protein has been noted in certain probe studies (reviewed in Rumsby & Crang, 1977), which suggests that part of the protein is exposed at the external face of the myelin lipid phase (Fig. 1). Other high-molecular-weight proteins in myelin are probably intrinsic components and are mainly located at the external surface.

The basic protein and the proteolipid protein occur in myelin from brain tissue in about equimolar proportions. The two proteins have distinctly different lipid-binding properties, and we tentatively suggest that they may associate in the membrane to form a 1:1 structural complex which would act as a nucleus for stabilizing the lipid phase. The complete lipid–basic protein–proteolipid protein complex would form an overall subunit [some 15nm×4nm (150Å×40Å)] which would occupy about 80% of the myelin bilayer, the remainder between each subunit being occupied by lipid that shows little affinity for either protein species and high-molecular-weight proteins.

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Reaction of the Covalently Binding Probes Trinitrobenzenesulphonic Acid and Fluorodinitrobenzene with Isolated Myelin-Sheath Preparations

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Covalently binding probe molecules have been used extensively to investigate the surface architecture of membranes (reviewed by Hubbard & Cohn, 1976). Results from probe studies have provided evidence for the asymmetric organization of lipid and protein in membranes and that certain intrinsic proteins have sequences of their polypeptide chains exposed at both faces of the membrane system.

Trinitrobenzenesulphonic acid and fluorodinitrobenzene have been used as probes which bind covalently with amino groups on lipid and protein at the surface of a membrane. In particular, these probes have been applied to the erythrocyte by
Marinetti and colleagues (Gordesky et al., 1972, 1975; Gordesky & Marinetti, 1973) and their reaction properties and binding characteristics examined. Under alkaline conditions (pH 8.2–8.6) in bicarbonate buffer, N$_3$phs* is non-permeant to membranes whereas N$_2$ph-F is permeant, as some labelling of haemoglobin can be detected on interaction with intact erythrocytes (Marinetti & Love, 1976). Amino group-containing lipids, such as phosphatidylethanolamine and phosphatidylserine, in membranes both show some reaction with N$_3$phs and N$_2$ph-F. However, Papahadjopoulos & Weiss (1969) have reported that pure phosphatidylethanolamine and phosphatidylserine, dispersed in balanced salt solution at pH 7.2–7.6, do not readily react with N$_3$phs. Addition of phosphatidylcholine enhances reaction of phosphatidylethanolamine with N$_3$phs but not that of phosphatidylserine.

We are using these probes to examine the surface structure of the myelin sheath in health and disease. For such work the membrane must present a uniform surface to the probe. This is so for myelin both in isolated forms and those in situ. When myelin is exposed to hypo-osmotic solutions in situ the lamellae separate and swell apart exclusively at the external apposition region, the intraperiod dense line of electron microscopy (Finean & Burge, 1963; McIntosh & Robertson, 1976). The cytoplasmic apposition (the main dense line) remains opposed. Electron micrographs of isolated myelin samples reveal that, during purification, splitting of the multilamellar membrane structure occurs at the external apposition and that a uniform and constant surface, the external face, is presented to the medium (C. Linington & M. G. Rumsby, unpublished work). Small probe molecules, such as N$_3$phs [1 nm x 0.9 nm (10 Å x 9 Å)] and N$_2$ph-F [1 nm x 0.7 nm (10 Å x 7 Å)], may even be able to penetrate the external apposition region in multilamellar sections of isolated myelin samples.

N$_3$phs and N$_2$ph-F both bind covalently to phosphatidylethanolamine and phosphatidylserine under alkaline conditions of reaction (pH 8.2–8.4) in bicarbonate buffer (120 mm-NaHCO$_3$ + 40 mm-NaCl) as shown in Fig. 1. At room temperature (20°C) both probes labelled some 60% of phosphatidylethanolamine over a 3 h reaction period. On longer exposure more phosphatidylethanolamine was labelled with N$_3$phs but not with N$_2$ph-F. With phosphatidylserine vesicles, N$_2$ph-F was more reactive than N$_3$phs with 60 and 30% of the total lipid becoming labelled in 3 h respectively. Both probes slowly labelled more phosphatidylserine over extended periods of reaction. These findings, especially the results with N$_3$phs, are contrary to those of Papahadjopoulos & Weiss (1969), who found little or no reaction of N$_3$phs with phosphatidylethanolamine or phosphatidylserine at pH 7.2–7.6. The requirement for alkaline reaction conditions has been confirmed and our data indicate that pure phosphatidylethanolamine and phosphatidylserine react with the probes, although not to completion. The lack of 100% labelling of lipid dispersed in an aqueous system presumably reflects such features as accessibility of probe to each lipid molecule, packing characteristics of lipid molecules in vesicles and probe geometry. These studies are being extended by mixing phosphatidylethanolamine and phosphatidylserine with other purified lipids typical of myelin and noting how N$_3$phs and N$_2$ph-F binding to the amino group-containing lipids is affected.

With isolated myelin preparations (Rumsby et al., 1970) both lipid and protein acquire label on interaction with either probe. The reaction of myelin with N$_3$phs has been most closely studied to date (Fig. 2). The $A_{337}$ of the solvent-soluble material from myelin reflects total N$_3$phs binding to accessible amino groups on lipid and protein from the membrane. In the acid two-phase stopping system, myelin basic protein collects at the boundary between the solvent and aqueous layers and can be easily collected. Labelling of myelin is slower at 4°C than at room temperature (20°C), as would be expected (Fig. 2). The binding of N$_3$phs in the membrane seems to occur at two rates. There is an initial rapid binding of probe over the first 30 min and this is followed by a slower binding over the next 6 h. Free probe was still present after exposure for 20 h. Presumably the different binding rates reflect the accessibility of amino groups in the membrane, the

* Abbreviations: N$_3$phs, trinitrobenzenesulphonic acid; N$_2$ph-F, fluorodinitrobenzene.
initial uptake being by immediately exposed amino groups on lipid and protein followed by a lower rate as the probe penetrates into the multilamellar structure of the membrane. Analysis of lipid extracts from labelled myelin preparations (Fig. 2) shows that some 60% of the total phosphatidylethanolamine in the membrane reacts with the probe. The shape of the binding curve with phosphatidylethanolamine and N₃phs indicates that labelling is very rapid. Thus this proportion of the phosphatidylethanolamine in myelin is readily accessible for reaction with the probe, perhaps being located at the external surface of the membrane. If this is so then it seems that a much higher proportion of phosphatidylethanolamine is arranged on the external surface of the membrane in myelin than is found with the erythrocyte (Marinetti & Love, 1976; Zwaal et al., 1973). Phosphatidylserines does not appear to react to any appreciable extent with N₃phs in isolated myelin preparations, as has been found for its reaction with

![Fig. 1. Interaction of N₃phs (a) and N₃ph-F (b) with pure phosphatidylethanolamine and phosphatidylserine dispersed in bicarbonate buffer](image)

Phosphatidylethanolamine (●) and phosphatidylserine (○) were dried down from solvent under N₂. Bicarbonate buffer (120 mM NaHCO₃ + 40 mM NaCl), pH 8.3, was added and the lipid suspension was sonicated for 60 min under N₂ and at a constant 20°C. Probe was then added at 2 mM concentration. Samples (1 ml) were withdrawn and added to 10 ml of chloroform/methanol (2:1, v/v) + 1 ml of 0.1 M-HCl to stop the reaction. After mixing and centrifugation, the lower chloroform phase containing lipid was recovered and A₃₃₇ measured against chloroform. Blanks for each probe, less lipid, were put through the same procedure. Lipid and probe-lipid products were resolved by t.l.c. on silica gel G or H in a solvent system of chloroform/methanol/acetic acid/water (50:15:5:2, by vol.). Phosphorus was determined by the procedure of Bartlett (1959).
Myelin, isolated from bovine central-nervous-system tissues as previously described (Rumsby et al., 1970), was finally suspended in distilled water. Samples were made 120 mM with respect to NaHCO$_3$ and 40 mM with respect to NaCl. This brought the pH to 8.3. After taking a zero-time sample, N$_3$phs was added to 2 mM and the reaction allowed to proceed at 4°C (▴) or 20°C (●) in the dark. Samples were removed when required, and the reaction was stopped as described for Fig. 1, unchanged probe partitioning into the upper phase. $A_{337}$ of the lower chloroform phase was measured against pure chloroform. Subsequently, unchanged lipid and N$_3$ph-lipid products were resolved by t.l.c. as described for Fig. 1 to obtain the percentage total myelin phosphatidylethanolamine (●) labelled with N$_3$phs.

dansyl chloride (Crang & Rumsby, 1977). Lack of interaction between this lipid and probe in myelin may be because the probe does not penetrate to sites where the lipid is located. It may also be, however, that phosphatidylerine is involved with other membrane components. Some information to resolve this problem may be gained by studying N$_3$phs binding to myelin lipids dispersed in aqueous systems. Further, reaction of myelin with N$_2$ph-F, which is more active with phosphatidylerine in vesicle form than is N$_3$phs (Fig. 1), will also help to resolve this problem.

These preliminary results reveal that reaction of N$_3$phs and N$_2$ph-F with myelin can give data which may be useful in defining the arrangement of lipid and protein in the membrane system.

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