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THE INTERACTION OF FLUORESCENCE AND NUCLEAR MAGNETIC RESONANCE PROBES WITH EYRTHROCYTE MEMBRANE PROTEIN FRACTIONS

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SUMMARY

(1) Two water-soluble fractions (I, II) of erythrocyte membrane proteins accounting for 30% of the total membrane protein have been separated by the aqueous washing procedures of ROSENBERG AND GUIDOTTI (*J. Biol. Chem.*, 224 (1969) 5118). The fractions had similar chemical properties to those described previously, particularly in the relatively low proportions of non-polar amino acids compared with the residual membrane proteins.

(2) The separation of all the erythrocyte membrane proteins by butanol from the membrane lipid results in the exposure of many new protein binding sites for probe molecules, which are not accessible in the intact membrane. These abnormal protein binding sites are readily detected and estimated by fluorescence and NMR probes. In contrast, no significant amounts of abnormal binding sites are exposed in the protein Fractions I and II separated by aqueous washing solutions, or in the residual membranes from which these protein fractions have been removed.

(3) When the residual membranes are pretreated with lytic concentrations of benzyl alcohol, a full complement of abnormal protein binding sites is exposed. Similar treatment of Fraction I produces no evidence for abnormal binding sites, while Fraction II carries less than half the full set of binding sites, as judged by both fluorescence and nuclear magnetic resonance techniques. These probe experiments confirm the chemical evidence that protein Fractions I and II are distinct from the proteins in the residual membranes, and also suggest that Fractions I and II are not essential for the structural integrity of the membrane.

INTRODUCTION

When the separated lipid and protein components of erythrocyte membranes are assembled into the intact membrane structure many of the binding sites for probe molecules which are available on the separated components become inaccessible[2]. This applies to fluorescence probes, spin labels, and to small organic molecules used as probes in NMR experiments. For example, approximately half of the total com-

Abbreviation: ANS, 1-anilino-napthaline-8-sulphonate.

plement of binding sites available to 1-anilino-napthaline-8-sulphonate (ANS) on the separated components are inaccessible in the intact membrane structure[3], and a similar proportion of binding sites is excluded to benzyl alcohol, used as an NMR probe[4]. Analysis of the binding of these probes and their spectroscopic properties shows that most of the extra binding sites are located on the membrane proteins, and that these protein binding sites are lost on assembly of the proteins in the membrane. The disruption of the membrane structure which exposes the new protein binding sites may be followed by adding increasing concentrations of a perturbing agent, such as benzyl alcohol, to the membranes[5]. Up to the critical (lytic) concentration, the alcohol binds to non-essential binding sites in the membrane and the interaction is fully reversible. Above the lytic concentration, the partition coefficient increases as the new binding sites are exposed, until in the limit, the membrane interacts with the alcohol essentially as the sum of its separated components[2]. Both the separated lipid and protein components have higher binding capacities than the intact membrane in the prelytic concentration range, by a factor of 1.3 and 2.6, respectively. The abnormal protein binding sites are taken as a marker of the disruption of essential interactions between components in the intact membrane structure, since the effect on the structure is irreversible. The abnormal binding sites are clearly distinguished from the sites available to probe molecules in the intact membrane by the spectroscopic properties of the bound probe molecules, which provide a convenient assay for the state of both the membrane structure and of separated membrane proteins[6].

A recent paper by ROSENBERG AND GUIDOTTI[1] describes the removal of up to 50 % of the proteins of the erythrocyte membrane by mild aqueous washing solutions. Fraction I, consisting of approximately 11 % of the total membrane protein was released by dialyzing against an aqueous solution of 1 mM EDTA and 50 mM 2-mercaptoethanol, and Fraction II (approx. 41 %) was separated by washing with 0.8 M NaCl. In this paper we have used a similar fractionation procedure to determine whether the separated protein Fractions I and II carry the abnormal binding sites, or whether the sites are restricted to a distinct set of proteins in the residual membranes from which I and II have been separated. The abnormal binding sites were estimated by fluorescence intensity and nuclear magnetic relaxation measurements of the interaction of ANS and benzyl alcohol with the protein Fractions I and II, and with the original and residual membranes. The experiments were also used to determined whether the fractionation procedure itself exposes abnormal binding sites on the separated protein fractions or the residual membranes. In previous studies, the use of organic solvents or detergents has always exposed the abnormal protein binding sites irreversibly.

MATERIALS AND METHODS

Haemoglobin-free human erythrocyte membranes were prepared from blood less than 7 days old (Group O, rhesus positive) by the method of ROSENBERG AND GUIDOTTI[1]. The plasma and buffy coat were removed and the cells washed 3 times in 310 mosM Tris–HCl (pH 7.5, 4°). The packed cells were lysed in at least 10 vol. of 15 mosM Tris–HCl (pH 7.5, 4°) for approximately 1 h. The cells were centrifuged and

rewashed 3 or 4 times in the same buffer until free of haemoglobin (< 0.5 %, w/w). These membranes are referred to as total membranes.

The water soluble protein Fractions I and II were prepared from total membranes according to the general scheme of ROSENBERG AND GUIDOTTI (Fig. 1). The total membranes (approx. 10 mg/ml) were suspended in 5 vol. of solution which finally contained 1.0 mM EDTA, 50 mM 2-mercaptoethanol, 15 mosM Tris–HCl, pH 7.5, at 4°. The suspension was stirred gently for 24 h at 4° and centrifuged at 75000 × *g* for 40 min. The protein Fraction I in the supernatant was concentrated to 10–20 mg/ml in an Amicon concentrator (UM10 filter) and dialysed into the standard buffer for probe experiments (45 mM NaCl; 30 mM sodium acetate; 5 mM sodium phosphates; 1 mM NaN_3; pH 7.4; in ^2H_2O for NMR experiments, and in H_2O for fluorescence experiments). The membrane pellet was resuspended in an equal volume of the same EDTA–mercaptoethanol solution containing also 0.8 M NaCl. The suspension was again stirred for 24 h at 4° and centrifuged at 75000 × *g* for 40 min. The supernatant (Fraction II) was concentrated and dialysed into standard buffer as for Fraction I. The residual membranes after removal of Fractions I and II were also dialysed against the standard buffer solution.

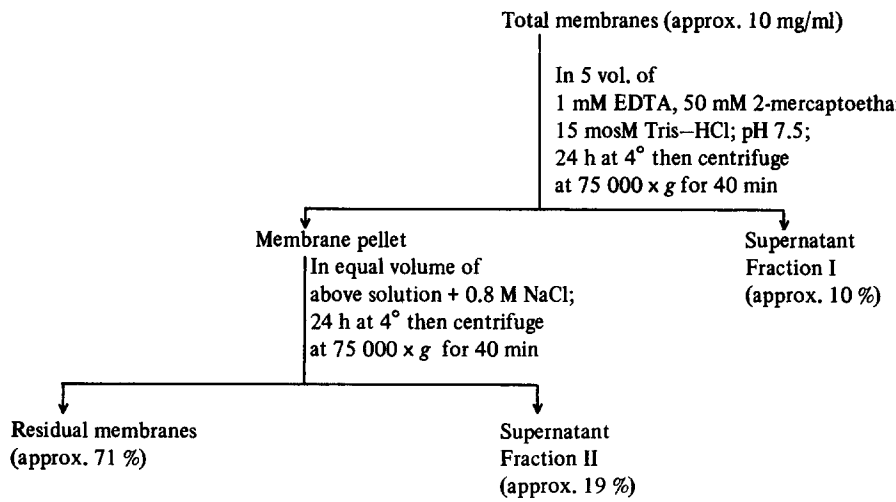

Fig. 1. The fractionation procedure.

Erythrocyte membrane lipids and proteins in the total and residual membranes were separated as described previously[5] by the butanol fractionation method of MADDY[7] as modified by REGA *et al.*[8]. The yield of membrane protein in all samples used for probe experiments was at least 95 %, and the protein was dialysed into the standard buffer solution in which it remained soluble.

Membrane and protein concentrations were determined by drying to constant weight at 105° and corrected for the dry weight of the standard buffer determined in the same way. Protein was also determined by the method of LOWRY *et al.*[9] using crystallised bovine serum albumin as a standard. Residual haemoglobin in total membranes was determined by the haemochromogen method as adapted by DODGE *et al.*[10]. Phosphorus was determined according to FISKE AND SUBBAROW,[11] and the

amount of phospholipid was calculated assuming an average phospholipid molecular weight of 800 and that no other source of phosphorous was present. Cholesterol was determined using the $FeCl_3$ reagent of ZLATKIS *et al.*[12] on lipids dissolved in acetic acid after extraction from the membranes or protein fractions with chloroform–methanol (1:1, v/v) followed by light petroleum. To determine sialic acid, samples were first hydrolysed in 0.05 M H_2SO_4 before estimation by the method of WARREN[13]. Amino acid analysis was performed on a Locarte amino acid analyser on membrane and protein samples thoroughly delipidated with chloroform–methanol (1:1, v/v), which removed less than 5 % by weight of the protein present (some of which may correspond to Fraction III of ROSENBERG AND GUIDOTTI[1]).

Acrylamide gel electrophoresis in 0.1 % sodium dodecyl sulphate was performed by the method of SHAPIRO *et al.*[14] and the gels were stained with Coomassie brilliant blue.

Fluorescence experiments were performed exactly as described previously[3], using the sodium salt of ANS recrystallized 3 times from acetone–ether (1:1, v/v). In all experiments the membrane preparation concentration was 200 μg/ml, the ANS concentration was $2.0 \cdot 10^{-5}$ M. Fluorescence was excited at 388 nm and emission was measured at 480 nm from 1.0-ml samples in a Zeiss PM Q11 spectrofluorimeter at 25°. Fluorescence intensities from different experiments were compared with a freshly prepared reference sample of ANS in ethanol.

For some fluorescence experiments, membranes (total and residual) and protein Fractions I and II were pretreated with a lytic concentration of benzyl alcohol (usually $>$ 200 mM). The appropriate volume of 300 mM benzyl alcohol was added to a concentrated sample of membranes or protein (approx. 10 mg/ml) and allowed to equilibrate for at least 4 h at 25°. Aliquots from this stock sample pretreated with benzyl alcohol were diluted with standard buffer solutions containing appropriate concentrations of ANS and benzyl alcohol to give the required final concentration of 200 μg/ml of membrane preparation and $2.0 \cdot 10^{-5}$ M ANS for fluorescence measurements.

Nuclear magnetic resonance linewidths of the phenyl protons of benzyl alcohol in the presence of membrane preparations were measured at 25° on a Varian HA 100-15 NMR spectrometer. The phenyl resonance was swept at 0.4 Hz/sec at radio-frequency power levels which caused no detectable saturation of the signal. Instrumental broadening was corrected for using the linewidth of the internal acetate reference present in the standard buffer, which was not significantly broadened by the presence of the membrane preparations. The concentration of the various membrane preparations was between 0.3 and 1.0 % (w/w) and for comparison all the linewidth measurements were corrected to 1.0 % (w/w) by the procedure described previously[4].

For the NMR experiments, concentrated samples of protein Fractions I and II (approx. 10 mg/ml) were pretreated with lytic concentrations of benzyl alcohol by dialysing against the required volume of 300 mM benzyl alcohol. The alcohol was then reduced to less than 5 mM by dialysis against the standard buffer solution.

RESULTS

Properties of the protein fractions

To determine whether defined amounts of protein were released from the membrane in Fractions I and II, serial 24 h washes at 4° with each solution were

performed. The results in Fig. 2 show that the major part of both fractions is released by a single wash and that the yield from subsequent washes rapidly decreases to the level obtained in 15 mosM Tris–HCl alone. The data suggest that either Fractions I and II are distinct sets of membrane proteins, or that the ease with which these protein components are released varies according to their environment in the membrane, implying a heterogeneous structural organisation.

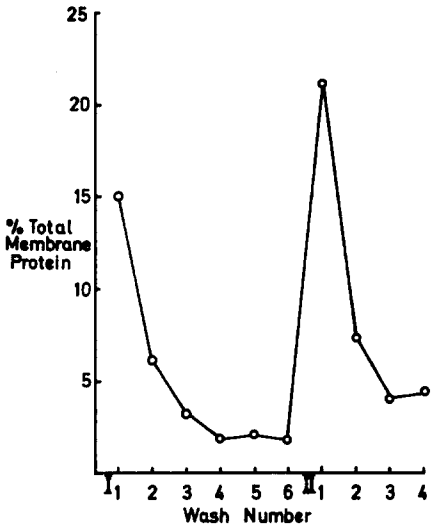

Fig. 2. The yield of protein Fractions I and II in a serial washing experiment at 4°.

TABLE I

Sample	*% of protein in total membranes*	*Phospholipid* (%, *w/w*)	*Cholesterol: protein* (*w/w*)	*Sialic acid* (%, *w/w*)	*Amino acids*** (*moles/100 moles*)	
					*Acidic****	*Non polar*§
I	10 (11)*	3.1	0.05	0.04	25 (30)*	37 (36)*
II	19 (41)*	1.7	0.02	0.003	26 (29)*	35 (35)*
Residual membranes	71	36.1	0.27	0.97	18 (21)*	42 (44)*
Total membranes	100	29.1	0.22	0.87	—	—

* Taken from ROSENBERG AND GUIDOTTI[1].
** Cys and Trp were not determined.
*** Asp; Glu.
§ Ala; Val; Met; Ile; Leu; Phe.

The average yields of Fractions I and II (15 preparations) were 10 % and 19 %, respectively, from single 24 h washes at 4° under the defined volume ratio of cells to solution described above, compared with yields of 11 % (I) and 41 % (II) reported by ROSENBERG AND GUIDOTTI[1] (Table I). The relatively low yield of Fraction II in our experiments may be due to the high volume ratio of cells to washing solution compared with the earlier experiments. ROSENBERG AND GUIDOTTI obtained a

greatly reduced yield of 20 % for Fraction II when the prior extraction of Fraction I with EDTA/2-mercaptoethanol was omitted. However in a systematic experiment on a single sample of erythrocytes under the conditions defined above, there were no significant differences in the total yield of extractable protein (Fractions I + II) with any of the possible sequences or combinations of solutes, which all gave a total yield of 30 ± 5 % protein. This is the only qualitative differences between our data for Fractions I and II and that of ROSENBERG AND GUIDOTTI[1]. There was, however, considerable variation in yields from individual experiments (for example, compare the data in Fig. 1 and Table I).

Fractions I and II contain little phospholipid (< 5 %), cholesterol, or sialic acid, and there is a corresponding enrichment of each of these components in the residual membranes compared with the total membranes (Table I). The amino acid composition of Fractions I and II is relatively high in acidic residues and low in non polar residues compared to the protein remaining in the residual membranes. This is in agreement with the data of ROSENBERG AND GUIDOTTI[1], although the percentages of acidic residues are consistently lower than in the previous work (Table I).

On gel electrophoresis, Fraction I consists mainly of the slowest moving major bands of the total membrane pattern with a mol. wt. of approx. 200000 (ref. 15) and there is a corresponding reduction in intensity of the corresponding band in the residual membrane pattern. There are also approx. 6 minor faster moving bands which can be detected in Fraction I. Fraction II by contrast contains no significant amount of the slowest moving band in Fraction I, but gives at least 9 well-defined bands corresponding to components in the total membranes. The fractions therefore appear to consist of discrete membrane components corresponding in electrophoretic mobility with some of the bands present in the total membrane pattern. Similar bands, altered in relative intensity, are still observed in the residual membranes. It is not clear whether these remaining bands are the same proteins as in Fractions I and II which are incompletely removed, or are different proteins with similar molecular weights. It is important to note that while Fraction I and in particular Fraction II are mixtures of several defined membrane components, they are not the products of random proteolysis, which readily occurs in this system[16]. Very similar gel patterns were obtained in the presence of the protease inhibitor phenylmethane sulphonyl fluoride.

Fluorescence of ANS

The fluorescence of ANS in the presence of total membranes and residual membranes with increasing concentrations of benzyl alcohol is shown in Figs. 3a and 3b. The curves are very similar in form with a decrease in fluorescence up to approximately 70 mM benzyl alcohol, followed by a pronounced upswing in the lytic concentration range. Both curves are characteristic of an intact membrane structure, as described previously[3]. The immediate conclusion is that the removal of Fractions I and II has not exposed the abnormal protein binding sites on the residual membranes. These are exposed in both the residual membranes and the total membranes in the lytic alcohol concentration range as shown by the pronounced upswing above 70 mM. Pretreatment of either total or residual membranes with 210 mM benzyl alcohol results in the irreversible exposure of the binding sites and produces enhanced fluorescence of the pretreated membranes over the entire alcohol concentration range

(Fig. 3). The fluorescence intensities from both pretreated membranes are similar, and the curves from the membrane proteins solubilised by butanol extraction from both total membranes and residual membranes are also similar. The total and residual membrane preparations may differ in their residual Ca^{2+} concentrations due to the EDTA treatment, and since ANS binding is known to be very sensitive to the concentration of divalent cations[17], detailed quantitative comparisons between total and residual membranes and their derived preparations are avoided. However, we take the fluorescence intensities from the butanol extracted protein fractions of both total and residual membranes to represent all the abnormal protein binding sites available under the conditions to which the proteins have been exposed, since they comprise virtually all the proteins in their respective membranes.

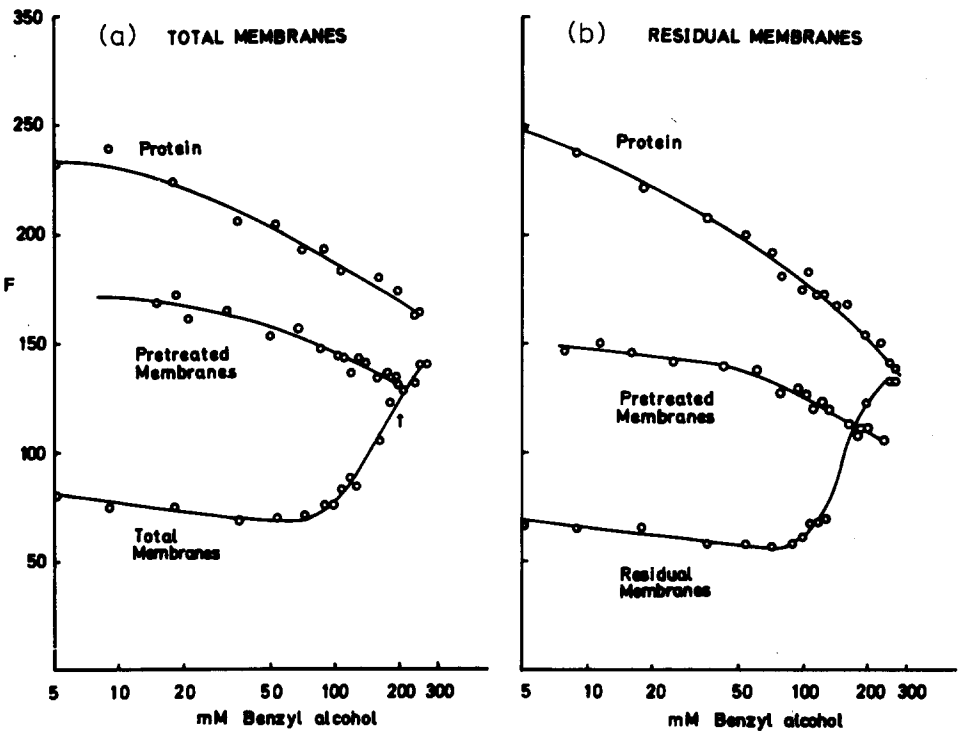

Fig. 3. The fluorescence intensity of ANS ($2.0 \cdot 10^{-5}$ M) in the presence of various membrane preparations (200 μg/ml) at 25°. (a) Total membranes; total membranes pretreated with 210 mM benzyl alcohol; and membrane protein extracted from total membranes with butanol. (b) Residual membranes; residual membranes pretreated with 210 mM benzyl alcohol and membrane protein extracted from residual membranes with butanol.

In contrast, the soluble Fractions I and II both produce fluorescence intensities significantly lower even than the intact membranes (Figs. 4a and 4b), showing that either these proteins do not carry abnormal binding sites, or that they have not been exposed by separation from the membrane. To distinguish these alternatives, the proteins were pretreated with 210 mM benzyl alcohol. Previous work has established that this pretreatment causes the same maximal exposure of abnormal binding sites on the membrane proteins as extraction with butanol[2]. The results are quite clear-cut

in that Fraction I shows no significant change in fluorescence after pretreatment, whereas the fluorescence from Fraction II is increased about 5-fold at low alcohol concentrations (Figs. 4a and 4b). However, Fraction II is still clearly distinguished from the butanol extracted protein in that even after pretreatment the fluorescence intensity is only 40 % that of the complete protein component.

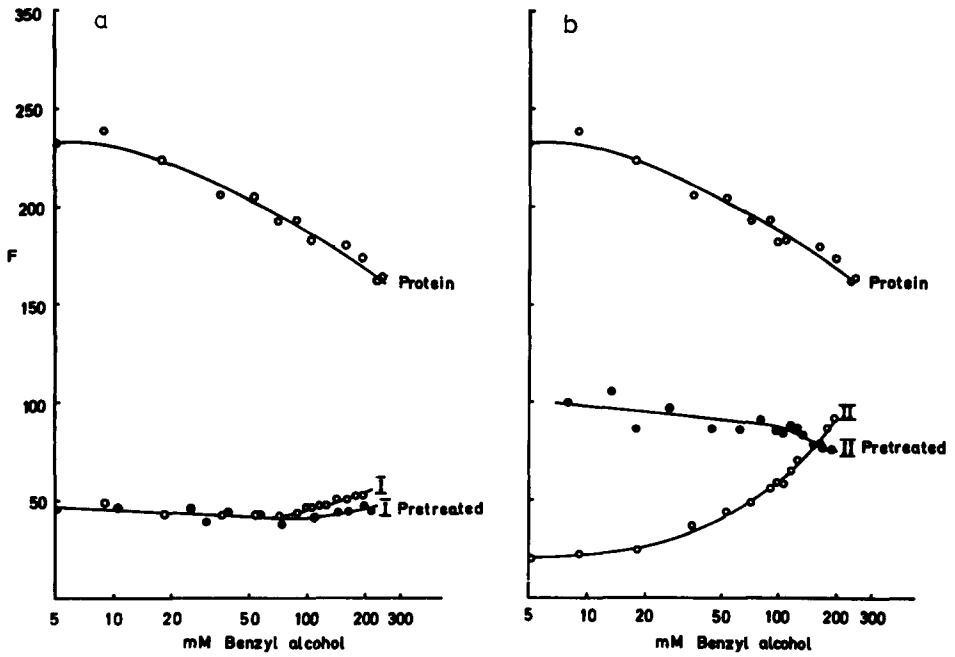

Fig. 4. The fluorescence intensity of ANS ($20 \cdot 10^{-5}$ M) in the presence of membrane protein fractions (a) Fraction I; Fraction I pretreated with 210 mM benzyl alcohol; and butanol-extracted protein from total membranes (b) Fraction II; Fraction II pretreated with 210 mM benzyl alcohol; and butanol-extracted protein from total membranes.

Magnetic resonance

The interaction of ANS with the protein fractions can be compared directly with the magnetic resonance data from benzyl alcohol. The line width of the phenyl proton resonance depends on the fraction of the alcohol molecules which are bound and the steric interactions of the alcohol molecules with the binding sites[4]. The abnormal protein binding sites are characterised by being strongly immobilised compared with the sites in the intact membrane. This is shown in Figs. 5a and 5b where the linewidths of the phenyl resonances in the presence of 1.0 % membrane preparations are compared for total and residual membranes. The two sets of curves are very similar and show the characteristic biphasic response of an intact membrane as described previously. The broadening due to the separated membrane proteins is much greater than for the corresponding membranes, and the protein curves decrease monotonically with increasing alcohol concentration. Membranes pretreated with lytic alcohol concentrations show increased broadening over the whole concentration range, and it has been shown previously that this broadening lies close to the weighted mean value for the separated lipid and protein components calculated according to the

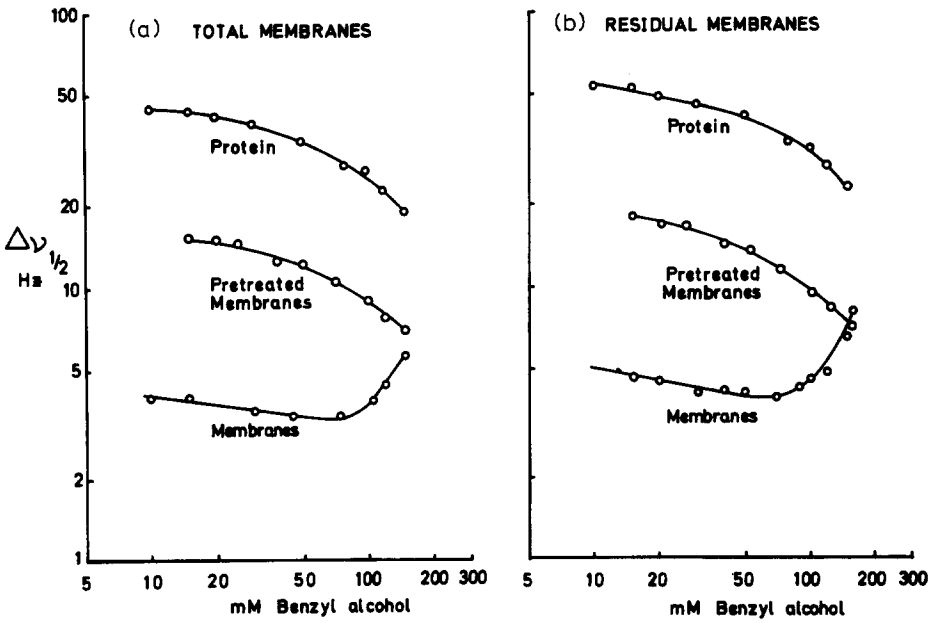

Fig. 5. The linewidths of the aromatic protons of benzyl alcohol in the presence of 1.0% membrane preparations (a) Total membranes; total membranes pretreated with 210 mM benzyl alcohol; and butanol-extracted protein from total membranes. (b) Residual membranes; residual membranes pretreated with 210 mM benzyl alcohol; and butanol-extracted protein from residual membranes.

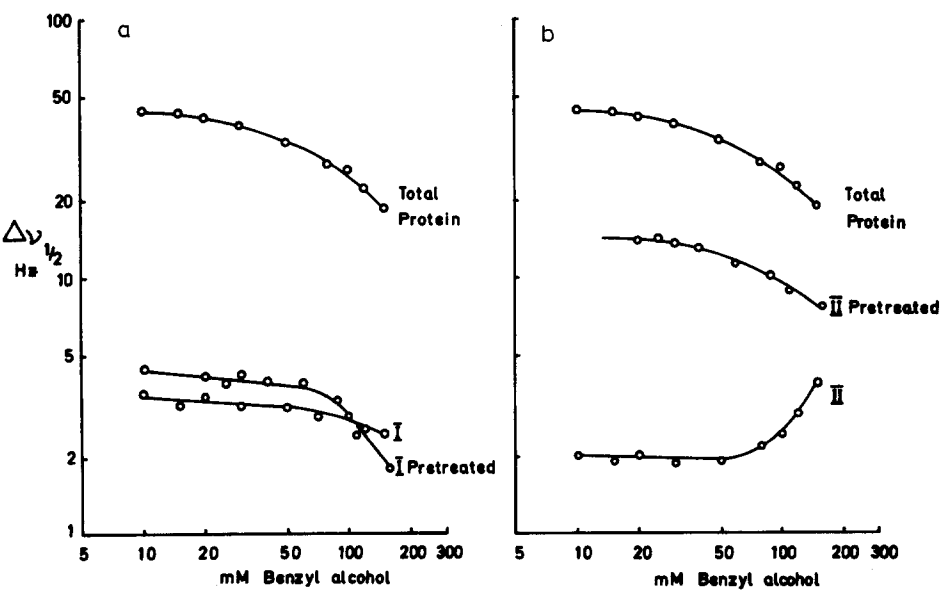

Fig. 6. The linewidths of the aromatic protons of benzyl alcohol in the presence of membranes protein fractions (a) Fraction I; Fraction I pretreated with 210 mM benzyl alcohol; and butanol-extracted protein from total membranes. (b) Fraction II; Fraction II pretreated with 210 mM benzyl alcohol; and butanol-extracted protein from total membranes.

composition of the intact membrane[4]. Since the separated lipid causes very little broadening[4,5], it is clear that it is the abnormal protein binding sites which make the major contribution to the broadening in the pretreated membranes, and that there is no significant exposure of abnormal protein binding sites in the residual membranes due to the removal of protein Fractions I and II.

The curves in Figs. 5a and 5b are similar in form to the corresponding fluorescence curves in Figs. 3a and 3b for reasons which have been discussed in detail elsewhere[3,4]. The NMR linewidth curves for Fractions I and II also resemble the corresponding fluorescence curves (Figs. 6a and 6b). Both fractions cause less broadening than the intact membrane (approx. 80 % Fraction I and approx 50 % Fraction II), and clearly do not carry significant proportions of the abnormal protein binding sites on separation. The effect of pretreatment with 210 mM benzyl alcohol is to increase the linewidth of Fraction I only slightly, whereas the linewidth from Fraction II is increased substantially over the whole concentration range. However, even this increased linewidth is only 30 % of that of the full membrane protein fraction (Fig. 6).

DISCUSSION

The properties of Fractions I and II used in these experiments are generally very similar to those described by ROSENBERG AND GUIDOTTI[1]. The soluble protein fractions have a higher percentage of acidic amino acid residues than the proteins in the residual membranes, and very low proportions of sialic acid which remains in the residual protein fraction. The phospholipid extracted with the soluble proteins was less than 5 % by weight of either Fraction I or II and similar amounts of cholesterol were detected. The serial washing experiments with the aqueous solutions used to obtain Fractions I and II indicate that the two groups of proteins are distinct fractions at least in the ease with which they are removed from the membrane. Unless the membrane structure is markedly heterogeneous in this respect they probably represent distinct groups of proteins from each other and from the residual membrane proteins. ROSENBERG AND GUIDOTTI[1] suggested from the amino acid compositions that Fractions I and II could not be composed of different proportions of the peptides present in the complete set of protein components, although the evidence by itself was not conclusive. The electrophoretic patterns in sodium dodecyl sulphate gels show that Fraction II consists of at least 9 polypeptides distinguished by their molecular weights, and that there is considerable overlap in their molecular weights with the proteins in the residual membranes. This suggests that many of the bands in the gel pattern of total membrane proteins may consist of several peptides.

Indirect evidence that Fractions I and II are probably located on the inside of the membrane is implied in the labelling experiments of BRETSCHER[15] which showed that there were only two major protein components resolved by sodium dodecyl sulphate gel electrophoresis on the outside surface of the membrane. Since one of these carries most of the sialic acid associated with the membrane protein, there is only one major band without sialic acid on the outside of the membrane. Fraction I, which carries little sialic acid, consists mainly of the slowest moving component on gel electrophoresis, and does not correspond to the only major band detected by BRETSCHER[15] on the outside surface of the membrane which lacks sialic acid (band a, ref. 15). Fraction II also carries very little sialic acid, but gives a

multiple band pattern on electrophoresis, so that most of these bands must presumably be derived from the inside surface of the membrane.

The above evidence that Fractions I and II are chemically distinct from the residual membrane proteins is supported by the probe experiments with ANS, which distinguish the proteins by their complement of abnormal binding sites. In these experiments, we have simply used the ANS fluorescence intensities produced by the various protein preparations under directly comparable conditions as a measure of the abnormal protein binding sites. It is assumed that abnormal protein binding sites have similar fluorescence characteristics whether they are situated in a membrane pretreated with benzyl alcohol or on proteins separated from lipid and other membrane proteins. This is strongly supported by the close similarity between the pretreated membrane fluorescence curve and the weighted mean fluorescence of the separated membrane components over the entire benzyl alcohol concentration range[3]. It should be noted that pretreatment with benzyl alcohol of the total protein component extracted by butanol causes no change in fluorescence, so that the fluorescence intensities from the proteins extracted by butanol can be directly compared with those obtained by pretreatment with benzyl alcohol. The intrinsic fluorescence per ANS molecule bound and the number of binding sites for the various membrane and protein preparations used will be described elsewhere.

Pretreatment with 210 mM benzyl alcohol exposes the full complement of abnormal protein binding sites in the membrane, but has no effect on the fluorescence of Fraction I, which remains at approx. 20 % of the total membrane protein fluorescence. We take this to indicate that Fraction I carries few, if any, abnormal binding sites and that any sites it has have already been exposed by the separation procedure. Fraction II differs in that on pretreatment its fluorescence increases from 8 to 40 % of the total protein fluorescence, so that while very few binding sites have been exposed by the separation process, a significant number are exposed by pretreatment. However, these new sites produce only 40 % of the fluorescence of the total protein fraction, so that in this respect Fraction II is intermediate between Fraction I and the total protein. It is consistent with the conclusion that there is no significant exposure of abnormal binding sites in Fractions I and II on separation from the membrane, that the residual membrane retains the normal biphasic fluorescence curve of an intact membrane. As far as the experiments allow, we conclude that there is no gross conformational change in the proteins of Fractions I and II or in the residual membrane during the fractionation process.

The nuclear magnetic relaxation curves for benzyl alcohol for the same set of membrane preparations are very similar to the ANS fluorescence curves and the reasons for this have been discussed previously[3]. Here we would emphasise that the NMR curves for Fractions I and II and the effects of pretreatment maintain a detailed consistency in the data from the two techniques, strongly reinforcing the above interpretation of the fluorescence data. In particular, the NMR data again shows that Fraction II has very few abnormal protein binding sites exposed when separated from the membrane, while pretreatment of Fraction II produces approximately half the broadening of the phenyl resonance of the total protein component.

The experiments using both techniques also suggest that Fractions I and II are not essential to the integrity of the membrane structure. All the previous evidence from both techniques has shown that the full set of abnormal binding sites can only

be exposed when essential interactions between the membrane components are irreversibly disrupted. In contrast, the abnormal binding sites of Fraction II are exposed by pretreatment with benzyl alcohol on the isolated protein fraction, independent of its separation from the membrane. The accessibility of these sites is not therefore controlled directly by interaction with other membrane components. As yet, the subsequent extraction of the residual membrane proteins by any technique has simultaneously exposed the abnormal binding sites. Taken together with the relative ease of extraction of Fractions I and II, their distinct chemical composition and limited complement of abnormal binding sites, we consider that Fractions I and II are not an essential part of the intact membrane structure, and that the interactions between membrane components which are critical for structural integrity are conserved in the residual membrane.

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