Solubilization of Isolated Central-Nervous-System Myelin Preparations by the Anionic Detergent Sodium Dodecyl Sulphate

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The mechanism for the solubilization of isolated central-nervous-system myelin by sodium dodecyl sulphate was studied in detail. The release of protein and phospholipid to the $100000g \times 1\text{ h}$ supernatant fraction is dependent on the total amount of detergent relative to the amount of membrane present and on the ionic strength of the solubilization system. Gel-filtration analysis of supernatant fractions indicate that at suboptimal concentrations of detergent these contain lipid-protein complexes. The complete dissociation of the individual protein components from lipid is dependent on the total amount of sodium dodecyl sulphate present in the system. The results indicate that for the analysis of membrane components in sodium dodecyl sulphate it is essential that sufficient detergent is present.

The anionic detergent SDS has been used extensively to dissociate biological membranes (Helenius & Simons, 1975; Tanford & Reynolds, 1976). The complete separation of the protein components of membranes from the lipids is an essential prerequisite for the analysis of membrane proteins by electrophoretic techniques (Maddy, 1976). There are, however, few detailed studies on the specific interactions that occur between SDS and individual membrane systems to achieve the dissolution of the lipid and protein components (Engleman *et al.*, 1967; Kirkpatrick *et al.*, 1974; Becker *et al.*, 1975). The details of how the myelin membrane system is solubilized by SDS have not been considered previously.

Details on the binding of SDS to water-soluble proteins have been established. Most water-soluble proteins bind approx. 1.4 g of SDS/g of protein (Pitt-Rivers & Impiombato, 1968) to form uniform rod-like structures with length proportional to the molecular weight of the protein (Reynolds & Tanford, 1970). It is often assumed that the binding of SDS to membrane proteins is quantitatively identical with that established for water-soluble proteins. There are, however, indications that this assumption may be invalid for the intrinsic protein components of membranes (Simons & Kääriäinen, 1970; Spatz & Strittmatter, 1971, 1973; Kyte, 1972; Rubin & Tzagoloff, 1973; Greffrath & Reynolds, 1974). Furthermore the lipid phase of biological membranes also possesses considerable potential for binding the detergent. For myelin, with its characteristic low protein/lipid composition ratio, the binding of SDS by the lipid phase may quantitatively exceed that by the protein components.

Abbreviation used: SDS, sodium dodecyl sulphate.

As part of our work on the molecular architecture of the myelin sheath (Rumsby & Crang, 1977) we have undertaken a detailed study of how SDS achieves the solubilization of isolated central-nervous-system myelin preparations. The results reveal that for myelin the amount of SDS added to the membrane system is critical in determining the extent of solubilization of the component lipid and protein molecules. The present paper also indicates that the relative amounts of detergent and membrane should be considered when techniques involving detergent solubilization are applied to the study of membrane systems.

Materials and Methods

Bovine brain tissue was collected and placed on ice within 20 min of slaughter. Myelin was prepared from the white matter by the method described by Rumsby *et al.* (1970). The purified myelin was finally resuspended in glass-distilled water, pH 7.2. Basic protein was prepared from purified myelin by the method of Banik & Davison (1973). SDS (specially pure; BDH, Poole, Dorset, U.K.) stock solutions were prepared freshly for each experiment at a concentration of 200 mg/ml in glass-distilled water. Samples of SDS stock solution were added to purified myelin suspensions in glass-distilled water, pH 7.2 at 22°C. These samples were allowed to equilibrate with the added SDS for 1 h before optical measurements or subsequent separation and analytical procedures. The amount of SDS added to each sample is expressed as a solubilization ratio (the weight ratio of SDS added to total myelin dry weight per sample). For initial studies a working criterion

for solubilization was the inability to sediment material by centrifugal forces of $100\,000g_{av}$ for 60 min.

Solubilized and partially solubilized myelin samples were separated on linear sucrose density gradients (3–30%, w/v) by centrifugation at $57\,000g_{av}$ for 90 min at 22°C. Fractions (approx. 1 ml) were collected from the bottom of the gradient tube. The $100\,000g$ supernatant fractions and total myelin samples in SDS were analysed by gel-exclusion chromatography on Bio-Gel A-5m (Bio-Rad Laboratories, Bromley, Kent, U.K.) and on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) by using a number of different buffered elution systems containing 0.2% (w/v) SDS. Polyacrylamide-gel electrophoresis was carried out by the method described by Greenfield *et al.* (1971) by using 7.5% (w/v) acrylamide gels with 1% SDS in both the gel and the electrode buffers.

Total lipid extracts of myelin were prepared by the method of Folch *et al.* (1957), but by using 0.1 M-tripotassium citrate instead of 0.1 M-KCl to precipitate the major myelin protein components (Webster & Folch, 1961; Banik & Davison, 1973). The major phospholipid classes in myelin were separated by t.l.c. on silica gel H (Merck, Darmstadt, Germany) by using chloroform/methanol/water (65:25:4, by vol.). Lipid P was measured by the method of Bartlett (1959), cholesterol by the method of Zlatkis *et al.* (1953) and total glycolipid by the method of Roughan & Batt (1968). Protein was measured by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (BDH) as the standard.

Measurements of light-scattering by suspended myelin particles were made with a Zeiss M4 QIII/PM QII spectrophotometer (Carl Zeiss, Oberkochen, West Germany) with samples in 10 mm path-length quartz cells. In spectral regions devoid of absorption peaks (beyond 350 nm for isolated myelin samples) the turbidity T of a suspension, determined with a spectrophotometer as apparent absorbance, conforms to the relationship $T \propto 1/\lambda^n$, where λ is the wavelength and n is the wavelength exponent or scattering power. The wavelength exponent for suspended particles ranges typically between -2 and -4 , depending on the size and shape of the particles (Koch, 1961) and in some cases may have a value of less than -2 (Winder & Gent, 1971).

Results

Typical analyses of the purified myelin preparations used in these studies gave 27% protein and 73% lipid by weight. The lipid component comprised 33 mol% phospholipid, 43 mol% cholesterol and 24 mol% galactolipid (cerebroside + sulphatide).

When SDS is added to a suspension of isolated myelin in water at pH 7.2 the turbidity of the system

Fig. 1. Decrease in the turbidity of isolated myelin with the addition of sodium dodecyl sulphate

SDS was added to samples of purified myelin in suspension (3.5 mg of myelin in 3.0 ml of glass-distilled water, pH 7.2) to give solubilization ratios between 0 and 2.0 as described in the Materials and Methods section. The turbidity (apparent A_{560}) was measured after equilibration of the samples with the added detergent.

Fig. 2. Increase in the light-scattering power of isolated myelin suspensions with the addition of SDS

The light-scattering power for myelin suspensions equilibrated with SDS at solubilization ratios between 0 and 2.0 was calculated from the gradient of the plot of log absorbance against log wavelength for absorbances measured at 380, 460 and 560 nm.

decreases in a characteristic multistep manner (Fig. 1). A constant minimum turbidity is observed at an SDS solubilization ratio of approx. 1.8. These changes in the light-scattering properties of the myelin fragments

with the addition of SDS were examined by estimating the scattering power for myelin suspensions over a range of SDS solubilization ratios. The scattering power of the myelin suspension, calculated from the gradient of the plot of log absorbance against log wavelength, increases from -0.6 at zero SDS to a maximum constant value of -2.5 at a solubilization ratio of 1.8 (Fig. 2). The scattering power estimated for myelin in a number of other solvents is -2.0 and -1.8 for 5mM-NaOH and chloroform/methanol

Table 1. *Effect of ionic strength on the co-release of phospholipid and protein to the 100000g × 1 h supernatant*

The ratio of phospholipid/protein co-released by SDS to the 100000g × 1 h supernatant fraction is calculated from the data in Fig. 4 for SDS solubilization ratios of less than 1.3. Below this solubilization ratio there is a linear relationship between the amount of protein and phospholipid recovered in the supernatant.

[NaCl] (mM)	Phospholipid-protein co-released (μ mol of phospholipid/mg of protein)
0	0.8
16	0.8
32	0.7
64	0.6
128	0.5
256	0.3

(2:1, v/v) respectively. A 40000g × 1 h supernatant fraction from myelin solubilized in SDS at a solubilization ratio of 1.3 had a scattering power of -3.1 .

The solubilization of protein and phospholipid components from myelin preparations by SDS was studied by examining the release of these constituents to the 100000g × 60 min supernatant fraction as a function of the amount of SDS added (Fig. 3). Over the 10-fold range of myelin concentrations studied the release of protein from myelin exhibits a characteristic dependence on the SDS solubilization ratio. All the membrane protein is recovered in the 100000g supernatant fraction at a solubilization ratio of 1.8 (Fig. 3a). The release of phospholipid from myelin exhibits a similar dependence on the solubilization ratio (Fig. 3b). However, for complete recovery of the myelin phospholipid in the supernatant fraction a solubilization ratio of at least 7 is required (Fig. 3b).

To investigate the involvement of ionic interactions in the solubilization of isolated myelin by SDS the effect of ionic strength on the solubilization profiles for phospholipid and protein was studied (Fig. 4): At zero ionic strength the release of protein and phospholipid from myelin exhibit their characteristic profiles established in Fig. 3. There is little change in the solubilization profiles with ionic strengths up to 64mM-NaCl. In 128mM-NaCl the initial release of protein at low concentrations of SDS appears to be

Fig. 3. *Solubilization of protein and phospholipid from isolated myelin*

The release of protein (a) and phospholipid (b) components from isolated myelin suspensions to the 100000g × 1 h supernatant fraction by SDS was examined as a function of the solubilization ratio. Composition of myelin samples per ml was: 1, 14.3mg of myelin, 4.4mg of protein, 6.2 μ mol of phospholipid; 2, 7.1mg of myelin, 2.2mg of protein, 3.1 μ mol of phospholipid; 3, 2.9mg of myelin, 0.88mg of protein, 1.24 μ mol of phospholipid; 4, 1.4mg of myelin, 0.44mg of protein, 0.62 μ mol of phospholipid.

Fig. 4. *Effect of ionic strength on the solubilization of protein and phospholipid from isolated myelin by sodium dodecyl sulphate*

The percentage recovery of myelin protein (a-d) and phospholipid (e-h) components in $100\,000 \times 1\text{ h}$ supernatant fractions was examined as a function of the solubilization ratio. All samples were suspended in glass-distilled water, pH 7.2, containing: no NaCl (a and e); 16 mM-NaCl (b and f); 128 mM-NaCl (c and g); 256 mM-NaCl (d and h).

Fig. 5. *Release of the major phospholipid components from isolated myelin by sodium dodecyl sulphate*

The percentage recovery of the major phospholipid classes of myelin in $100\,000 \times 1\text{ h}$ supernatant fractions was examined as a function of the solubilization ratio. Total lipid extracts were prepared from supernatant fractions and the major phospholipid classes were separated by t.l.c. and assayed as described in the Materials and Methods section. (a) Ethanolamine phospholipids; (b) serine and inositol phospholipids; (c) phosphatidylcholine; (d) sphingomyelin.

inhibited (Fig. 4a). This trend of inhibition in the release of protein at low solubilization ratios is continued in 256mM-NaCl. The data for release of phospholipid from myelin by SDS show a similar inhibition of phospholipid solubilization at ionic strengths greater than 64mM-NaCl. However, overall, the SDS/myelin dry-weight ratio required for complete solubilization of myelin protein and phospholipid decreases as the ionic strength of the system increases (Fig. 4). From these data the amount of phospholipid initially co-released with the protein by SDS may be calculated. These results are expressed as phospholipid/protein ratios in Table 1.

The release of the major phospholipid classes from myelin by SDS was studied by t.l.c. analysis of lipid extracts prepared from 100000g × 60 min supernatant fractions. The results, plotted as percentages of total phospholipid class released against solubilization ratio, indicate similar solubilization profiles for phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, as shown in Fig. 5. These components require solubilization ratios of between 2 and 3 for their total release from isolated myelin (Fig. 5). In contrast, phosphatidylserine + phosphatidylinositol require solubilization ratios of 13 for their complete recovery in the supernatant fraction (Fig. 5).

The details for the release of protein and phospholipid components from myelin were further examined by analysing myelin at a range of SDS solubilization ratios on sucrose density gradients. Isolated myelin samples were equilibrated with SDS at solubilization ratios between 0 and 4.6 for 1 h at 22°C. Samples were

then fractionated by velocity gradient centrifugation through linear 3–30% (w/v) sucrose gradients buffered with 10mM-Tris/HCl, pH 7.2. Isolated myelin (zero solubilization ratio) sediments to between 16 and 21% sucrose (Fig. 6a). This broad band presumably reflects heterogeneity within the myelin sample arising principally from differences in the size of the membrane fragments (discussed further in Rumsby & Crang, 1977). At a solubilization ratio of 0.37 (Fig. 6b) a significant proportion of protein and phospholipid is detected in the top fraction of the gradient. A residual lipid-rich, protein-depleted fraction that sediments on the gradient to the position of untreated myelin remains (Fig. 6b). With increasing SDS solubilization ratios the protein-depleted myelin is further degraded to give phospholipid–protein–SDS fragments that are detected in the top fractions of the density gradient (Figs. 6c–6e). These phospholipid-rich fragments in the upper fractions of the density gradient are further degraded by adding SDS such that at a solubilization ratio of 4.6 most of the protein and phospholipid from myelin is released to the top three fractions of the density gradient.

A more detailed analysis of the protein–phospholipid fragments released from myelin by SDS was performed by gel-exclusion chromatography of the 100000g × 60 min supernatant fractions of solubilized myelin. A typical elution profile from Bio-Gel A-5m (nominal exclusion limit 5×10^6 daltons) of a supernatant from myelin at a solubilization ratio of 1.0 (Fig. 7) indicates that most of the protein constituents

Fig. 6. Velocity gradient-centrifugation analysis of myelin and partially solubilized myelin. Samples of myelin at solubilization ratios between 0 and 4.6 were placed on 3–30% linear sucrose gradients and centrifuged at 57000 g_{av} for 90 min as described in the Materials and Methods section. Samples (approx. 1 ml) were collected and assayed for total protein and phospholipid. Solubilization ratios were: a, zero; b, 0.37; c, 0.74; d, 1.85; e, 4.6.

Fig. 7. Analysis of sodium dodecyl sulphate-solubilized myelin by gel-exclusion chromatography

A 100000 $g \times 1$ h supernatant fraction of isolated myelin prepared at an SDS solubilization ratio of 1.0 was applied to a Bio-Gel A-5m column (2.5 cm $\times$ 90 cm) and eluted with 0.2% SDS in glass-distilled water, pH 7.2. Fractions (10 ml) were collected and assayed for protein; total lipid extracts were prepared and separated by t.l.c. for determination of cholesterol, cerebroside and phospholipids as described in the Materials and Methods section. Elution coefficient = $(V_e - V_0)/(V_c - V_0)$, where V_e is the elution volume of separated component, V_0 is the void volume of the column and V_c is the column volume.

enter the gel and are recovered in fractions eluted beyond the void volume. Most of the lipid components are recovered in the void-volume fraction. However, detectable amounts of cholesterol, phospholipid and galactolipid are co-eluted with the major protein peak and some protein is recovered in the void-volume fraction.

The effect of the SDS solubilization ratio on the distribution of protein and phospholipid between the

Fig. 8. Effect of sodium dodecyl sulphate solubilization ratio on the gel-filtration analysis of solubilized myelin. Supernatant fractions (100000 $g \times 1$ h) were prepared from isolated myelin at solubilization ratios between 0.5 and 2.0 and were analysed by gel-exclusion chromatography (column dimensions 2.5 cm $\times$ 90 cm). Fractions were assayed for protein (●) and for phospholipid (○). (a) Solubilization ratio 0.5, elution from Bio-Gel A-5m with 0.2% SDS, pH 7.2. (b) Solubilization ratio 1.0, elution from Bio-Gel A-5m with 0.2% SDS, pH 7.2. (c) Solubilization ratio 2.0, elution from Sephadex G-200 with 0.2% SDS, pH 7.2. (d) Solubilization ratio 2.0 in 40 mM-NaCl; elution from Sephadex G-200 with 0.2% SDS in 40 mM-NaCl, pH 7.2.

void-volume fraction and the major protein fraction was examined further. The elution profiles for protein and phospholipid in 100000g supernatant fractions from Bio-Gel A-5m (Figs. 8a and 8b) and from Sephadex G-200 (Figs. 8c and d) are presented. At a solubilization ratio of 0.5 the void-volume fraction comprises most of the phospholipid (over 70%), but only a small proportion of the protein (less than 13%). A solubilization ratio of 1.0 decreases the proportions of phospholipids and protein in the void-volume fraction to less than 32% and 5% respectively. With the improved resolution of Sephadex G-200 and a solubilization ratio of 2.0 the protein components entering the gel are fractionated and give a multicomponent elution profile. However, there is significant co-elution of phospholipid with the major protein fractions. The addition of 40 mM-NaCl to the solubilizing and elution buffer results in the recovery of most (over 95%) of the phospholipid in a single peak that is eluted just beyond the low-

molecular-weight protein components. The protein elution profile shows three major components. These have been characterized by gel electrophoresis and represent, in order of elution from the gel, the high-molecular-weight proteins, the proteolipid protein and the basic protein.

Discussion

The results of this study show that the solubilization of isolated myelin by SDS is a complex multistep process. The characteristic decrease in turbidity of myelin suspensions with added SDS is similar to that reported briefly by Hulcher (1963). The process also has the same general character as the solubilization profiles of isolated myelin by lysophosphatidylcholine as described by Gent *et al.* (1964). However, the stoichiometries for the solubilization of myelin by SDS and lysophosphatidylcholine differ. The minimum amount of lysophosphatidylcholine required to give a minimum constant turbidity for myelin is 36mg/100mg dry wt. of myelin (Gent *et al.*, 1964). The value for SDS is 184mg/100mg dry wt. Taking into account the approximate 2-fold difference in the molecular weights of the two detergents, solubilization of myelin apparently requires at least 10 times more SDS than lysophosphatidylcholine on a molar basis. These differences probably reflect the state of solubilization of the myelin components in the two different detergents and the contrasting nature of the molecules involved in terms of charge, head-group size and hydrocarbon chain length. However, the minimum turbidity observed in the present study clearly does not indicate total solubilization of the membrane system by SDS. The same is probably true for lysophosphatidylcholine also.

Some indication of the size of the fragments of myelin released by SDS can be gained from our results. The increase in scattering power (Fig. 2) for isolated myelin with increasing SDS concentration indicates that there is a gradual decrease in mean particle size. A constant maximum value for the wavelength exponent of -2.5 for myelin in SDS is observed at a solubilization ratio of about 1.8. Atherton & Peters (1953) have examined the relationship between particle size and light-scattering power. From their calculations a wavelength exponent of -2.5 corresponds to a mean particle size of about 230nm. Myelin dispersed in chloroform/methanol (2:1, v/v) and in 5mM-NaOH has wavelength exponents of about -2 suggesting that the membrane fragments are significantly larger. Our present studies have shown that a protein-rich SDS supernatant fraction from myelin has a wavelength exponent of -3.1 that corresponds to a particle of mean diameter of about 150nm. This size of particle is larger by about an order of magnitude than SDS-

protein complexes (Reynolds & Tanford, 1970) and SDS-lipid micelles (Tanford & Reynolds, 1976). The observation is consistent with the gel-filtration data (Fig. 8), which resolves a significant proportion of high-molecular-weight components that are excluded from the gel at a solubilization ratio of 2.

The release of protein and phospholipid components from isolated myelin to a $100000g \times 1h$ supernatant fraction exhibits a characteristic relationship with the total amount of SDS added to the system. Complete solubilization of myelin takes place below the critical micellar concentration of SDS in agreement with studies on the solubilization of the Semliki Forest virus membrane with the same detergent (Becker *et al.*, 1975). These data suggest that the critical micellar concentration for SDS-lipid mixed micelles is significantly less than for pure SDS. The solubilization ratio required for the complete release of protein and phospholipid from myelin (Fig. 4) decreases as the ionic strength of the system increases. This observation can be ascribed principally to the stabilization of lipid-SDS complexes. The final amount of SDS bound by proteins is independent of ionic strength (Reynolds & Tanford, 1970), though the ionic strength clearly influences initial SDS interaction with basic proteins such as the myelin basic protein (Jones & Rumsby, 1978) and ferricytochrome *c* (Burkhard & Stolzenberg, 1972). The critical micellar concentration of SDS in water is 8.2mM, whereas in 0.5M-NaCl it is 0.52mM (Mukerjee & Mysels, 1971). This effect is demonstrated clearly by the gel filtration of SDS-treated supernatant fractions of myelin in the presence and absence of 40mM-NaCl. Without salt there is a considerable co-elution of phospholipid with the protein components. In the presence of salt most of the phospholipid is recovered as a single sharp peak that is eluted from Sephadex G-200 just beyond the basic protein. This co-elution of phospholipid with protein at low ionic strengths may arrive from the ionic association of phospholipid with protein that has been shown to be decreased with increasing ionic strength.

However, the co-elution of two components in a single system is not adequate evidence that they are present in a single complex.

The resolution of the protein components of myelin by gel-exclusion chromatography clearly requires complete monomerization of the protein components and the total removal of any bound phospholipid. This is especially important for the proteolipid protein of myelin, which has a marked tendency to aggregate even in the presence of SDS (Lees & Paxman, 1974; Morell *et al.*, 1975). The conditions necessary to achieve complete monomerization and removal of bound phospholipid from myelin proteins have been established. The results indicate that problems for subsequent analyses and

determination can be introduced by the use of sub-optimal concentrations of SDS. Measurement of the major protein components of myelin separated on Sephadex G-200 by using 40mM-NaCl in the elution buffer agree well with the proportions of the major myelin proteins established by other techniques (Eng *et al.*, 1968; Mehl & Halaris, 1970). A similar gel-filtration analysis of myelin proteins reported by Reynolds & Green (1973) demonstrated a large proportion of high-molecular-weight (over 35000) protein components present. The results from our study would suggest that such high-molecular-weight protein material is principally protein-protein and protein-lipid complexes formed in a system containing insufficient detergent to achieve complete monomerization.

From the present study it is clear that the solubilization of isolated myelin by SDS passes through a continuum of lipid-protein-SDS complexes and that the 100000g supernatant fractions contain a number of lipid-protein-SDS complexes that achieve monomerization to form SDS-protein complexes and SDS-lipid mixed micelles only when sufficient SDS is available. The observation that phosphatidylserine + phosphatidylinositol require significantly higher SDS solubilization ratios than the other myelin phospholipids for their release to the 100000g supernatant does not necessarily indicate that these negatively charged lipids are not associated structurally with specific myelin proteins in the intact membrane system. Indeed the myelin basic protein has a strong affinity for negatively charged lipids (reviewed in Rumsby & Crang, 1977). SDS will probably displace phosphatidylserine and phosphatidylinositol from sites of ionic interaction on proteins because of its sulphate group, which is more strongly acidic than the phosphate or carboxy groups of such phospholipids (Abramson *et al.*, 1967). These sites where SDS displaces phospholipids may be the initial points for the interaction of the detergent with the membrane system. For the isolated myelin basic protein the initial interaction with SDS is ionic (Jones & Rumsby, 1975, 1978). Negatively charged phospholipids such as phosphatidylserine and phosphatidylinositol would be expected to form mixed micelles with SDS less readily than the zwitterionic lipids such as phosphatidylcholine and sphingomyelin because their interaction with the detergent is presumably inhibited by charge-charge repulsion. The phospholipids co-released with myelin proteins at solubilization ratios below 3 in low-ionic-strength buffers (less than 16mM) are principally the zwitterionic phospholipids (Fig. 5).

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