Sialic Acid in Crude Myelin Fractions from Rat Brain

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(Received 11 October 1974)

Protein- and lipid-bound sialic acid was assayed in myelin fractions isolated by four different methods from rat brain homogenates. The extent to which non-myelin cellular membranes contaminate these fractions was assessed by electron microscopy and marker-enzyme assays. Small amounts of sialic acid found in the least-contaminated myelin fractions are considered to be constituents of axonal and satellite cell plasma membranes known to be present. The data are discussed with reference to the ultrastructural appearance of myelin.

Two characteristics of glycoproteins present on the mammalian cell surface which may exert a considerable influence on the nature of cellular interactions have been defined by recent investigations. It is evident from data on the erythrocyte membrane that some glycoproteins span the lipid bilayer and moreover extend their oligosaccharide chains into the surrounding extracellular space (Bretscher, 1971; Marchesi *et al.*, 1972). A second important feature of certain glycoproteins is the negative charge they bear under normal physiological conditions owing to their constituent sialic acid residues (Cook, 1968). Sialic acid residues are almost invariably located terminally in the oligosaccharide chains of sialoglycoproteins (Winzler, 1970) and a proportion of such groups is exposed in the outermost layers of the cell periphery, where they may be expected to exert the greatest electrostatic effect on the cellular environment. There has been considerable debate about the exact role of charge in cell-to-cell interactions. On one hand it has been suggested that the 'normal' separation distance of 20 nm (200 Å) observed in the electron microscope between plasma membranes of adjacent cells is due to stabilization in a secondary energy minimum resulting from the combination of attractive London–Van der Waal's interactions and electrostatic repulsion (Curtis, 1964). Weiss (1967, 1968), on the other hand, has argued that the secondary energy minimum is too small to afford such a stabilizing effect and that cell contacts are stabilized in primary minima between components of the cell periphery separated by only a few nanometres. The surface ionogenic groups involved in such contacts may extend a considerable distance from the osmophilic unit membrane: these are not detected in the electron microscope after routine tissue preparation and consequently give rise to the appearance of an intercellular electron-lucent space.

Myelin is a notable exception to the general rule. The external aspects of adjacent spiral satellite cell

membrane are separated by an order of magnitude less than found elsewhere (Napolitano & Scallen, 1969; Revel & Hamilton, 1969) or appear to be fused into a single 'intraperiod line'. It has been suggested from data on peripheral nerve that the absence from myelin of acidic glycoproteins could explain this close approach of adjacent membranes (Langley, 1971). The likelihood of this suggestion is further examined here with data from myelin fractions isolated from rat brain. Both lipid- and protein-bound sialic acid has been assayed in myelin samples isolated by different procedures. The extent to which non-myelin cellular membranes contaminate these fractions has also been assessed by electron microscopy and marker-enzyme assays.

Experimental

Tissue preparation

Whole brains of male Wistar rats (250–350 g) were homogenized in ice-cold unbuffered sucrose solutions (0.32 M or 0.88 M; tissue concentration about 15%, w/v) in a glass homogenizer with a mechanically driven Teflon pestle (clearance 0.15–0.25 mm; speed of rotation 1000 rev./min; four or five strokes; time 45 s). The homogenate was further diluted with fresh sucrose solution (final concentration 10%, w/v).

Myelin preparation

Sedimentation methods. (a) From postnuclear supernatant. After removal of the nuclear fraction at 1000g (10000g·min) the combined postnuclear supernatant and the nuclear wash supernatant were underlaid with ice-cold 0.88 M-sucrose and centrifuged at 99000g (6×10^6 g·min). The interfacial material was removed, diluted with water to 0.32 M-sucrose and re-centrifuged in the same way. The interfacial material was aspirated and a portion centrifuged at 99000g (3×10^6 g·min) after tenfold dilution with 0.32 M-sucrose or water.

(b) From mitochondrial and microsomal fractions. Mitochondrial and microsomal pellets, isolated at $11\,500g$ ($2.3 \times 10^5 g\text{-min}$) and $99\,000g$ ($6 \times 10^6 g\text{-min}$) respectively, were resuspended in $0.32M$ -sucrose and the myelin was then isolated as outlined above.

Flotation methods. (a) From whole brain homogenate. Brain homogenates in $0.88M$ -sucrose were centrifuged ($6 \times 10^6 g\text{-min}$) on a discontinuous gradient with $0.32M$ -sucrose. The interfacial material was collected, made up with sucrose to $0.88M$ and re-centrifuged in a similar manner. Subsequent manipulation of the material was identical with that for the other preparations.

(b) From mitochondrial and microsomal fractions. Mitochondrial and microsomal pellets resuspended in $0.88M$ -sucrose were used as starting materials for the isolation of myelin fractions by the above method.

Analytical methods

Lipid extraction. KCl was added to samples (final concentration $0.1M$), since the extractability of gangliosides has been found to be dependent on univalent-cation concentration (Spence & Wolfe, 1967). Lipids were then extracted with chloroform-methanol by the method of Folch *et al.* (1957).

Gangliosides. These were isolated by partition from the lipid extract (Folch *et al.*, 1957) and assayed for sialic acid after acid hydrolysis with $0.05M$ - H_2SO_4 (2 h at $80^\circ C$) by the method of Warren (1959). Since sucrose interferes in the Warren assay, it was removed before acid hydrolysis by dialysis or washing in trichloroacetic acid (10% , w/v). The absorption spectra of both reference sialic acid and hydrolysed extracted gangliosides closely resembled each other. The standard used was *N*-acetylneuraminic acid purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Cholesterol. This was assayed on the lipid extract after removal of gangliosides by the method of Searcy *et al.* (1960).

Protein. This was assayed by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard.

Sialoprotein. Protein-bound sialic acid was measured by the thiobarbituric acid method (Warren, 1959) on the chloroform-methanol-insoluble residue after acid hydrolysis in $0.05M$ - H_2SO_4 (1 h at $80^\circ C$).

$Na^+ + K^+$ -activated ATPase*. This was determined by method of Bonting (1970).

5'-Nucleotidase. Samples were incubated in media containing Mg^{2+} ($5mM$), potassium tartrate ($10mM$) and buffered at pH 7.7 with Tris-HCl ($50mM$). Released P_i was determined as in the assay of ATPase.

* Abbreviation: ATPase, adenosine triphosphatase.

Electron microscopy

Small samples of washed and unwashed myelin were fixed overnight in sodium phosphate-buffered (pH 7.2) glutaraldehyde (4% , w/v) at $4^\circ C$ and post-fixed in 1% (w/v) buffered OsO_4 . They were dehydrated in ethanol and embedded in Epon. Thin sections were stained with lead citrate (0.4% , w/v) and uranyl acetate [saturated solution in 50% (w/v) ethanol]. Two separate preparations of each myelin sample were examined.

Results

Sialic acid in myelin fractions

A considerable proportion of total brain sialic acid is found in crude myelin isolated by sedimentation of the postnuclear supernatant from rat brain homogenate. The recovery of protein-bound sialic acid was halved, however, after high-speed centrifugation of this material after dilution with sucrose solution. No appreciable further decrease in yield was achieved by subsequent aqueous washing of the pellet. The decrease in ganglioside content brought about by such procedures was somewhat smaller. No loss of sialic acid was noted when myelin samples obtained from isolated mitochondrial and microsomal fractions were washed in water or sucrose. Approximately two-thirds of both lipid- and protein-bound sialic acid sedimented at the interface of a discontinuous sucrose gradient from postnuclear supernatants derived from particles that sediment with the microsomal fraction. By contrast, over two-thirds of the ganglioside and 80% of the sialoprotein found in myelin prepared by flotation of brain homogenates was recovered by flotation of the isolated mitochondrial pellet.

The specific activities of both gangliosides and sialoproteins were lowest in material isolated from mitochondrial pellets and were similar for both flotation and sedimentation methods (Table 1). Simple flotation of whole brain homogenate, however, yielded material with lower concentrations than that obtained by sedimentation of postnuclear supernatants.

Enzyme activity of myelin fractions

Table 2 shows the specific activities of 5'-nucleotidase and the $Na^+ + K^+$ -activated ATPase of the various crude myelin preparations and the protein content of recovered material. Highest activities and yields were found with both sedimentation methods. No significant difference in enzyme activity was found between myelin derived by flotation from total brain homogenate and that obtained from isolated mitochondrial pellets. No significant loss in enzyme activity was found in myelin samples washed with water followed by high-speed centrifugation.

EXPLANATION OF PLATE I

Crude myelin fraction isolated by sedimentation of postnuclear supernatant (a) or crude myelin isolated by sedimentation of resuspended mitochondrial pellet (b)

For experimental details see the text. The bar represents 1 μ m.

EXPLANATION OF PLATE 2

Myelin obtained by flotation of whole brain homogenate

For details of preparation see the text. The bar represents 0.5 μm .

EXPLANATION OF PLATE 3

Unwashed myelin fraction illustrating axoplasmic organelles

For details of preparation see the text. The bar represents 0.5 μm .

EXPLANATION OF PLATE 4

Expanded membrane networks produced by exposure of myelin sample to distilled water

For details of preparation see the text. The bar represents 1 μm .

Table 1. *Sialic acid in myelin fractions*

Myelin fractions were prepared from postnuclear supernatant, whole brain homogenate, mitochondrial and microsomal pellets by sedimentation or flotation as described in the Experimental section. Results are means of two experiments; values in parentheses are percentages of total brain lipid- or protein-bound sialic acid; data for myelin from mitochondrial and microsomal fractions are on unwashed samples. The reproducibility was better than $\pm 5\%$, except on one occasion when it was $\pm 15\%$.

Source of fraction	Sialic acid concentration (μmol of <i>N</i> -acetyl neuraminic acid/mg of protein)	
	Lipid-bound	Protein-bound
Sedimentation method		
Postnuclear supernatant		
(unwashed)	0.0288 (31.8)	0.0156 (26.1)
(washed)	0.0337 (26.6)	0.0111 (13.3)
Mitochondrial pellet	0.0109 (8.4)	0.0038 (4.5)
Microsomal pellet	0.0363 (16.3)	0.0125 (8.5)
Flotation method		
Whole brain homogenate		
(unwashed)	0.0182 (10.4)	0.0060 (5.2)
(washed)	0.0176 (8.3)	0.0064 (4.6)
Mitochondrial pellet	0.0103 (4.5)	0.0048 (3.2)
Microsomal pellet	— (2.0)	— (0.6)

Table 2. *Specific activities of plasma membrane enzymes in myelin fractions*

Values are the means $\pm$ S.D. of three to five myelin samples. Values in parentheses are percentages of total brain enzyme activity recovered in different myelin fractions. * Significantly different from postnuclear supernatant ($0.05 > P > 0.02$). † Significantly different from mitochondrial pellet ($0.02 > P > 0.01$).

Source of fraction	Enzyme activity (μmol of P_i released/min per mg of protein)		Protein (mg)
	$\text{Na}^+ + \text{K}^+ - \text{ATPase}$	5'-Nucleotidase	
Sedimentation method			
Postnuclear supernatant	0.246 ± 0.077 (14.9)	0.080 ± 0.029 (19.7)	10.52
Mitochondrial pellet	$0.117 \pm 0.007^*$ (7.8)	0.082 ± 0.007 (22.2)	11.50
Flotation method			
Whole brain homogenate	$0.090 \pm 0.027^*$ (3.6)	$0.055 \pm 0.011^\dagger$ (8.9)	6.94
Mitochondrial pellet	$0.096 \pm 0.002^*$ (4.9)	$0.068 \pm 0.001^\dagger$ (14.0)	8.86

Electron microscopy

Myelin prepared by both sedimentation methods contained two populations of subcellular particles: large numbers of small membrane-bound vesicles were present in addition to larger profiles of compact lamellae characteristic of myelin. This was most clearly demonstrated when interfacial material isolated by sedimentation was diluted with water, and pelleted. Then myelin particles sedimented beneath a layer composed almost entirely of vesicles (Plates 1a and 1b). This phenomenon was not observed in frac-

tions obtained by flotation. Such myelin samples contained few isolated vesicles (Plate 2). Most myelin profiles in all samples not treated with hypo-osmotic solutions contained axoplasmic organelles and, therefore, axon and satellite cell membrane (Plate 3). In washed samples, axoplasmic contents were often not present in myelin particles: the compact lamellar structure was sometimes destroyed, and expanded membrane networks were produced (Plate 4). It was then not possible to identify axon and oligodendroglial cell membrane.

Discussion

The extent to which myelin has been studied in recent years owes much to the marked contrast in physical properties between this and other organelles present in brain homogenates. The low specific gravity, owing to its high lipid content, and the large size of myelin particles when prepared in the absence of electrolytes (Norton, 1971) both aid its isolation in high yield. The importance of these two factors in myelin isolation has therefore been examined with particular regard to carbohydrate components.

Purity of myelin fractions

Electron-microscopic examination of different myelin preparations enabled the relative purity of the fractions to be assessed. Myelin isolated by the simple sedimentation method contained a large number of small membrane-bound vesicles typical of those in microsomal fractions in addition to profiles characteristic of myelin. Moreover, these small vesicles may be separated from such myelin fractions by subsequent equilibrium sedimentation on a continuous sucrose gradient, when they are much denser than myelin (O. K. Langley, unpublished work). Such microsomal contamination is largely absent from electron micrographs of myelin prepared by flotation methods. In all preparations not exposed to hypo-osmotic solutions, however, axon and satellite cell membranes, recognizable by their contents, were a distinct feature of most myelin particles. After exposure of samples to water, followed by high-speed centrifugation, it was rarely possible to recognize either axolemma or oligodendroglial cell membranes in any of the preparations (though in fact the membrane-marker-enzyme activities were not significantly decreased by this treatment).

A quantitative assessment of the extent of contamination of isolated myelin fraction with non-myelin membranous elements has been based on well-documented evidence of 5'-nucleotidase and $\text{Na}^+ + \text{K}^+$ -activated ATPase as excellent specific markers for plasma membranes (Novikoff & Essner, 1962; Emmelot *et al.*, 1964; Torak & Barnett, 1964; Coleman & Finean, 1966; El-Aaser *et al.*, 1966; Coleman *et al.*, 1967; Song *et al.*, 1969; Steck & Wallach, 1970).

Lowest ATPase activities were found in the fractions prepared by flotation, with no significant difference between that derived in this way from isolated mitochondrial pellets or whole brain homogenates. This parallels the different extents of microsomal contamination of fractions observed by electron microscopy, which presumably result from the fact that such vesicles sediment more slowly than myelin isolated by sedimentation, which therefore forms a cohesive layer through which the denser

microsomal particles are 'force-filtered'. When myelin is prepared by flotation non-myelin membranes sediment through the 0.88M-sucrose to the bottom of the tube and myelin floats to the interface.

A similar qualitative distribution of 5'-nucleotidase was found, though the specific activities relative to whole brain were consistently higher, reflecting a considerable difference in the concentration of these enzymes in the plasma membranes contaminating the crude myelin preparations studied here. These data complement the findings of others (Hosie, 1965; Banik & Davison, 1969; Pilcher & Jones, 1970), who also detected relatively high concentrations in myelin samples not exposed to osmotic shock. If such enzyme activities truly reflect the extent of plasma-membrane contamination they serve to illustrate that myelin prepared by flotation (the least contaminated) contains 4-9% of the total rat brain plasma membranes.

Sialic acid and myelin

The question as to whether sialic acid, in the form of either gangliosides or glycoproteins, is a constituent of myelin or of the non-myelin membranes that contaminate these crude fractions may now be discussed. Although the increase in the specific activity of cholesterol was similar (about fourfold) for material prepared by either sedimentation or flotation, the sialic acid content of the latter was markedly lower. Two factors appear to be responsible for the decreased concentrations of sialic acid. It is evident that microsomal membranes contribute significantly to the sialic acid content of the fraction derived by sedimenting postnuclear supernatants. The microsomal fraction has been found in a separate study (O. K. Langley, unpublished work) to have a higher specific activity of sialic acid than have crude nuclear, mitochondrial and soluble fractions. The partial solubilization of more hydrophilic components containing sialic acid, produced by the additional sample manipulation involved in isolating myelin from resuspended mitochondrial pellets, must also be considered when comparing the different fractions. The loss of sialoprotein from crude myelin brought about simply by resuspension and centrifugation in sucrose serves to illustrate this point.

The myelin fraction isolated by flotation proved to be the least contaminated with cytomembranes as determined by enzyme assay and electron microscopy, though such preparations nevertheless contained a small but significant proportion of axon and glial membranes (see above).

The percentage of lipid- and protein-bound sialic acid recovered in myelin fractions isolated by flotation was either similar to or below the amount of plasma-membrane contamination for these fractions deduced from enzyme activities. It is thus possible to account

for the gangliosides and glycoproteins found in such preparations purely in terms of the non-myelin membranes present in them. It may therefore be concluded that sialic acid is not a normal constituent of the mature myelin sheath, though its presence in the incisures of Schmidt and Lantermann and in uncompacted paranodal regions is not excluded by the present data.

Classical methods for myelin isolation include attempts to remove cytomembrane contamination by exposure of crude myelin to hypo-osmotic shock (Autilio *et al.*, 1964; Cuzner *et al.*, 1965; Cuzner & Davison, 1968). The possible loss or redistribution of the more hydrophilic membrane components (such as glycoproteins and di- and tri-sialogangliosides) is an unfortunate consequence of such procedures. The data of Suzuki (Suzuki, 1967; Suzuki *et al.*, 1967, 1968) are interesting in this regard. He did not detect sialoproteins and found only the least hydrophilic of the gangliosides in his purified myelin preparations. The first stage in his isolation method involved centrifugation of postnuclear supernatants on discontinuous sucrose gradients. Clearly the large amount of microsomal contamination present in such crude myelin shown by the present study was considerably decreased by subsequent steps in Suzuki's (1967) procedure. It is possible, however, that complete elimination of the closely attached glial cell and axon membranes was not achieved even though axonal contents (in addition to membrane sialoproteins) were removed by exposure to distilled water, resulting in an inability to detect these non-myelin membranes with the electron microscope. Membrane fractions biochemically distinct from myelin have been isolated from crude myelin preparations, particularly from young animals (Davison, 1970), and there is doubt as to the effectiveness of distilled water in removing attached contaminating elements from subcellular fractions (Cotman & Matthews, 1971). Despite more recent metabolic studies (Suzuki, 1970) it remains possible from present observations that the mono-sialogangliosides detected in such 'purified myelin' represent constituents of neuronal and glial cell plasma membranes remaining attached to myelin lamellae after hypo-osmotic treatment.

In contrast with other membranes, sialic acid does not have a significant functional role in maintaining surface charge density at the intraperiod line of myelin. Myelin lipids provide a source of negative surface charge at this site, but this may be 'neutralized' by the basic protein also present (Dickinson *et al.*, 1970).

The situation here parallels the agglutination of erythrocytes by basic proteins (Forrester *et al.*, 1969). Normal erythrocyte morphology is lost and adjacent cells are flattened against each other. Moreover, cells depleted of their sialic acid by pretreatment with neuraminidase are still agglutinated by basic proteins.

The fusion of the adjacent lateral cell borders of gallbladder epithelial cells (Quinton & Philpott, 1973) produced by incubation in solutions of basic proteins provides a further example of the elimination of the apparent space between cells as a result of surface-charge neutralization. In myelin the absence of sialoproteins and presence of basic proteins combine to produce low surface-charge density at the intraperiod line and contribute to the compact appearance and relative physical stability of myelin under normal conditions. The extent of the gap normally observed between adjacent cell membranes in which sialoproteins are constituents may also reflect the ability of such macromolecules to protrude into the intervening extracellular space.

I thank Professor J. B. Cavanagh for his help and encouragement, Dr. D. N. Landon for help with the electron-microscopic examination of fractions and Mr. P. M. Freeman and Mr. P. M. Chandler for excellent technical assistance. The work was supported in part by a grant from the Multiple Sclerosis Society.

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