Effects of Pronase and Neuraminidase Treatment on a Myelin-Associated Glycoprotein in Developing Brain

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Rats (14 days old) were injected with [^{14}C]fucose and young adult rats with [^3H]fucose in order to label the myelin-associated glycoproteins. As previously reported, the major [^{14}C]fucose-labelled glycoprotein in the immature myelin had a higher apparent molecular weight on sodium dodecyl sulphate/polyacrylamide gels than the [^3H]fucose-labelled glycoprotein in mature myelin. This predominant doubly labelled glycoprotein component was partially purified by preparative gel electrophoresis and converted to glycopeptides by extensive Pronase digestion. Gel filtration on Sephadex G-50 separated the glycopeptides into several classes, which were designated A, B, C and D, from high to low molecular weight. The ^{14}C -labelled glycopeptides from immature myelin were enriched in the highest-molecular-weight class A relative to the ^3H -labelled glycopeptides from mature myelin. Neuraminidase treatment of the glycoprotein before Pronase digestion greatly decreased the proportion of glycopeptides fractionating in the higher-molecular-weight classes and largely eliminated the developmental differences that were apparent by gel filtration. However, neuraminidase treatment did not decrease the magnitude of the developmental difference revealed by electrophoresing the intact glycoprotein on sodium dodecyl sulphate gels, although it did decrease the apparent molecular weight of the glycoprotein from both the 15-day-old and adult rats by an amount comparable in magnitude to that developmental difference. The results from gel filtration of glycopeptides indicate that there is a higher content of large molecular weight, sialic acid-rich oligosaccharide units in the glycoprotein of immature myelin. However, the higher apparent molecular weight for the glycoprotein from 15-day-old rats on sodium dodecyl sulphate gels is not due primarily to its higher sialic acid content.

Glycoproteins are components of cell surfaces and are considered to be involved in recognition phenomena and in specific contact relationships between cells (Hughes, 1973; Spiro, 1973). A major glycoprotein of the erythrocyte membrane has been extensively characterized and many of its biological properties determined (Marchesi *et al.*, 1973). Membrane glycoproteins from other cells have not been nearly so well characterized, because of the limited amounts of membranes that can be obtained and difficulties in solubilizing and fractionating the glycoproteins. Nevertheless, there has been considerable interest in surface glycoproteins of other cells, particularly with regard to alterations in their carbohydrate moieties that occur during differentiation (Moscona, 1971; Glick *et al.*, 1973), cell division (Buck *et al.*, 1971; Burger, 1973) and oncogenic transformation (Warren *et al.*, 1973; Burger, 1973; van Beek *et al.*, 1973).

In the developing nervous system, cell surface glycoproteins are believed to be involved in the formation of specific interneuronal connections and in establish-

ing proper glial-neuronal relationships (Brunngraber, 1969). Investigation of brain glycoproteins has been hampered by the extreme cellular and sub-cellular complexity of neural tissue. It is possible, however, to isolate myelin, which is derived from the plasma membrane of the oligodendrocyte, in a high degree of purity. Recently, we have shown that purified myelin fractions contain several glycoproteins, and there is one major component (Quarles *et al.*, 1972). This predominant glycoprotein component is genuinely associated with myelin or myelin-related membranes in the central nervous system (Quarles *et al.*, 1973a; Matthieu *et al.*, 1973, 1974), and it may be most concentrated in the oligodendroglial plasma membrane and its region of transition to myelin (Matthieu *et al.*, 1973; Poduslo *et al.*, 1976). These findings raise the possibility that the glycoprotein could be involved in the recognition of axons to be myelinated by oligodendroglial processes or in contact relationships occurring between adjacent layers of myelin as they are compacted. Double-labelling experiments with radioactive fucose

and other sugar precursors showed that the myelin-associated glycoprotein, which is synthesized at the very early stages of myelination in the rat, has a slightly higher apparent molecular weight on sodium dodecyl sulphate/polyacrylamide gels than that synthesized by mature rats (Quarles *et al.*, 1973b; Matthieu *et al.*, 1975). In order to determine if the oligosaccharide units of the glycoprotein are involved in the developmental changes, we have partially purified the major glycoprotein component and studied the effects of pronase and neuraminidase degradation. The results indicate that there are complex developmental changes in the glycoprotein at least some of which involve the oligosaccharide moieties.

Experimental

Materials

L-[1,5,6-³H]Fucose and L-[¹⁴C]fucose were from New England Nuclear Corp. (Boston, MA, U.S.A.). Unlabelled fucose was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acrylamide and *NN'*-methylene-bisacrylamide were from Eastman Organic Chemicals (Rochester, NY, U.S.A.). The sodium dodecyl sulphate was Sequanol grade from Pierce Chemical Co. (Rockford, IL, U.S.A.). Sephadex G-50 (fine) was purchased from Pharmacia Fine Chemicals Inc. (Piscataway, NJ, U.S.A.). Osborn-Mendel rats were supplied by the Veterinary Resources Branch at NIH.

Injection of animals and preparation of myelin

[³H]Fucose (20 μ Ci) or [¹⁴C]fucose (8 μ Ci) was injected intracerebrally as previously described (Quarles & Brady, 1971). The animals received 160 nmol of fucose regardless of which isotope was injected. The specific activity of [¹⁴C]fucose was 50 μ Ci/mol, and the [³H]fucose was diluted with unlabelled fucose to a specific radioactivity of 125 μ Ci/ μ mol. The animals were killed 16–20 h after injection, and a 5% (w/v) homogenate in 0.32 M-sucrose was prepared. In most experiments, a doubly labelled, mixed homogenate was prepared by combining the brains of three 200–250 g rats, which had been injected with [³H]fucose, with the brains of eight 15-day-old rats, which had been injected with [¹⁴C]fucose, and homogenizing them together in a total volume of 250 ml. Myelin was then purified from 210 ml of the mixed homogenate, essentially by the procedure of Norton & Poduslo (1973). In the first discontinuous gradient 35 ml of the mixed homogenate was layered over 20 ml of 0.85 M-sucrose in each tube; two centrifugations were required to process 210 ml of homogenate. After the osmotic shocks the entire preparation was processed by one centrifugation in which 30 ml of myelin suspension was layered over 25 ml of 0.85 M-sucrose in each of three centrifuge tubes. In a few experiments myelin was

purified separately from rats of one age or the other by the procedure of Norton and Poduslo (1973).

Analytical polyacrylamide-gel electrophoresis

Solubilization of samples with sodium dodecyl sulphate and electrophoresis in 10 cm long, 5% (w/v) polyacrylamide gels was as previously described (Quarles *et al.*, 1972, 1973a). Gels were stained with Fast Green for proteins (Greenfield *et al.*, 1971) or with periodic acid-Schiff reagents for glycoproteins (Segrest & Jackson, 1972). Method B of Quarles *et al.* (1973a) was used for the determination of radioactivity in the gels after cutting 1 mm slices with a Mickle gel slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). In many experiments, radioactivity was only determined in the portion of the gel that contained the major myelin-associated glycoprotein, i.e. the region from 2 to 5 cm from the top. In order to best compare the positions of the peaks on doubly labelled gels, it was convenient to express the results as a percentage of the total radioactivity recovered in that portion of the gel. When this was done the total d.p.m. of ³H and ¹⁴C, which were recovered, are given in the Figure legends.

Partial purification of the major myelin-associated glycoprotein

Freeze-dried myelin (20–30 mg of protein) was treated with 20 ml of chloroform/methanol (2:1, v/v), and the insoluble residue was pelleted at 100 000 g. The pellet was washed with 10 ml of diethyl ether and then dried under N₂. The dried material was dissolved in 1.5 ml of 2.5% (w/v) sodium dodecyl sulphate 1% (w/v) Na₂CO₃/10% (v/v) 2-mercaptoethanol. It was then dialysed against 200 ml of 0.1% sodium dodecyl sulphate / 1.6 M-urea/0.01 M-sodium phosphate buffer (pH 7.2), containing 0.05% dithiothreitol. The sample was electrophoresed on a preparative polyacrylamide gel, which was the same as the analytical gels except that it was 1.5 cm in diameter. Electrophoresis was done in an apparatus made by the Biomedical Engineering Instrumentation Branch of NIH and specifically designed to hold gels of this size. Electrophoresis took about 6 h at 40 V and was terminated when a Bromophenol Blue tracking dye reached the bottom of the gel. The gel was cut into longitudinal wedge-shaped segments and sliced into 3.5 mm fractions with a razor blade. One portion of each fraction, representing about one-sixth of the gel in cross-sectional area, was used for radioactive counting by the same method as for analytical gels, and the other five-sixths of each fraction was stored overnight at 4°C. After determining the fractions that contained the major radioactive peak, the remaining five-sixths of the appropriate fractions were combined and extracted with water. Extraction was done at 4°C and consisted of disrupting the gel with a

homogenizer in about 30ml of water. The gel suspension was stirred for 15min, and the gel particles were removed by centrifugation. The sedimented gel particles were re-extracted by adding 20ml of cold water, stirring for another 15min, and centrifuging again. The supernatants from the two extractions were combined and freeze-dried to give the partially purified glycoprotein fraction.

Neuraminidase incubations

Samples of the partially purified glycoprotein were dissolved in water, and 0.5ml portions were dialysed overnight in the cold against 0.02% sodium dodecyl sulphate in 0.02M-sodium acetate buffer (pH5.4). After dialysis the samples were adjusted to 0.1M-sodium acetate buffer (pH5.4) and 0.01M-CaCl₂ in a total volume of 1.0ml. Neuraminidase incubations were carried out at 37°C for 8h in the presence of 0.02% sodium azide and saturating amounts of toluene. Incubated controls were treated in the same way except that 0.1M-NaCl was added instead of enzyme. Two different neuraminidases were used. Some experiments were done with the *Vibrio cholerae* neuraminidase, which was from Behring Diagnostics (Somerville, NJ, U.S.A.). The other neuraminidase was a gift from Dr. P. Fishman (National Institutes of Health, Bethesda, MD, U.S.A.) and was a preparation that had been highly purified from *Clostridium perfringens* by affinity chromatography (Cuatrecasas & Illiano, 1971). At the time of these experiments, the activity of both preparations was determined on fetuin to be between 3 and 4 μ mol/h per ml. In the incubations described here 0.1ml of the *V. cholerae* enzyme was added at the beginning, and two subsequent additions of 0.05ml were made after 2 and 5h. In the experiments with the highly purified *C. perfringens* enzyme, 0.025ml was added at the beginning followed by two additions of 0.01ml.

Pronase digestions

For Pronase digestions, the partially purified glycoprotein was first dialysed overnight at 4°C versus 0.02% sodium dodecyl sulphate. The sample was adjusted to 0.05M-Tris/HCl (pH7.8) and 0.01M-CaCl₂. If the sample had previously been incubated with neuraminidase, the dialysis step was not repeated, but the solution was titrated to pH7.8 with Tris base before the Tris/HCl buffer was added. Pronase digestions were for 5 days at 37°C in the presence of saturating amounts of toluene. The Pronase was the purified type VI protease from *Streptomyces griseus* (Sigma). At the beginning of the digestion 100 μ g of enzyme was added, followed by two subsequent additions of 50 μ g on days one and three.

Gel filtration on Sephadex G-50

After Pronase digestions, the samples were adjusted

to pH9.0 with Tris base in a total volume of 2.0ml containing 0.1% EDTA, 1% sodium dodecyl sulphate and 0.1% 2-mercaptoethanol. The sample was applied to a column (1.5cm $\times$ 90cm) of Sephadex G-50, which had been equilibrated with 0.1M-Tris/acetate buffer (pH9.0)/0.1% sodium dodecyl sulphate/0.01% EDTA/0.1% 2-mercaptoethanol (Buck *et al.*, 1970). The column was eluted with the same buffer at room temperature (22°C), and fractions were collected, which varied in size between 1.2 and 1.6ml, depending on the experiment. The excluded volume (V_0) was determined with Blue Dextran 2000 and the total volume (V_T) with [¹⁴C]fucose before applying samples to the column. Portions (1ml) of each fraction and 20 μ l of glacial acetic acid were added to 10ml of Aquasol (New England Nuclear Corp.) for the determination of radioactivity by liquid-scintillation counting.

Results

Developmental change in the electrophoretic mobility of the myelin-associated glycoprotein

Purified rat brain myelin contains a high-molecular-weight glycoprotein that can be labelled *in vivo* by intracerebral injection of radioactive fucose. Double labelling experiments were used to compare the newly synthesized glycoprotein in 15-day-old rats, which are just beginning to myelinate, with that in young adult rats. Details of these experiments are described in the Experimental section, but the general approach was to inject 14-day-old rats with [¹⁴C]fucose and young adult rats with [³H]fucose. The next day the brains from rats at the two ages were combined, homogenized together, and the myelin was purified from the mixed homogenate. As previously reported (Quarles *et al.*, 1973b), the labelled glycoprotein from the 15-day-old animals exhibited a slightly lower electrophoretic mobility, i.e. had a higher apparent molecular weight (Fig. 1). The ¹⁴C/³H ratio for each slice, which is plotted at the top of the figure, was near unity at positions far removed from the peak, but increased on the high molecular-weight side of the peak and decreased on the low-molecular-weight side. This suggests that the increase of high-molecular-weight material from the immature myelin was instead of material on the low-molecular-weight side of the peak and not in addition to it.

Developmental changes in glycopeptides derived from the major myelin-associated glycoprotein by pronase digestion

The major myelin-associated glycoprotein from a double-labelled myelin preparation was partially purified by preparative polyacrylamide-gel electrophoresis as described in the Experimental section and converted into glycopeptides by extensive Pronase digestion. The distribution of radioactivity on a

Fig. 1. Comparison of the major fucose-labelled glycoprotein in myelin from 15-day-old rats with that from adult rats

Myelin was purified from a mixed homogenate of [^{14}C]-fucose-labelled 15-day-old rat brains and [^3H]-fucose-labelled adult rat brains as described in the Experimental section. The myelin was freeze-dried, extracted with chloroform/methanol (2:1, v/v) and the residue was then solubilized in sodium dodecyl sulphate and electrophoresed. The gel was sliced into 1 mm fractions for radioactive counting; the Figure represents only that portion of the gel from 2 to 5 cm from the top, which contained the major labelled peak. The results are plotted as the radioactivity in each slice as a percentage of the total of ^3H (11 600 d.p.m.) or of ^{14}C (4050 d.p.m.) that was recovered in that portion of the gel. $\circ$, [^{14}C]-Fucose-labelled glycoprotein from 15-day-old rats; $\bullet$, [^3H]-fucose-labelled glycoprotein from adult rats. The ratio of the ^{14}C -percentage to the ^3H -percentage is plotted at the top of the Figure.

preparative gel is shown in Fig. 2. A slight divergence of ^{14}C and ^3H can be seen in the major peak, but it is not nearly as evident as in Fig. 1, because each fraction represented a threefold greater gel length and the resolution on preparative gels was not quite as high as on analytical gels. The labelled glycoprotein in fractions 12–15 was extracted and converted to glycopeptides. This partial purification of the glycoprotein before the formation of glycopeptides served to ensure that most of the glycopeptides were from the major fucose-labelled peak and kept interference by glycopeptides derived from other myelin-associated glycoproteins to a minimum. The doubly labelled glycopeptides were fractionated on Sephadex G-50, with the results shown in Fig. 3. The distribution of labelled glycopeptides on the column was rather complex, and the pattern was arbitrarily divided into fractions A, B, C and D in order to emphasize the developmental differences. In comparison to the ^3H -labelled glycopeptides from the glycoprotein of the adult rat, the ^{14}C -labelled glycopeptides from the

Fig. 2. Preparative polyacrylamide gel of a doubly labelled preparation

The method of purification of the myelin-associated glycoprotein is described in the Experimental section. This Figure shows the results when a longitudinal wedge-shaped segment of a preparative gel, representing about one-sixth of its total volume, was cut into 3.5 mm fractions for radioactive counting. The major labelled component in fractions 12–15 (shaded area) was extracted from the other five-sixths of the gel. $\circ$, [^{14}C]-Fucose-labelled glycoproteins from 15-day-old rats; $\bullet$, [^3H]-fucose-labelled glycoproteins from young adult rats.

Fig. 3. Gel filtration of glycopeptides

The doubly labelled glycoprotein fraction obtained as shown in Fig. 2 was converted into glycopeptides by extensive Pronase digestion. The glycopeptides were fractionated on a column of Sephadex G-50 in the presence of 0.1% sodium dodecyl sulphate. The fractionated glycopeptides were divided into fractions A–D in order to illustrate the developmental differences. $\circ$, [^{14}C]-Fucose-labelled glycopeptides from 15-day-old rats (7320 d.p.m.); $\bullet$, [^3H]-fucose-labelled glycopeptides from adult rats (5150 d.p.m.).

immature myelin were relatively enriched in fraction A, decreased in fraction B, slightly increased in fraction C and slightly decreased in the tailing fraction D.

Effects of neuraminidase treatment

In order to evaluate the role of sialic acid on the electrophoretic mobility of the glycoprotein on

Fig. 4. Effect of neuraminidase on the electrophoretic mobility of the glycoprotein from 15-day-old rats

Two separate preparations of the partially purified glycoprotein were obtained from 15-day-old rats, which had been injected with [^3H]fucose and [^{14}C]fucose. The [^3H]fucose-labelled glycoprotein served as a control in all cases. Portions of the [^{14}C]fucose-labelled glycoprotein were incubated with the neuraminidases from *C. perfringens* and *V. cholerae*. In each part of the Figure a portion of the [^{14}C]fucose-labelled glycoprotein (●) was electrophoresed on the same gel with the [^3H]fucose-labelled control glycoprotein (○). (a) Untreated [^{14}C]fucose-labelled glycoprotein; 5067 d.p.m. ^{14}C , 5558 d.p.m. ^3H . (b) [^{14}C]fucose-labelled glycoprotein incubated with *C. perfringens* neuraminidase; 3991 d.p.m. ^{14}C , 5154 d.p.m. ^3H . (c) [^{14}C]fucose-labelled glycoprotein incubated with *V. cholerae* neuraminidase; 3515 d.p.m. ^{14}C , 4620 d.p.m. ^3H .

sodium dodecyl sulphate gels and on the fractionation of glycopeptides on Sephadex G-50, experiments were done in which the partially purified glycoprotein was incubated with neuraminidase. These experiments were done both with neuraminidase from *V. cholerae* and with a neuraminidase from *C. perfringens*, which had been highly purified by affinity chromatography. Essentially the same results were obtained with the two neuraminidases.

The major fucose-labelled component was purified from myelin prepared from 15-day-old rats that had been injected with [^{14}C]fucose. Two portions of the fucose-labelled glycoprotein were incubated with the neuraminidases from *V. cholerae* and *C. perfringens*. Double-label gels were run comparing the neuraminidase treated glycoprotein with an intact [^3H]fucose-labelled glycoprotein that had been purified from another group of 15-day-old rats. The [^{14}C]fucose-labelled peak that had not been treated with neuraminidase, electrophoresed to the same position as the [^3H]fucose-labelled peak (Fig. 4a). However, treatment with either neuraminidase shifted the peak 2–3 mm down the gel (Figs. 4b and 4c). Thus neuraminidase treatment decreased the apparent molecular weight of glycoprotein by an amount equal to or greater than the developmental

Fig. 5. Effect of neuraminidase on gel filtration of glycopeptides derived from the glycoprotein in myelin from 15-day-old rats

Half of the incubation mixture containing [^{14}C]fucose-labelled glycoprotein and the *C. perfringens* neuraminidase described in Fig. 4 was adjusted to pH 7.8 with Tris base and digested with Pronase as described in the Experimental section. It was then mixed with a portion of the control [^3H]fucose-labelled glycoprotein that had also been converted into glycopeptides by Pronase digestion, and fractionated on Sephadex G-50 as described in the legend to Fig. 3. ○, Control [^3H]fucose-labelled glycopeptides, 3800 d.p.m.; ●, [^{14}C]fucose-labelled glycopeptides from neuraminidase-treated glycoprotein, 3540 d.p.m.

Fig. 6. Lack of an effect of neuraminidase on the developmental difference in the electrophoretic mobility of the glycoprotein

A doubly labelled glycoprotein from a mixed preparation was partially purified as described in Fig. 2. Portions of the glycoprotein were incubated with the neuraminidases from *V. cholerae* and *C. perfringens*, or under the same conditions in the absence of neuraminidase. Each portion and an untreated control were electrophoresed on 5% sodium dodecyl sulphate gels. The Figures show only the part of the gel from 2–5 cm from the top, which contained the major peak. ○, [^{14}C]Fucose-labelled glycoprotein from 15-day-old rats; ●, [^3H]Fucose-labelled glycoprotein from adult rats. (a) Untreated control; 3664 d.p.m. ^{14}C , 3881 d.p.m. ^3H . (b) Incubated control; 2644 d.p.m. ^{14}C , 2757 d.p.m. ^3H . (c) Incubated with *C. perfringens* neuraminidase, 2798 d.p.m. ^{14}C , 2780 d.p.m. ^3H . (d) Incubated with *V. cholerae* neuraminidase, 1965 d.p.m. ^{14}C , 2064 d.p.m. ^3H .

change in electrophoretic mobility shown in Fig. 1. Neuraminidase treatment also substantially altered the fractionation on Sephadex G-50 of the glycopeptides derived from the glycoprotein by pronase digestion (Fig. 5). The proportion of glycopeptides eluting in the position of fractions A and B was substantially decreased whereas the proportion in the position of fractions C and D increased.

These findings suggested that a higher sialic acid content in the glycoprotein from the immature rats could theoretically be responsible for the developmental differences described in the previous sections. In order to test this possibility, a doubly labelled partially purified glycoprotein similar to that described above was isolated from 15-day-old and young adult rats. As in previous experiments, the ^{14}C -labelled peak from the 15-day-old rats had a higher apparent molecular weight on sodium dodecyl sulphate gels than the ^3H -labelled peak from the mature rats (Fig. 6a). The difference between the electrophoretic mobilities of ^{14}C - and ^3H -labelled peaks survived incubation both in the absence of neuraminidase (Fig. 6b) and in the presence of the neuraminidases from *V. cholerae* (Fig. 6c) or *C. perfringens*

(Fig. 6d). Thus although neuraminidase treatment caused a decrease in the apparent molecular weight of the labelled glycoprotein, which was as large or larger than the developmental difference (cf. Figs. 4 and 6), the treatment affected the glycoproteins from rats of both ages to about the same degree and the developmental difference persisted.

In some experiments, one-half of the incubation mixtures, which were used to determine the effect of neuraminidase on the developmental difference in the intact glycoprotein, were digested with Pronase in order to determine the effect of sialic acid removal on the developmental differences in the glycopeptides. The glycopeptides in controls and incubated controls (Fig. 7a) were similar and closely resembled those previously shown in Fig. 3. Fig. 7(b) shows that neuraminidase treatment largely eliminated the glycopeptides, which eluted in the positions of fractions A and B, from animals of both ages. Developmental differences in the glycopeptides obtained from the neuraminidase-treated glycoprotein were considerably less evident than with those obtained from the untreated glycoprotein, although a slight shift of the ^{14}C -labelled glycopeptides towards higher molecular

Fig. 7. Effects of neuraminidase treatment of the glycoprotein on the developmental differences observed by gel filtration of glycopeptides

Portions of the partially purified doubly-labelled glycoprotein were incubated in the presence of *V. cholerae* neuraminidase and under the same conditions, but in the absence of neuraminidase. After extensive Pronase digestion the glycopeptides were fractionated on Sephadex G-50 as described in the legend to Fig. 3. ○, [¹⁴C]-fucose-labelled glycopeptides from 15-day-old rats; ●, [³H]-fucose-labelled glycopeptides from adult rats. (a) Incubated control, 2439 d.p.m. ³H, 3553 d.p.m. ¹⁴C. (b) Neuraminidase incubation, 2370 d.p.m. ³H, 3373 d.p.m. ¹⁴C.

weight was still apparent. In summary then, neuraminidase treatment did not substantially affect the developmental difference revealed by electrophoresis of the glycoprotein on sodium dodecyl sulphate gels, but the treatment largely eliminated the differences seen by gel filtration of the glycopeptides obtained by Pronase digestion.

Discussion

We have used double-labelling techniques with radioactive fucose, glucosamine and *N*-acetylmannosamine to show that the newly synthesized glycoprotein in myelin from 12- to 15-day-old rats has a higher apparent molecular weight on sodium dodecyl

sulphate gels than that in adult rats (Quarles *et al.*, 1973b; Matthieu *et al.*, 1975). The magnitude of this shift of the glycoprotein towards higher apparent molecular weight is greatest at 12 days, when myelination is just beginning, and decreases gradually with age until about 25 days, when the position of the glycoprotein on gels coincides with that in adult rats (Matthieu *et al.*, 1975). Although the higher apparent molecular weight for the glycoprotein in immature myelin was first revealed by the sensitive double-labelling technique, the difference can also be shown by periodic acid-Schiff staining (Matthieu *et al.*, 1975). This indicates that the difference is not restricted to the newly synthesized glycoprotein, but rather most of the glycoprotein molecules in the immature myelin are of higher apparent molecular weight. The experiments reported here were designed to obtain more information about the properties of the major glycoprotein component of myelin and the nature of the developmental changes.

Pronase digestion of the partially purified glycoprotein yielded a heterogeneous population of fucose-labelled glycopeptides that could be separated into several classes by gel filtration on Sephadex G-50. The results with the glycopeptides taken alone appear to be fairly straight forward. The glycoprotein in immature myelin yielded a greater proportion of glycopeptides in the highest-molecular-weight class. Since essentially all of the polypeptide chain is destroyed by exhaustive Pronase digestion, this finding indicates that the oligosaccharide units of the major glycoprotein component change during development. Further, neuraminidase treatment substantially reduced the developmental differences revealed by gel filtration of glycopeptides, suggesting that the relatively greater yield of high-molecular-weight glycopeptides from the glycoprotein in immature myelin is due mostly to a greater sialic acid content. These findings are very similar to the reported enrichment of high-molecular-weight fucose-labelled glycopeptides in virally transformed cells in comparison to normal cells, which is also due to a higher sialic acid content (Warren *et al.*, 1973; van Beek *et al.*, 1973). Indeed, the effect of neuraminidase on the distribution of myelin glycopeptides during gel filtration is very similar to the published results on normal and virally transformed cells. With regard to the development of neural tissue, it is of interest that an enrichment of high-molecular-weight fucose-labelled glycopeptides has also been reported in undifferentiated neuroblastoma lines in comparison to more differentiated lines (Glick *et al.*, 1973). On the other hand, the dialysable fucose-labelled glycopeptides of whole rat brain are shifted in the opposite direction in immature brain, i.e. towards smaller molecular weight (Margolis & Gomez, 1974). This developmental difference in the glycopeptides of whole brain was also eliminated by neuraminidase treatment.

Unlike the other investigations mentioned above in which the glycopeptides were prepared from a very heterogeneous population of glycoproteins, the present study was done with the major glycoprotein component of myelin after separation from the other glycoproteins. This also enabled us to determine the effect of neuraminidase treatment on the apparent molecular weight of the undegraded glycoprotein. The results indicate that the developmental changes in the glycoprotein are more complex than just a change in sialic acid content. Although removal of sialic acid did decrease the apparent molecular weight of the glycoprotein on sodium dodecyl sulphate gels by an amount comparable to the developmental change, it altered the glycoproteins from 15-day-old and adult rats by about the same amount, so that the magnitude of the developmental difference was not substantially affected. Therefore, unlike the developmental differences revealed by gel filtration of glycopeptides, the decrease in apparent molecular weight of the intact glycoprotein during development does not appear to be due primarily to a lower sialic acid content. It could be due to a proteolytic shortening of the polypeptide chain or to a decreased content of sugars other than sialic acid. It should be noted that a very small shift of the glycopeptides from immature myelin toward higher molecular weight did persist after neuraminidase treatment (Fig. 7), and this could reflect differences in other sugar moieties. Another possible explanation for the higher apparent molecular weight for the glycoprotein from immature myelin is that there are two or more different glycoproteins in the major component on sodium dodecyl sulphate gels, and their relative amounts change with age. This would result in a modification of carbohydrate residues on myelin or myelin related membranes as would a change in the nature and extent of glycosylation of a single glycoprotein. These changes in sugar residues might be important for the formation and maturation of myelin. These studies indicate that the developmental changes occurring in the predominant glycoprotein component associated with brain myelin are complex, and some involve the oligosaccharide units. The use of radioactive fucose as a marker and the fractionation techniques described here will be useful in further studies to more precisely define the chemical basis for these changes and their physiological significance.

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