Recent studies have demonstrated the involvement of two polysialyltransferases in neural cell adhesion molecule (N-CAM) polysialylation. The availability of cDNAs encoding these enzymes facilitated studies on polysialylation of N-CAM. However, there is a dearth of detailed structural information on the degree of polymerization (DP), DP ranges, and the influence of embryogenesis on the DP. It is also unclear how many polysialic acid (polySia) chains are attached to a single core N-glycan. In this paper we applied new, efficient, and sensitive high pressure liquid chromatography methods to qualitatively and quantitatively analyze the polySia structures expressed on embryonic and adult chicken brain N-CAM. Our studies resulted in the following new findings. 1) The DP of the polySia chains was invariably 40–50 throughout developmental stages from embryonic day 5 to 21 after fertilization. In contrast, glycopeptides containing polySia with shorter DPs, ranging from 15 to 35, were isolated from adult brain. 2) Chemical evidence showed glycan chains abundant in Neu5Acα2,8Neu5Ac were expressed during all developmental stages including adult. 3) Levels of both di- and polySia were found to show distinctive changes during embryonic development.

Polysialic acid (polySia) is a structurally and functionally unique glycotope expressed on the surface of living cells (1). In higher vertebrates, the α2,8-linked homopolymer of Neu5Ac is the only reported structure of polySia, although diverse structures of polySia differing in C-5 substitution of Sia residues and in the inter-residue linkages have been discovered in bacteria, invertebrates, and lower vertebrates (2). In embryonic vertebrate brain, the neural cell adhesion molecule (N-CAM) is a major carrier protein of polySia. A hypothesis that the presence of polySia on N-CAM attenuates the adhesive function of this molecule (3, 4) is supported by temporally regulated expression of polySia on embryonic N-CAM, its spatially limited expression in the olfactory bulb, hippocampus, and cerebellum of adult mammalian brain (where continuous plasticity is required). PolySia is also an oncodevelopmental antigen that is re-expressed on a number of human tumors, including neuroblastomas (5) and Wilms tumor (6). The presence of polySia on N-CAM not only functions as a negative regulator of N-CAM-mediated homotypic cell-cell adhesion but also decreases interactions with other cells. Although the molecular details of how polySia affects cell interactions has not been fully elucidated, it has been hypothesized to depend on the physical properties of this negatively charged and heavily hydrated polymer (7, 8).

Recent studies have shown that two polysialyltransferases (polySTs), designated PST-1 (PST/ST8SiaIV) and STX (ST8SiaII), catalyze the polysialylation of N-CAM. The genes encoding both enzymes have been cloned from several species and sequenced (9–13). The availability of cDNAs encoding these enzymes has facilitated new approaches to study the function, mechanism, and regulation of polysialylation of N-CAM (13–16). The properties and developmentally regulated expression of polyST activity in the membrane fraction of embryonic chicken (17, 18) and rat (19) brain have been studied. Despite extensive studies on the expression and function of polySia on N-CAM, there is a dearth of structural information on the degree of polymerization (DP) and, importantly, how the chain length may change during embryonic development. The overall structure of polysialylated glycan chains is also poorly understood, although the structure of core glycans in the embryonic chicken brain N-CAM was extensively examined and shown to have several unusual features (20). The presence of α2,8-linked oligo/polySia in glycopeptides isolated from developing rat brain was initially established by the susceptibility to Vibrio cholerae sialidase and methylation analysis, coupled with gas chromatography-mass spectrometry (21). In this pioneering work, it was shown that 8–12 Sia residues were linked to bi-, tri-, and tetra-antennary N-glycan chains. In more recent studies, evidence for the presence of polySia in neuronal tissues was based primarily on the susceptibility to a bacteriophage-induced poly(→ Neu5Acα2→) endo-N-acetylneuraminidase (Endo-N) (22) and reactivity to equine polyclonal antibody H.46 (23) or mouse monoclonal antibody 735 (24). The functional and
biosynthetic studies of polysialylation on N-CAM were stimulated and promoted by these sensitive and selective biological probes during past 15 years. However, although these specific reagents can be used for the diagnostic identification of polysialylated N-CAM, they are more effective for smaller oligoSia groups, i.e., 5 for Endo-N and 8–10 for H.46 and mouse monoclonal antibody. Consequently, certain ambiguity is inevitable in the results obtained with these reagents when polySia chains are analyzed.

The chemical and physicochemical determination of the DP of polySia chains contains many inherent problems which must be overcome. There are few reports on the determination of the DP of polySia on N-CAM by HPLC-based methods, and the published values vary widely, depending on the technique used. The initial evidence for the presence of extended polySia chains was based on the HPLC on a MonoQ column for the glycopolypeptides isolated from $[3H]$GlcNAc-labeled human neuroblastoma cells after brief treatment with Endo-N (5). Although the chromatograms seem to indicate the presence of extended polySia chains up to DP of 55, the resolution for DP $> 45$ was poor, and furthermore, the peaks at high DP region were not explicitly identified as polyNeu5Ac chains. In contrast, average DP obtained for a sample of N-CAM from embryonic chick brain, based on the separation and quantitation of non-reducing terminal and internal sialic acid residues, was 18 (25). However, this value may be an underestimate, as the molecule contains monoSia residues in addition to polySia chains.

Since the chain length-dependent physicochemical properties of polySia may determine its physiological role, a more accurate estimation of the DP of polySia chain expressed on embryonic N-CAM and the change in DP, if any, during development are essential for understanding the regulatory effects of polySia residues on N-CAM-associated physiological events. Information on the range of DP of polySia chains is also useful in understanding the biosynthetic reactions of polysialylation, and in clarifying how many sialyltransferases are involved in the formation of polySia N-CAM. To gain understanding to these problems, we addressed the following issues. First, a new analytical method was used to determining the DP of polySia chains of DP $> 50$ more accurately. Second, new methods were developed for isolation of polySia-N-CAM that eliminate or minimize unwanted cleavage of the inter-residue linkages of extended polySia chains, which are known to be more labile than shorter oligo/polySia (26). Third, two highly sensitive analytical methods were used for selective detection of monoSia, diSia, oligoSia, and polySia residues. The advantages of these recently developed HPLC-based analytical methods are twofold. First, high performance anion-exchange chromatography with pulsed electrochemical detector (HPAEC-PED) (27, 28) method accomplishes a highly efficient separation of underivatized oligo/polySia chains with DP ranging from 2 to as high as 80. Second, high performance liquid chromatography on a MonoQ column with fluorometric detection (HPLC-FD) method (28, 29) is a highly sensitive and selective method to measure fluorescence-tagged oligo/polySia residues (DP up to about 30). In the present study, these methods were used in tandem with an improved method for isolating and purifying polySia glycopolypeptides from chicken brain, so that stage-dependent changes in the DP and level of polySia expressed in embryonic and adult chicken brains can be determined. We thus can conclude that the DP narrowly ranges between 40 and 50 Sia residues (average DP = 45) in embryonic chicken brain. Surprisingly, both the DP range and the average DP values showed little variation during developmental stages, E5 to E21. On the other hand, the total amount of polySia expressed per brain exhibited large differences, with maximum expression around E14, as reported previously (18). One of the most unexpected findings was that no glycopolypeptides bearing short (5 to 30) polySia chains were isolated from embryonic chicken brains, although diSia residues were present in a fraction separated from polySia glycopolypeptides. Thus, polysialylation profile in the embryonic brain was in sharp contrast to that of the adult chicken brain, which showed polySia glycopolypeptides with polydispersity ranging from DP = 15 to 35. In addition, we also isolated glycopolypeptide fractions expressing the $\alpha 2,8$-linked diSia glycotype and proportionally lower levels of triSia and tetraSia residues in adult brain.

**EXPERIMENTAL PROCEDURES**

**Preparation of Sialylglycopeptide Fractions from Embryonic Chicken Brains**—Fertilized eggs were purchased from Taiwan Animal Health Research Institute and incubated at 38 °C under humidified conditions. Lyophilized homogenates of the brain prepared from chicken embryo at early developmental stages were prepared at University of California, Davis (18). Adult brains were purchased at the local market at Taipei soon after chicken (3 months old) were sacrificed. Brain tissues were stored at $-30$ °C or $-80$ °C for less than 1 month before further processing. Brains were homogenized at $4$ °C by either of the following methods: (i) with a 2-ml Kontes glass homogenizer in 50 mM MES buffer
(pH 6.1) containing 500 kallikrein-inactivating unit/ml aprotinin, 40 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride; (ii) with a Polytron homogenizer (Kinematica, Littau, Switzerland) in 10 mM Tris-HCl buffer (pH 8.0). No detectable difference was found between these two methods. Homogenates (fresh or after lyophilization) were delipidated with chloroform-methanol as described previously (20). The delipidated material was air-dried and exhaustively digested with bacterial protease, *Streptomyces griseus* proteinase (type

**FIG. 2.** HPLC-FD elution profiles of oligo/polyNeu5Ac and colominic acid from MonoQ HR 5/5 column. Samples of oligo/polySia and colominic acid were subjected to DMB-derivatization for 2 h at 50 °C. Oligo/polyNeu5Ac and high molecular weight colominic acid samples were first prepared by anion-exchange chromatography on a MonoQ HR 10/10 column from commercial colominic acid without pre-hydrolysis. a, the 7th peak; b, the 17th peak; c, the 26th peak, eluted from the MonoQ column; d, high molecular weight colominic acid, eluted after the 32nd peak from the MonoQ column. 100–400 ng of Neu5Ac was injected. Peaks were labeled with DP.
XIV, Sigma) as described previously (20). After digestion, an equal volume of cold acetone was added and the mixture was kept at −20 °C overnight, and precipitate (50% acetone precipitate) that contained high molecular weight compounds) was separated by centrifugation. Small glycopeptides remaining in the supernatant were precipitated by adding one more volume of acetone (75% acetone precipitate). Both the 50% and 75% acetone precipitates were subjected to size fractionation on Sephacryl S-200 columns (1.6 × 134 cm) equilibrated and eluted with 10 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl. The elution was monitored by $A_{230}$ nm and by determination of Neu5Ac using the fluorometric HPLC method, after hydrolysis in 0.1 N HCl for 2 h at 80 °C.

Sialoglycopeptides in the 50% acetone precipitate were separated into fractions H (tube numbers 38–48, $M_r \approx 100,000–20,000$), and L (tube numbers 52–70, $M_r \approx 12,000–3,000$). Sialoglycopeptides in the 75% acetone precipitate were eluted at a position similar to the L fraction. All fractions were dialyzed against MilliQ water in the cold and lyophilized.

Purification and further fractionation of the sialoglycopeptides were carried out on a MonoQ HR 10/10 column (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated with 10 mM Tris-HCl (pH 8.0) and eluted with a 0–0.7 M NaCl gradient in 10 mM Tris-HCl (pH 8.0) at 2 ml/min. Neu5Ac-containing fractions (monitored by the 1,2-diamino-4,5-methylenedioxybenzene (DMB) method after hydrolysis) were pooled and desalted on a Sephadex G-10 column.

**Sialic Acid Analysis**—Free Neu5Ac was quantitated by the fluorometric HPLC method after derivatization for 2.5 h at 55 °C with DMB (Dojindo Laboratories, Kumamoto, Japan) as described previously (30, 31). The reaction mixture contained 2.7 M DMB, 9 mM sodium hydrosulfite, 0.5 M β-mercaptoethanol, and 0.02 M trifluoroacetic acid. Samples were hydrolyzed in 0.1 M trifluoroacetic acid for the time required to obtain the maximum yield of free Neu5Ac, usually 4 h for polySia, and 1 h for diSia, and evaporated under vacuum to remove the acid. To monitor the elution profile of Neu5Ac-containing material after column chromatography, samples were hydrolyzed in 0.1 N HCl for 2 h at 80 °C. For oligo/polySia analysis by the HPLC-FD method, samples (100–500 ng of total Neu5Ac) were derivatized with the DMB reagent for 2 h at 50 °C without pre-hydrolysis, and the reaction mixture was neutralized with 1 M NaOH before injection. A Hewlett-Packard HPLC system series 1100 with a fluorescence detector 1046 (set at 373 nm for exci-
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RESULTS

Preparation of Glycopeptide Fractions for Di-, Oligo-, and PolySia Analysis—A published method (20) was modified to minimize hydrolytic cleavage of polysial chains that often occurs during prolonged treatments such as solubilization of polysial-containing glycopeptides from the precipitate with quaternary pyridinium ion (21), and concentration of the compounds from dilute solutions. As it is imperative to keep polysial chains intact and to accurately quantify the polysial, we exhaustively digested membrane-associated polysial-containing glycopeptides from delipidated homogenates of intact whole chicken brains with nonspecific bacterial protease to bring about solubilization. During proteolysis (0.1 M Tris-HCl, pH 8.0), chemical and enzymatic cleavage of sialyl linkages was negligible, as no significant amounts of free sia or free oligo/polySia chains were detected in any step of purification. Addition of equal volume of acetone to the material solubilized by proteolysis effectively precipitated all polysial-containing glycopeptides, which were readily re-solubilized in the small volume of buffer used in the next step.

Separation of High Molecular Weight PolySia Glycopeptides as Compounds Larger than Colominic Acid—The polysial glycopeptides in the 50% acetone-precipitated fraction at each developmental stage was fractionated by Sephacryl S-200 chromatography. The chromatographic profile (monitored for total Neu5Ac content) revealed two peaks H (high M<sub>n</sub>) and L (low M<sub>n</sub>) (Fig. 1). The column was calibrated using sialoglycoproteins of known M<sub>n</sub> isolated from fish eggs (36, and S. Inoue, unpublished results). The peak H (M<sub>n</sub> range 100,000–20,000, peak 45,000), decreased during the early stages of development and reached a peak value around day E14 after fertilization (designated E14) and then gradually decreased. This finding confirms the previous findings based on a polysial antibody (18). The H fraction from adult chicken brain eluted in a lower molecular weight range than that of embryonic H fractions (range 72,000–15,000, peak 27,000). Oligo/polySia analysis by the HPLC-FD method of H and L fractions showed that polysial was present only in the H fraction. In L fractions and the fraction soluble in 50% acetone, disia residues were present at all developmental stages, although the major portion of Neu5Ac occurred as monoSia residue. It is noted that the colominic acid sample was eluted in a more polydisperse peak than the H fractions, with M<sub>n</sub> values ranging from 72,000 down to 5,000, with a peak at 15,000.

Oligo/PolySia Analysis by HPLC-FD Method—The HPLC-FD method is a sensitive and selective method (28, 29), and was used in this study. Under the conditions required for derivatization with the DMB reagent, some polysial underwent partial hydrolysis to result in the characteristic elution ladders of (Sia)<sub>α</sub> (n = 1–n) useful in detection and identification of oligo/polySia. After such treatment, an authentic higher oligomer of Neu5Ac showed a parent peak, which is always of higher yield than the rest of the oligomer peaks produced during derivatization (Fig. 2, a–c). Thus, the peak of the highest DP can be regarded as the maximum size of the polysial chain under study. In contrast, for polydisperse materials like colominic acid, no distinct highest peak was yielded, but a range of peaks up to DP ~ 25 was shown (Fig. 2d).
other conditions of acid and temperature for derivatization examined did not improve the yield of highest DP peak. When colominic acid was treated under similar conditions (0.02 M trifluoroacetic acid for 2 h at 50 °C), oligo/polymers of Neu5Ac up to DP 40 were detected by HPAEC-PED. In the chromatographic separation on a MonoQ column (similar to HPLC-FD method) for underivatized polySia, resolution of peaks in the region of DP 30–40 could be improved by changing salt gradient. However, for DMB-polySia, no such improvement has so far been achieved. Thus, the HPLC-FD method cannot be used for the determination of DP values >30.

Characterization of the PolySia Chains in H Fraction and DiSia in L Fraction—The DP of the Sia chains in the H and L fractions from embryonic and adult chicken brains was determined by HPLC-FD method. First, sialoglycopeptides (containing 300–700 ng of total Neu5Ac) isolated from H fraction after Sephacryl S-200 chromatography (Fig. 1) were treated with the DMB reagent and analyzed on a MonoQ HR 5/5 column. Fig. 3 shows a representative profile for samples obtained at three stages of development: E5, E14, and adult. Profiles nearly identical to that shown for E14 (panel b) were observed for E8, E12, E16, E18, and E21. These profiles, when compared with that of colominic acid (Fig. 2d), indicate that polySia chains in the samples from these developmental stages are large in DP. The results indicate that polySia chains were also present in an early developmental stage as E5 (panel a), and in the adult (panel c). It is noted, however, that the proportion of monoSia with respect to the higher DPs was large in the E5 and adult samples. In contrast to H fraction, no polySia chains were detected in L fraction when examined by the same HPLC-FD technique. Rather, this fraction contained a small amount of diSia, which as described below, was present in larger amount in the soluble fraction of the 50% acetone fractionation.

HPLC on a MonoQ Column of H and L Fractions—To obtain information on the DP range of the polySia chains, the H and L fractions from each developmental stage were subjected to HPLC on a MonoQ HR 10/10 column, pre-equilibrated with 0.01 M Tris-HCl (pH 8.0), and eluted with a NaCl gradient. Essentially all sialoglycopeptides in the H fractions obtained from E5 to E21 were eluted in a peak at 0.45–0.55 M NaCl (Fig. 4). This elution position was slightly delayed in comparison to that of colominic acid, which eluted at 0.4–0.5 M NaCl (Fig. 4). It is noted that in samples obtained from late (>E18) stages, a small proportion of sialyl compounds eluted earlier than the major peak (e.g. Q2 for E21). In contrast, sialoglycopeptides isolated from adult brain showed a different elution profile (Fig. 4). In addition to peak Q1, which eluted slightly before the embryonic Q1 material, a larger portion of the adult derived sialoglycopeptides eluted over a broad region under partially separated multiple peaks (Q2–Q4). The DP analysis of these peaks by HPLC-FD showed that Q1 and Q2 contained polySia as expected from the elution position (Fig. 3c). The Q3 and Q4 fractions, eluted at lower NaCl concentration, also contained polySia but with DPs of 15–20 (Fig. 5), significantly shorter than the chains derived from embryonic polySia. When the L fractions were fractionated on a MonoQ HR 10/10 column, the major proportion of Neu5Ac-containing molecules eluted with NaCl at less than 0.2 M, and much smaller proportions (depending on the developmental stage) eluted at NaCl concentration between 0.3 and 0.55 M. Interestingly, those fractions eluted at the lower NaCl concentrations contained only monoSia residues, as expected, whereas the fractions that eluted at the higher NaCl concentrations contained significant levels of diSia (Fig. 6). In some fractions, the amount of diSia was as much as 30% of the total Neu5Ac content, and in those fractions small amounts of oligoSia (DP ≈ 3) were observed. As shown in
Fig. 6. HPLC-FD detection of diNeu5Ac as a major oligoNeu5Ac components in the L fractions of embryonic chicken brains. The L fractions (Fig. 1) were further fractionated by MonoQ HR 10/10 chromatography as described under "Experimental Procedures." Fractions containing sialo-glycopeptide were subjected to HPLC-FD analysis as described in Fig. 3 to detect oligo/polySia. Panels a and b are elution profiles for diSia-containing fractions from E10 and E21, respectively. The numbers refer to the DP.

Acid Stability of the Sialyl Linkages in PolySia-containing Glycopeptides from Chicken Brain—To characterize sialyl linkages in the polySia glycopeptides isolated from embryonic and adult chicken brains, the rate of release of free Neu5Ac by acid hydrolysis was determined, and compared with that of authentic mono-, oligo-, and polySia control compounds (Table I). The DMB-method was used to quantitatively determine the amount of Neu5Ac liberated. Under the conditions of DMB-derivationization for free Sia assay (0.02 N trifluoroacetic acid, 2.5 h at 55 °C), approximately 60% of the Neu5Ac \( \alpha_2,3 \) and Neu5Ac \( \alpha_2,6 \)-Gal linkages in a fetuin N-linked glycan were cleaved, showing the greater lability of these linkages compared with the \( \alpha_2,8 \) linkage in the \( \alpha_2,8 \)-linked diSia-containing glycopeptides. The liberation of free Neu5Ac from polySia such as authentic (Neu5Ac)\( _{25} \) and colominic acid occurred at a much slower rate as shown in Table I. The stability of \( \alpha_2,8 \) linkage of Neu5Ac under milder acidic conditions than those used in this study has been published previously (26). The rate of liberation of free Neu5Ac from polySia-containing glycopeptides derived from the embryonic brain (E10–E21) and adult Q1 was even slower than that of colominic acid, suggesting that polySia chains attached to these glycopeptides were limited to those having large DPs. Although the colominic acid sample used was the fraction that eluted after the DP 32 peak in anion-exchange chromatography, it also contained some species with DP 20–30 due to incomplete separation. Low yields of free Neu5Ac released from the embryonic brain sialoglycopeptides at zero time and at 1 h of hydrolysis are noteworthy, and suggested that short chains of Neu5Ac were negligible in these samples. Furthermore, low Neu5Ac values at zero time suggested the absence or infrequent occurrence of monomeric Neu5Ac-linked \( \alpha_2,3 \). Our previous study also failed to show the release of free Neu5Ac from polySia glycopeptides isolated from the E14 chicken brain by digestion with the Neu5Ac-2-3Gal-specific sialidase (20). A rate of liberation of free Neu5Ac from the E5 sialoglycopeptidase and adult Q2–Q4 is indicative of the presence of a larger proportion of monomeric Neu5Ac and/or di- and short oligoSia chains. The time course of the release of free Neu5Ac from the polySia glycopeptides and colominic acid also revealed that the release of monomer retained their high values after it reaching the maximum level. This result indicated that the total amount of Neu5Ac in these samples was higher than the maximum amount liberated as monomer.

Determination of the DP of PolySia Chains Expressed in Embryonic and Adult Chicken Brains—It is difficult to accurately determine the exact DP of polySia chains on glycopeptides, unless methods to selectively cleave the sialyl linkage between the proximal Sia residue and core oligosaccharide chain are developed, and methods for separation and determination of the size of the highly extended polySia chains are established. Separation of highly polymerized polySia chains (DP 70 to >90), liberated by controlled hydrolysis of polySia chains by HPAEC and detection by PED, appeared to be an excellent method to determine polySia DP (27, 28). We thus applied the method to determine the DP of polySia expressed in embryonic and adult chicken brains and compared the results with DP values determined for colominic acid. Partial chromatograms for polySia glycopeptides from representative stages of embryonic development and colominic acid are shown in Fig. 7. Under these conditions, the DP values of the latest
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Each tube containing a sample of oligo/polySia (50–100 ng NeuAc in 100 μl of 0.1 M TFA) was incubated at 80 °C. After reaction, acid was removed by centrifugation under vacuum and NeuAc monomer was determined by a reverse phase HPLC as the DMB-derivative (see text). Values are expressed as percentage of the maximum.

### Table I

| Hydrolysis time (h) | Embryonic polySia glycopeptides | Adult polySia glycopeptides |
|---------------------|---------------------------------|-------------------------------|
|                     | E5     | E10    | E14    | E18    | E21    | Q1     | Q2     | Q3     | Q4     |
| 0                   | 6.6    | 1.3    | 2.0    | 1.1    | 1.3    | 2.5    | 4.8    | 3.8    | 8.3    |
| 1                   | 43     | 13     | 13     | 10     | 10     | 15     | 26     | 18     | 26     |
| 2                   | 87     | 72     | 54     | 44     | 78     | 55     | 63     | 75     | 72     |
| 4                   | 96     | 89     | 97     | 83     | 100    | 100    | 94     | 85     | 100    |
| 6                   | 100    | 100    | 100    | 100    | 85     | 100    | 100    | 87     |        |
| 8                   | 98     | 70     | 70     | 100    | 62     | 91     | 88     | 67     | 73     |

| Hydrolysis time (h) | Fetuin Glycopeptide | DiSia-Glycopeptide | (NeuAc)₂ | (NeuAc)₄ | (NeuAc)₁₀ | (NeuAc)₁₂⁵ | Colominic acid |
|---------------------|---------------------|---------------------|-----------|-----------|-----------|------------|---------------|
| 0                   | 64                  | 37                  | 37        | 16        | 9.4       | 4.1        | 3.6           |
| 0.5                 | 98                  | 52                  | 56        |           |           |            |               |
| 1                   | 100                 | 68                  | 76        | 72        | 45        | 41         | 29            |
| 2                   | 100                 | 99                  | 100       | 100       | 86        | 68         | 57            |
| 4                   | 92                  | 100                 | 92        | 92        | 100       | 100        | 97            |
| 6                   | 79                  | 97                  | 94        |           |           |            |               |
| 8                   | 100                 |                     |           |           |           |            |               |

A compound under the 25th peak of (NeuAc)₄ was tentatively identified as a triantennary N-glycan chain in which NeuAc residue is linked α2,3 (30%) and α2,6 (70%) to each terminal Gal residue.

The results of amino acid analysis for embryonic polySia glycopeptides were expressed as a molar ratio relative to Asx, and summarized in Table III. The ratios of amino acid residues not listed in the table were much smaller than 1.0. In all samples, ratios of GlcNAc were about 5, suggesting that Asx represented the glycosylated asparagine residue and the high yield of Ser may indicate high frequency of polysialylation at the fifth glycosylation site (39). The molar ratios of Neu5Ac (maximum value by mild acid hydrolysis) to Asx showed good agreement with the values obtained from carbohydrate analysis. Taken together, the results indicated that each polySia glycopeptide sample obtained after exhaustive proteolysis contained single glycan chain and single asparagine residue. The presence of relatively high proportion of Glx and Gly in all samples cannot be accounted for by the reported amino acid sequence near glycosylation sites of N-CAM (39), and may possibly be ascribed to contaminants. The presence of significant amount of GalNAc residues that were also detected by gas-liquid chromatography in all samples should also be accounted for by future study.

Determination of the Level of DiSia and PolySia in Embryonic and Adult Chicken Brains—PolySia was found only in the H fraction, where polySia accounted for a major part of the total Neu5Ac (the DMB method, 4 h in 0.1 M trifluoroacetic acid at 80 °C). The results in Table IV show notable changes in polySia levels relative to developmental stages. There was a sharp increase during early stages that reaches the maximum at about E14, followed by a gradual decrease in the later stages. DiSia, which was found in L fraction and 50% acetone-soluble fraction, was estimated from the peak area in the HPLC-FD analysis (cf. Fig. 6) and recorded in Table IV. It is noted that the level of diSia was also dependent on developmental stage. The DiSia-containing glycopeptides were hitherto unreported in chicken brain, and further characterization of diSia-containing glycopeptides is an important future task.

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DISCUSSION

In this study, we purified oligo/polySia glycopeptides solubilized from delipidated whole brain of embryonic and adult chicken after exhaustive proteolysis, and analyzed their oligo/polySia structures. Past studies have shown that N-CAM is the major carrier of polySia in embryonic chicken brain although polySia is also reported in the α-subunit of the sodium channels in adult rat brain (40), and polySTs PST and STX are autopoly-sialylated in vivo and in vitro (41, 42). No data are available on the quantitative analysis of polySia at different stages of chicken brain development. Our results show highest polySia expression during E12–E16. The amounts of polySia were determined to be 1.9–26 μg/g of tissue during E5 through E21 in our study. The relevant data for the level of polySia reported in the previous papers are: 11 μg/g of tissue for E14 chicken brain (43), 25–37 μg/g for developing mouse brain and 19–28 μg/g for fetal human brain (44) (all values were recalculated and expressed in μg/g tissue based on data given by the authors).

DP Analysis of PolySia Chains and Its Relevance to the Previous Studies—We used two HPLC-based methods for the DP analysis of polySia chains. The HPLC-FD method is more sensitive (>20-fold) and more selective for Sia compared with HPAEC-PED method (28). However, for the resolution of polySia with DP greater than about 30, we had to depend on the latter method although about a 10-μg (as Neu5Ac) quantity of purified material was needed to be injected to obtain a high
quality chromatogram. The important conclusions thus obtained are the highest DPs of polySia chains expressed in developing chicken brain are unchanged during embryonic development (E5–E21) and within a range of 40–50, which is smaller than colominic acid used.

The Sephacryl S-200 elution volumes of polysialoglycopeptides derived from embryonic chicken brain suggested that these compounds were significantly larger than that of colominic acid. By HPLC on a MonoQ column, with reference to the retention times of authentic oligo/polyNeu5Ac peaks, the polySia glycopeptides derived from embryonic chicken brain showed a peak at DP = 52 with a range of 40–70, and the DP of colominic acid was 40 at peak position with DPs ranging from 20 to 70. The higher DP values for embryonic chicken brain samples estimated by these methods than those obtained by the HPAEC-PED method can be ascribed to the presence of the core N-glycan chain. The molar ratios of Neu5Ac relative to 3 Man for the embryonic polySia-glycopeptide was ~50. The elution of adult Q1 fraction from both Sephacryl S-200 and MonoQ column was similar to colominic acid but the DP values estimated from HPAEC-PED for this sample was much smaller than colominic acid. The molar ratio of Neu5Ac relative to 3 Man residues was only 20, showing that this is the average number of Neu5Ac residues present in an N-glycan chain of the polySia glycopeptide derived from adult N-CAM. In our previous study on fish egg polysialoglycoproteins, we have experienced that the elution of oligoSia-containing glycans in DEAE-Sephadex anion-exchange chromatography was affected by the structure of core glycan chains and cannot simply be correlated with homologous oligo/polySia chains (27, 35). Contrarily, in a case when the structure of the core glycan chain is short and simple, the DP value estimated from the elution of oligo/polySia glycan chains from a MonoQ column coincided with the value obtained by HPAEC-PED analysis after controlled acid

### TABLE II

| Sugars | Man | Gal | GalNAc | GlcNAc | NeuAc |
|--------|-----|-----|--------|--------|-------|
| E10    | 3.0 | 3.1 | 5.2    | 4.3    | 23    |
| E14    | 3.0 | 3.0 | 3.2    | 4.0    | 20    |
| E21-Q1 | 3.0 | 3.0 | 3.7    | 3.7    | 25    |
| Adult-Q1 | 3.0 | 3.0 | 3.1    | 4.2    | 18    |
| NeuAc (maximum) | 56 | 43 | 47 | 20 | |

* Maximum values for NeuAc liberated by hydrolysis and determined by reverse phase HPLC as DMB derivatives (see Table II).

### TABLE III

| Axs | GalNAc, GlcNAc, and NeuAc relative to Axs |
|-----|-----------------------------------------|
| E10 | E14 | E18 |
| Asx | 1.0 | 1.0 | 1.0 | 1.0 |
| Gln | 0.68 | 0.68 | 0.68 | 0.76 |
| Ser | 1.0 | 1.0 | 1.1 | 1.2 |
| Gly | 0.86 | 0.80 | 0.86 | 1.1 |
| GalNAc | 7.5 | 7.6 | 7.6 | 4.3 |
| GlcNAc | 6.8 | 5.4 | 5.4 | 5.0 |
| NeuAc (maximum) | 54 | 50 | 44 | 40 |

### TABLE IV

Amounts of poly- and diSia expressed in embryonic and adult chicken delipidated brains

| PolySia | DiSia | % of Total NeuAc |
|---------|-------|------------------|
| µg/g tissue | µg/brain | µg/g tissue | µg/brain | % of Total NeuAc |
| E5 | 1.9 | 0.09 | 3.8 | 1.5 | 0.07 | 2.6 |
| E6 | 4.1 | 0.23 | 7.5 | 1.0 | 0.06 | 1.8 |
| E8 | 7.1 | 0.85 | 11.3 | 4.2 | 0.50 | 6.7 |
| E10 | 9.3 | 1.4 | 16.5 | 1.1 | 0.19 | 2.0 |
| E12 | 21.9 | 5.7 | 20.7 | 11.3 | 2.9 | 10.7 |
| E14 | 25.4 | 8.7 | 28.9 | 8.5 | 3.0 | 9.7 |
| E16 | 30.0 | 12.2 | 21.9 | 14.5 | 5.8 | 12.2 |
| E18 | 19.7 | 9.4 | 19.9 | 13.9 | 6.7 | 14.0 |
| E19 | 16.7 | 9.5 | 15.8 | 12.5 | 7.1 | 11.9 |
| E21 | 16.2 | 11.6 | 16.2 | 7.9 | 5.7 | 7.9 |
| Adult | 3.6 | 10.8 | 1.8 | 3.9 | 11.7 | 2.0 |

* Mean values of two independent experiments.

b Amounts of polySia with lower DP were included.
hydrolysis (28, 45). When the DP of oligo/polySia is determined by HPLC on a MonoQ anion-exchange column, it is also important to confirm that the retention time of each peak coincides with that of authentic oligo/polySia. Clearly the previous estimation of the minimum DP of 55 may include possible errors (5). These authors counted a number of molecular forms including (a) glycopeptides having oligo/polySia chains with different DPs or net negative charges and different core structures, and (b) free oligo/polySia chains with varying DPs which were formed upon such mild brief digestion with Endo-N, all of which must have appeared in different positions on HPLC chromatogram (5). Further, our recent study has revealed that the core glycans of the embryonic form of chicken brain N-CAM have sulfated lactosaminyl antennae, which contribute to additional net negative charges to cause delayed elution (20).

In our HPAEC-PED analysis, we compared the retention time of oligo/polySia peaks from chicken brain glycopeptides with those from colominic acid generated under the same conditions of controlled acid hydrolysis.

Although it still remains an important challenge to determine exactly the structural features of sialylation and polysialylation on N-CAM glycan chains, our estimation of the highest DP, based on HPAEC-PED, of invariably 40–50 throughout developmental stages and the data of carbohydrate analysis, Man:Neu5Ac = 3.40–60, together with the result of methylation analysis strongly indicate that (a) only one of the 3 antennae is polysialylated, (b) one of the remaining two antennae is either disialylated or oligosialylated with lower DP, and (c) the third antenna is not sialylated but terminated by the unsubstituted Gal residue (cf. Ref. 20). This conclusion of the presence of a single polySia chain and an oligoSia with lower DP (most likely diSia) is rather compatible with the previous finding that average DP obtained for a sample of embryonic chicken brain N-CAM based on the separation and quantitation of non-reducing terminal and internal sialic acid residues was 18 (25).

The major functional properties proposed for polySia on N-CAM have been ascribed to those of negatively charged and hydrated linear polymers rather than recognition phenomena by this unique glycoyte. The DP 40–50 of polySia chains may be long enough and fit for such down-regulatory purpose. In contrast, glycopeptides derived from adult chicken brain contain polySia with a broader range of DP: 15–35. Further studies on chemical structures of oligo/polySia-containing N-CAM and oligo/polySialyltransferases in adult chicken brain are in progress in our laboratory.

Occurrence of DiSia Residues in the N-CAM—The occurrence of diSia residues as the almost exclusive form of lower oligomer, throughout all developmental stages including adult, is also a new finding to be emphasized, and supports the multiplicity of oligo/polySialyltransferase in chicken brain. Studies on functional significance of the expression of these enzymes and their products, oligo/polySia, is urgently needed. Recently, the co-existence of polyST, PST and STX, which are involved in polysialylation of N-CAM in cooperative manner, became known, and the developmentally regulated expression of their gene transcripts was shown in mammalian systems. The expression of each enzyme was differently regulated by their gene transcripts was shown in mammalian systems.

It should be emphasized that these diSia residues were not inadvertent products generated during the preparation. Although free diSia is the most stable product obtained by mild acid hydrolysis of polySia, the α2–3 linkage of Neu5Ac to the proximal Gal is much more labile than α2–8 linkages in polySia chains as described above, and further, we have never found any detectable amount of free oligo/polySia chains in our samples. Throughout the embryonic stages, the amount of Neu5Ac as diSia was 12–75% of that occurred in polySia, and the proportion of diSia residue was by far more abundant than the level of other oligoSia residues, i.e. diNeu5Ac >> triNeu5Ac >> tetraNeu5Ac. Isolation, from embryonic and adult chicken brains, of tri/tetra-antennary N-glycans that contained diSia (as well as tri- and tetraSia in smaller proportions), and shared structural characteristics with the core N-glycan chain originated from embryonic chicken brain N-CAM (20) can be taken as a supporting evidence that at least a part of diSia is associated directly with N-CAM. Identification of glycoprotein(s) containing diSia is now in progress in our laboratory.

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