Chemical Characterization of a Neural Cell Adhesion Molecule Purified from Embryonic Brain Membranes*

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A neural cell adhesion molecule (N-CAM) was purified in milligram quantities from detergent extracts of embryonic chick brain membranes. N-CAM has an unusual carbohydrate content and structure, is polydisperse in solution, and is associated with proteolytic activity leading to its spontaneous cleavage. The carbohydrate composition of N-CAM includes 13 mol of sialic acid but only 1.4 mol of galactose/100 mol of amino acids, suggesting the presence of a sialic acid to protein linkage not previously observed in higher organisms.

N-CAM appears to be an integral membrane protein in that its extraction from membranes required detergent. Although soluble, the purified molecule was aggregated (M₀ = 0.5 to 1.2 × 10⁶) and polydisperse in detergent-free solutions. N-CAM from brain also migrated as a broad but continuously stained region from M₀ = 200,000 to M₀ = 250,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the molecule from retina was similar but had a somewhat faster mobility. Desialation of N-CAM did not significantly change its behavior in solution, but converted both brain and retinal N-CAM to components migrating on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as material of about M₀ = 140,000. Despite the apparent heterogeneity, amino acid sequence analysis and comparison of proteolytic fragments suggest that all forms of the glycoprotein are derived from the same polypeptide chain.

On prolonged incubation at neutral pH, N-CAM undergoes apparent proteolysis to yield a polypeptide that contains little sialic acid and has a M₀ = 65,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a separate sialic acid-rich component, and a variety of small peptides. The 65,000-dalton polypeptide appeared to contain all of the antigenic determinants of intact N-CAM that neutralize the adhesion-blocking ability of anti-retinal cell Fab' fragments.

Adhesive interactions among nerve cells and their neurites are believed to be an important factor in the assembly of nerve tissue and the interconnection of different parts of the nervous system (1). Adhesion between vertebrate cells has been studied for several decades, and various substances have been proposed to be involved in the process (2–10), but the molecular structures and functions necessary for this binding have not been determined.

For the past several years, we have attempted to identify and characterize molecular components of the adhesive mechanism responsible for aggregation of chick embryo nerve cells in vitro. These studies have led to the identification of a cell surface molecule, called N-CAM, that is required for calcium-independent aggregation of retinal and brain cells (5, 11). The previous procedure for isolation of N-CAM (5, 12), began with culture supernatants of intact retinal tissue and yielded only microgram quantities of the purified molecule. This quantity was sufficient for the production of anti-(N-CAM) antibodies in rabbits and the estimation of its size on SDS-PAGE. Fab' fragments of anti-(N-CAM) antibodies were shown not only to block the aggregation of nerve cells in suspension, but also to perturb the normal patterns of histotypic differentiation in cultured cell aggregates (13) and retinal tissue (14), to inhibit the fasciculation of neurites (15), and to alter the response of ganglionic processes to nerve growth factor (16). A protein that cross-reacts antigenically with N-CAM has been found in the rat nervous system (17).

Despite this progress, the limited quantities of N-CAM that were obtained made it difficult to analyze its chemical nature. Moreover, this purified material did not demonstrate any binding affinities that might reveal its function in the formation of cell-cell bonds. The present report describes two new fractionation procedures that use embryonic brain membranes as a source of N-CAM. The first scheme gave increased quantities of N-CAM and made possible the preparation of monoclonal antibodies against N-CAM. The monoclonal antibodies in turn allowed a rapid, affinity purification of the molecule in milligram quantities. Purified N-CAM has been found to display binding affinity for nerve cells (18), and the chemical characterization of N-CAM reported here indicates that it has a number of unusual properties that may be related to its role in cell-cell adhesion.

MATERIALS AND METHODS

Reagents—The following reagents were purchased from the sources indicated in parentheses: Sepharose CL-2B, Sepharose CL-6B, protein A, and protein A-Sepharose (Pharmacia); Bio-Beads SM-1. The abbreviations used are: N-CAM, neural cell adhesion molecule; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; salt/P, buffer, 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH₂PO₄, 0.15 g of Na₂HPO₄/liter (pH 7.4); CMF, calcium-magnesium-free medium, 8 g of NaCl, 0.3 g of KCl, 0.05 g of NaH₂PO₄, 0.025 g of KH₂PO₄, 1 g of NaHCO₃, 2 g of glucose/liter; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

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2, Bio-Gel P-2 and P-10, and materials for SDS-PAGE (Bio-Rad); octyl-agarose, Staphylococcus aureus V8 protease, rabbit anti-mouse IgG (200 units/ml containing 40 mg/ml CMF) supplied by Miles Laboratories (Elkhart, IN); Clostridium perfringens neuraminidase and carboxypeptidase A and B (Millipore, Bedford, MA); Vibrio cholerae neuraminidase and lactoperoxidase (Calbiochem-Behring); trypsin (Mobay Chemical, New York, NY); thin layer chromatography plates coated with cellulose (CEL 300-10) or Silica Gel 60 (Brinkmann Instruments); DEAE-cellulose ion exchange support (DE52, Whatman); Perkin-Elmer P-40 (BDH Chemicals, Poole, England); carrier-free 125I and [3H]iodoacetic acid (New England Nuclear).

Preparation of Membrane Extracts of Chick Embryo Brains—Brains were removed from 600 14-day chick embryos and washed with cold CMF. The tissue was homogenized in 100 brains at a time, in 1 ml CMF containing 40 mg/ml CMF supplied by Miles Laboratories (50 mg/ml solutions in a capillary viscometer). The homogenate was centrifuged for 10 min at 20,000 rpm in a Sorvall SS34 rotor at 4 °C. The pellets (160 ml) were resuspended with 320 ml of 2.25 M sucrose in salt/Pi buffer, buffer, placed in 12 centrifuge tubes, each aliquot overlaid with 10 ml of 0.8 M sucrose, and centrifuged in a Beckman Ti-75 rotor at 4 °C in a Beckman Ti 75 rotor. The material at the interface between the two sucrose concentrations was collected, washed twice with salt/Pi buffer, resuspended in the same buffer, and stored at -20 °C.

To extract proteins for ion exchange chromatography (see below), the membranes obtained from 600 brains were thawed, washed with 10 mM Tris, 5 mM EDTA (pH 8.2), and suspended in 600 ml of the same buffer plus 0.5% Nonidet P-40. For immunoaffinity chromatography (see below), the thawed membranes from 600 brains were suspended in 600 ml of salt/Pi buffer plus 1 mM EDTA and 0.5% Nonidet P-40. In both cases, the extracts were centrifuged for 40 min at 55,000 rpm to remove undissolved material.

Purification of N-CAM by Ion Exchange Chromatography and Hydrophobic Chromatography—The membrane extract was stirred for 1.5 h at 4 °C with 120 g of DEAE-cellulose which was then collected on a Buchner funnel and washed with 10 mM Tris, 5 mM EDTA (pH 8.2), and suspended in 600 ml of the same buffer plus 0.5% Nonidet P-40. For immunoaffinity chromatography (see below), the thawed membranes from 600 brains were suspended in 600 ml of salt/Pi buffer plus 1 mM EDTA and 0.5% Nonidet P-40. In both cases, the extracts were centrifuged for 40 min at 55,000 rpm to remove undissolved material.

Immunoprecipitation of radiolabeled proteins was performed by boiling for 3 min with mercaptoethanol. Gels were calibrated with the following 40 kDa standard: ferritin (Mr = 220,000), β-galactosidase (Mr = 130,000), phosphorylase b (Mr = 94,000), bovine serum albumin (Mr = 66,000), catalase (Mr = 59,000), ovalbumin (Mr = 43,000), carbonic anhydrase (Mr = 29,000), and cytochrome c (Mr = 15,000). For two-dimensional separations, the SDS-PAGE was followed by isolation of the relevant bands from the gels and by isolation of antigen-antibody conjugates using protein A-Sepharose. Primary proteins of N-CAM were generated and resolved by the method of Cleveland et al. (25) using S. aureus V8 protease.

The molecular weight of N-CAM was estimated by sedimentation equilibrium ultracentrifugation in 10 mM ammonium bicarbonate, 0.15 M sodium chloride (pH 8.3) or 6 M guanidine hydrochloride, 50 mM sodium phosphate (pH 7.4) using a Beckman, Model E analytical ultracentrifuge and the long column method of Yphantis (26). Intrinsic viscosities of proteins in 10 mM Tris hydrochloride, 0.15 M sodium chloride were determined by measuring the flow rate of 5.0, 3.0, 1.5, and 0 mg/ml solutions in a capillary viscometer.

Carbohydrate compositions were determined using several methods. Sialic acid was determined using the thiobarbituric acid assay following hydrolysis in 0.1 N H2SO4 at 80 °C for 4 h (30), and by the
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resorcinol assay (31). N-Acetyleneuraminic acid was used as a standard. Uronic acids (32) and fucose (33) were assayed colorimetrically. Quantitative analysis of hexosamine was performed on a Beckman 121M amino acid analyzer (34) following hydrolysis in vacuo in 4 N HCl for 2, 4, 8, and 16 h at 100 °C using p-fluorophenylalanine added before hydrolysis as an internal standard. Neutral sugars were analyzed as their O-trimethylsilyl derivatives after release by methanoly- sis in 0.1 M methanolic HCl at 85 °C for 2, 4, 8, 16, and 24 h (35) using myoinositol as an internal standard. Samples were resolved on a Beckman GC-65 gas chromatograph equipped with 2-mm inner diameter, 6-foot-long glass columns containing 0.1% OV-17 on 120 to 140 mesh GLC-110. The chromatogram was developed with a temperature gradient from 90-220 °C and the elute detected by flame ionization.

In order to determine the specie(s) of sialic acid in N-CAM, sialic acid was released from the protein by neuraminidase treatment (see below) and analyzed by thin layer chromatography on cellulose coated plates (CEL 300-10) using N-acetyleneuraminic acid and N-glycolylneuraminic acid as standards (36). Spots were visualized with the thiobarbituric acid spray reagent (37).

For lipid analysis, N-CAM in water was extracted with 20 volumes of 2:1 or 1:2 chloroform/methanol and the extracts were analyzed by thin layer chromatography on silica gel coated plates in 60:35:8 chloroform/methanol/H2O. Lipids were detected by charring after spraying the plates with 0.6% potassium dichromate in 65% (w/w) sulfuric acid or with a phospholipid stain (38).

Protein was determined by the method of Lowry et al. (40). Moisture was measured by drying in vacuo at 78 °C over P2O5, and ash was determined following complete pyrolysis (Rockefeller University Microanalytical Service).

Amino Acid Sequence Determinations—NH2-terminal residues were determined by treatment with dansyl chloride in 8 M urea, 0.5 M NaHCO3 followed by hydrolysis and chromatography on polyamide sheets (41).

NH2-terminal sequences were determined as described (42) on material that had been first reduced and alkylated in urea (43). 5-Dimethylaminonaphthalene sulfonamide was used as an internal standard.

Carboxyl-terminal sequences were determined by digestion with carboxypeptidases A and B in 0.2 M N-ethylmorpholine (pH 8.0) (44) or carboxypeptidase Y in 0.1 M ammonium acetate (pH 5.5) for 2, 5, 10, 30, 60, and 120 min followed by analysis of free amino acids (45).

Treatments of N-CAM—To fully desialate N-CAM, 1 mg was treated with 0.05 IU of V. cholerae neuraminidase at 37 °C for 24 h in 1 ml of 2 M CaCl2, 0.2 M EDTA, 50 mM sodium acetate (pH 5.0). Similar results were obtained with C. perfringens neuraminidase only after protease contamination was removed by affinity chromatography on N-(p-aminophenyl)-oxamic acid-agarose (46).

N-CAM was reduced and alkylated in 6 M guanidine hydrochloride as previously described (28).

Autoxidation of N-CAM occurred during a 96-h incubation at 37 °C of a 1 mg/ml solution in 10 mM ammonium bicarbonate, 10 mM sodium azide. After addition of 0.1 volume of 10 times concentrated salt/P, buffer, the sample was subjected to the immunoaffinity fractionation as described above but without detergent.

Isolation of N-CAM from Different Sources—Membranes from brain and retinal tissue were prepared as described above; retinal tissue required the use of a nitrogen cavitation bomb (10 min, 1000 p.s.i.) to achieve efficient cell disruption. Cultured retinal cells were prepared as previously described (12). Cells (4 × 106/ml in CMF) and membranes (2 mg of protein/ml in salt/P, buffer) were radiiodinated using 690 μCi/ml of lactoperoxidase, 1.5 mM CaCl2 of Na2I, and 0.05% H2O2. (For additions of 1 μl of 0.1 volume of 10 times concentrated salt/P, buffer, added to buffer plus 20 mM KI, and extracted with Nonidet P-40 as described above. N-CAM was immunoprecipitated from the extracts using either monovalent Fab antibodies coupled to Sepharose CL-2B or rabbit anti-N-CAM followed by protein A-Sepharose to isolate the antibody-antigen conjugates. Immunoprecipitates were washed five times with salt/P, buffer containing 0.5% Nonidet P-40 and 1 mM EDTA and analyzed by SDS-PAGE.

RESULTS

Purification of N-CAM—In order to purify N-CAM in sufficient quantities for structural and functional studies, it was necessary to obtain a richer source of the protein as well as a more rapid and efficient purification scheme. N-CAM has been shown to be a component of cell and neurite plasma membranes in all nervous tissues examined (13) and the nonionic detergent, Nonidet P-40, was found to extract N-CAM efficiently from membrane preparations at a detergent concentration of 0.5% and a membrane protein concentration of 2 mg/ml or less. The amount of N-CAM per mg of protein, as quantitated by the Fab' neutralization assay, was similar for membrane preparations from brains from 10- to 14-day embryos; 14-day brains were chosen as the source of N-CAM because of their larger size and ease of dissection.

The strategy used for isolation of N-CAM was first to obtain an enriched fraction by conventional chromatographic techniques and to use that material to produce, screen, and characterize monoclonal antibodies against N-CAM. Immunoaffinity fractionation using monoclonal antibodies attached to Sepharose CL-2B was then used for purification of N-CAM directly from Nonidet P-40 extracts.

N-CAM was partially purified from Nonidet P-40 extracts of embryonic chick brain membranes by ion exchange chromatography on DEAE-cellulose followed by hydrophobic chromatography on octyl-agarose (Fig. 1). This purification scheme resulted in a 29-fold purification with a 9% yield (Table I) and produced material clearly enriched in certain proteins observed on SDS-PAGE as compared to the unfraccionated Nonidet P-40 extract (Fig. 2A, lanes 1 and 2). In both steps of this purification, N-CAM was not released from the column support unless the eluting buffer contained Nonidet P-40. Detergent-free solutions containing high concentrations of salt or ethylene glycol could therefore be used to elute contaminants respectively from the DEAE-cellulose and octyl-agarose. Attempts to purify N-CAM further from these preparations were unsuccessful despite the use of a variety of chromatographic methods and solvents.

The major protein on SDS-PAGE of this partially purified material was represented by a broad but continuously stained major band of 140,000 daltons, which migrated at the position of N-CAM (Fig. 2A, lane 3) and was designated N-CAM by the Fab' fragment of an antibody that inhibited retinal growth in vitro and in vivo (28).

FIG. 1. Purification of N-CAM by hydrophobic chromatography on octyl-agarose. N-CAM, partially purified on DEAE-cellulose, in 1200 ml of 1 M KCl, 0.2 mM EDTA, 1 mM Tris (pH 8.2) was shaken with 200 ml of octyl-agarose for 1 h at 4 °C. The octyl-agarose was collected in a column and further eluted with: Arrow I, 1600 ml of 75% (v/v) ethylene glycol, 0.15 M KCl, 0.2 mM EDTA, 1 mM Tris (pH 8.2); Arrow II, 1000 ml of 0.2 mM EDTA, 1 mM Tris (pH 8.2); and Arrow III, a 1500 ml linear gradient of 0 to 0.4 M KCl in 0.5% Nonidet P-40, 0.2 mM EDTA, 1 mM Tris (pH 8.2). N-CAM was eluted in the final gradient and the fractions were pooled as indicated.

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The amount of N-CAM in a sample was estimated by its ability to neutralize the adhesion-blocking ability of Fab' prepared from the sera of rabbits immunized with retinal cells. One unit of N-CAM activity is defined as the amount of N-CAM required to reduce the inhibition of aggregation by 25% (12). N-CAM was purified from 14-day brain membranes by Nonidet P-40 (NP40) extraction followed by sequential batch chromatography on DEAE-cellulose and octyl-agarose; or affinity purified on a (monoclonal anti-(N-CAM))-Sepharose CL-2B column as described under “Materials and Methods.”

Affinity-purified N-CAM 16.5 314,000 19,030 78 114

Table I

| Fraction                  | Protein | Activity units | Specific activity | Yield % | Purification fold |
|---------------------------|---------|----------------|-------------------|---------|-------------------|
| NP40 extract              | 2420    | 403,000        | 167               | 100     | 1                 |
| Ion exchange chromatography purified N-CAM | 48     | 100,000        | 2,083             | 25      | 12.5              |
| Hydrophobic chromatography purified N-CAM | 7.7    | 37,000         | 4,805             | 9       | 28.8              |
| Affinity-purified N-CAM   | 16.5    | 314,000        | 19,030            | 78      | 114               |

The relative composition of individual amino acids and carbohydrates in N-CAM is shown in Table II. Over 90% of the weight of the lyophilized material was accounted for by amino acids, carbohydrates, water, and ash. No lipid was detected in chloroform/methanol extracts of the purified protein either by staining with a phospholipid stain or by charring. The molecule did contain enough phosphate to suggest the possible presence of one or more phosphate groups per mol of protein.

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The relative composition of individual amino acids and carbohydrates in N-CAM is shown in Table III. The amino acid composition is not unusual (see Ref. 47). In order to obtain optimal yields of half-cysteine, it was necessary to oxidize N-CAM with performic acid prior to acid hydrolysis.

No free half-cysteine was present in N-CAM as indicated by the failure of [3H]iodoacetic acid to label N-CAM in the absence of reducing agents; after reduction, sufficient label was incorporated into the protein to account for all of the half-cysteine determined by amino acid analysis of the performic acid-oxidized protein. The migration of N-CAM on SDS-PAGE was not altered by the inclusion of reducing agents in the sample (Fig. 2B, compare lanes 1 and 3) suggesting that N-CAM is not composed of multiple chains and does not contain interchain disulfide bonds. The fact that the mobility of neuraminidase-treated N-CAM was slightly decreased in the presence of reducing agents (Fig. 2B, compare lanes 2 and 4) is consistent with the hypothesis that at least some of the half-cysteine in N-CAM is involved in intrachain disulfide bonds (48).

The carbohydrate composition of N-CAM is marked by the high content of sialic acid, 13 mol/100 mol of amino acids (Table III). The sialic acid appears to be covalently linked to N-CAM because it cannot be separated from the protein by gel filtration in 6 M guanidine hydrochloride or extracted as glycolipid. The sialic acid in N-CAM is also unusual in its slow release by neuraminidase or by acid (Fig. 4) relative to the release of sialic acid from fetuin under the same conditions. The rate of release of sialic acid from bovine submaxillary glycoprotein was also appreciably faster than that of N-CAM.

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![Graph](image)

**Fig. 3.** Purification of N-CAM by immunoaffinity chromatography. **A,** 900 ml of Nonidet P-40 extract from the membranes of 600 embryonic chick brains were shaken with 30 ml (monoclonal IgG)-Sepharose CL-2B for 1.5 h at 4 °C. The Sepharose was collected in a column, washed with 100 ml of 0.5% Nonidet P-40, 1 mM EDTA in salt/Pi buffer and then eluted with 0.5% Nonidet P-40, 1 mM EDTA, 50 mM diethylamine. The first 60 ml of eluate were pooled and neutralized with 6 ml of 1 M potassium phosphate (pH 7.0). The eluate from two preparations as in A were combined and again loaded on a column containing 30 ml (monoclonal IgG)-Sepharose CL-2B. The column was washed and the bulk of the protein then eluted with diethylamine; fractions containing N-CAM were pooled and neutralized.

**TABLE II**

| Constituent | Weight % |
|-------------|----------|
| Amino acids | 57.6     |
| Carbohydrates | 26.3   |
| Lipid       | <0.2     |
| Phosphate   | 0.15     |
| Moisture    | 4.4a, 7.5a |
| Ash         | 3.2a, 0.7a |

Moisture and ash determinations on two independent preparations.

Physicochemical Properties of N-CAM—The molecular weight and hydrodynamic properties of N-CAM in solution were investigated by analytical ultracentrifugation, gel filtration, and intrinsic viscosity studies. The weight average molecular weights calculated from ultracentrifugation data varied with the speed of centrifugation (Table IV) indicating that the sample was heterogeneous in size. Treatment of N-CAM with neuraminidase or the use of 6 M guanidine hydrochloride as solvent did not reduce the dependence of the apparent molecular weights on centrifuge speed (Table IV) and did not alter the relative intensity of the two bands in the doublet varied from preparation to preparation except for moisture and ash.

Neuraminidase-treated 99% of the sialic acid from N-CAM but did not otherwise affect its composition. In addition, the activity of neuraminidase-treated N-CAM in the Fab'-neutralization assay was similar to that of N-CAM. Neuraminidase-treated N-CAM, however, migrated much differently than N-CAM on SDS-PAGE (Fig. 2C, compare lanes 1 and 5), appearing as a closely spaced doublet with an apparent molecular weight of about 140,000. The relative intensity of the two bands in the doublet varied from preparation to preparation. The migration of N-CAM on SDS-PAGE also changed when the sample was boiled. Three minutes of boiling (Fig. 2C, lane 2) increased its mobility and the width of the region of continuous staining; however, after 10 or 30 min boiling (Fig. 2C, lanes 3 and 4), N-CAM migrated with a reduced heterogeneity and a further increased mobility, as was the case with neuraminidase-treated N-CAM. Assays showed that sialic acid was gradually released from N-CAM during boiling until after 30 min, 95% of the sialic acid had been released. The material released did not appear to be free sialic acid but because it had a larger apparent molecular weight on gel filtration on Bio-Gel P-2 and P-10 and because hydrolysis with acid or neuraminidase was required for optimal yield in the thiobarbituric acid assay (30) used to estimate free sialic acid. Prolonged boiling had no effect on the migration of neuraminidase-treated N-CAM on SDS-PAGE.

**TABLE III**

| Constituent | mol/100 mol amino acids |
|-------------|-------------------------|
| Amino acids |                         |
| Asp         | 9.5                     |
| Thr         | 7.2b                    |
| Ser         | 9.1b                    |
| Glx         | 12.5                    |
| Pro         | 7.1                     |
| Gly         | 5.9                     |
| Ala         | 7.9                     |
| Cys         | 1.7b                    |
| Val         | 8.4b                    |
| Met         | 1.1b                    |
| Ile         | 6.4b                    |
| Leu         | 6.2                     |
| Tyr         | 2.7b                    |
| Phe         | 2.6b                    |
| Lys         | 6.4                     |
| His         | 1.1                     |
| Arg         | 2.7                     |
| Trp         | 1.6b                    |
| Carbohydrates |                 |
| Fucose      | 1.0b                    |
| Mannose     | 2.2b                    |
| Galactose   | 1.4b                    |
| N-Acetylglucosamine   | 2.7b   |
| N-Acetylgalactosamine | <0.1b  |
| N-Acetylneuraminic acid | 13.2b  |
| Uronic acids | 0.4                     |

Amino acid analysis was performed on a Beckman 121M amino acid analyzer. Values reported were averages for hydrolysis times of 24, 48, and 72 h, except where otherwise noted.

Values for serine, threonine, and tyrosine were extrapolated to 0 h hydrolysis time.

Cysteine and methionine were determined as cystic acid and methionine sulfone, following oxidation with performic acid and hydrolysis (27). Similar results were obtained for cysteine by reduction in guanidine hydrochloride and alkylation with [3H]iodoacetic acid (28).

Values for valine, isoleucine, and phenylalanine were taken at 72 h hydrolysis time.

Determined spectrophotometrically (29).

Determined by gas chromatography of the trimethylsilyl derivatives following methanolysis (35).

The cysteine-sulfuric acid assay (33) gave the same value for fucose as did gas chromatography.

Quantitative analysis of hexosamines was performed on the Beckman 121M amino acid analyzer following hydrolysis to glucosamine (34). The native forms of these sugars in N-CAM were assumed to be N-acetylglucosamine and N-acetylgalactosamine as found in other glycoproteins.

Determined by the thiobarbituric acid assay (30). The resorcinol assay (31) gave the same result.

Determined colorimetrically (32).
aliquots removed at intervals from and by acid.

2.2 mg of fetuin (open circles) in 2 ml of 2 mM CaCl₂, 0.2 mM EDTA, 50 mM sodium acetate (pH 5.0) during treatment with 0.06 IU of neuraminidase at 37 °C; and B, 0.5 mg of N-CAM (closed circles) or 1.1 mg of fetuin (open circles) during incubation in 1 ml of 0.1 N H₂SO₄ at 80 °C. Release of sialic acid from N-CAM and fetuin by neuraminidase reached 100% after 16 h of treatment (data not shown).

Table IV

| Sample          | Molecular weight (×10⁸) |
|-----------------|------------------------|
| N-CAM           | 7.2  11.8 ± 1.5        |
|                 | 13   7.1 ± 0.5         |
|                 | 18   4.9 ± 0.4         |
| Neuraminidase-treated N-CAM | 7.2  9.0 ± 0.7        |
|                 | 13   5.1 ± 0.4         |
|                 | 18   3.1 ± 0.4         |
| N-CAM in 6 M guanidine hydrochloride | 7.2  19.3 ± 1.8        |
|                 | 13   8.3 ± 0.6         |

a The sample was first reduced and alkylated in the presence of 6 M guanidine hydrochloride (28).

the observation that apparent molecular weights of N-CAM or neuraminidase-treated N-CAM in solution were several times their apparent molecular weights on SDS-PAGE. On gel filtration, N-CAM eluted well ahead of thyroglobulin (Mᵣ = 670,000) and ferritin (Mᵣ = 425,000), indicating that its apparent molecular weight is at least 1 × 10⁶ (Fig. 5). Again, neuraminidase treatment of the sample or use of 6 M guanidine hydrochloride as the solvent did not significantly alter the elution profile of N-CAM on gel filtration. The intrinsic viscosity of N-CAM in solution in 10 mM ammonium bicarbonate, 0.15 M NaCl was 30 ± 5 ml/g, a value comparable to that found for fibrinogen, a rod-like protein, and much greater than the intrinsic viscosity of globular proteins. This result suggests either that the molecule is not a typical globular protein or that it forms stable asymmetric aggregates.

In addition to its apparent dispersive molecular weight, N-CAM was also heterogeneous in charge as determined by isoelectric focusing. The molecule separated into two regions with average PI values of 4.4 and 4.6 (Fig. 6A). The distribution of N-CAM between these two regions varied from preparation to preparation. Whether the resolution of N-CAM into two zones indicated two distinct classes of N-CAM monomers is not clear because as indicated above, N-CAM may not exist as a monomer in solution. As expected, the pI of neuraminidase-treated N-CAM was more basic; both bands resolved by SDS-PAGE had a pI of about 5.5 (Fig. 6B).

Both NH₂-terminal and carboxyl-terminal analysis of N-CAM revealed predominant amino acid sequences, but did not rule out the possibility of some sequence heterogeneity. The NH₂-terminal sequence of N-CAM was determined to be NH₂-Leu-Gln-Val by automatic sequencing. Molar yields of 70% were observed for each of these residues. Leucine was also identified as the NH₂-terminal residue of N-CAM and neuraminidase-treated N-CAM by the dansyl technique (41).
Carboxypeptidase A treatment of N-CAM rapidly released alanine in 70 to 80% yield followed by a slower release of a small amount of threonine (10 to 20% yield) and lesser amounts of other amino acids that differed from experiment to experiment. Lysine (70% yield) and additional alanine were released by subsequent treatment with carboxypeptidase B. These results suggest that the carboxyl-terminal sequence of N-CAM is \( \text{Lys-Ala-COOH}. \)

As indicated above, N-CAM and neuraminidase-treated N-CAM are heterogeneous in their mobility on SDS-PAGE. This differential migration of various subfractions was found to be a stable property: N-CAM and neuraminidase-treated N-CAM were resolved on SDS-PAGE, the gel stained, and multiple segments of different migration were cut from the gel; on re-electrophoresis the protein in each segment migrated to its original position (Fig. 7A). All of these components, however, appeared to be derived from the same polypeptide chain. If, for example, the protein in these gel slices was instead treated with \( \text{S. aureus} \) V8 protease, and these digests resolved by SDS-PAGE (Fig. 7B) as described by Cleveland et al. (25), the patterns obtained from all regions of the N-CAM and neuraminidase-treated N-CAM gels were similar.

Proteolytic Activity in N-CAM Preparations and Regional Differentiation of the Molecule—Incubation of N-CAM in neutral buffer of low ionic strength resulted in a gradual loss of the 200,000 to 250,000 molecular weight species and the appearance of a 65,000-dalton polypeptide as well as smaller peptides as assessed by SDS-PAGE (Fig. 8A, lanes 1 to 6). This transformation was blocked by the inclusion of 0.5 M sodium chloride in the incubation mixture (Fig. 8A, lane 9) and was temperature-dependent (Fig. 8A, lanes 6 to 8). Proteolysis of N-CAM appeared to be occurring because new NH\(_2\)-terminal residues were detected by the dansyl technique. Partial degradation of N-CAM can also occur during routine purification as judged by the appearance of the 65,000-dalton polypeptide on SDS-PAGE of purified material.

When the incubated material was reapplied to the immunoaffinity column originally used to purify the molecule, 35 to 65% of the protein in the sample and only 1 to 4% of the sialic acid were bound to the column. (Variations among preparations are assumed to be due to differing amounts of residual undigested or partially digested N-CAM molecules.) The remaining protein and sialic acid were recovered in the unbound fraction. The bound and unbound fractions were also compared by SDS-PAGE (Fig. 8B) and in the Fab'-neutralization assay. Greater than 90% of the Fab'-neutralization activity recovered was found in the bound fraction. The primary species in the bound fraction was the 65,000-dalton polypeptide (Fig. 8B, lane 2). In preliminary experiments, we have found that greater than 90% of the sialic acid in the unbound fraction chromatographed as a peak of molecular weight greater than 100,000 as estimated by gel filtration. No major polypeptide species were detected, however, by SDS-PAGE in the unbound fraction (Fig. 8B, lane 3).

To verify that the 65,000-dalton polypeptide is derived from N-CAM, the 65,000-dalton polypeptide and intact N-CAM were cut from SDS gels and the fragments produced by proteolytic digestion were compared. At least four proteolytic fragments were common to the digest of both N-CAM and the 65,000-dalton polypeptide (Fig. 9, lanes 2 and 3), although they occurred in different relative amounts. Other fragments were found in only one of the two samples. It is possible that other portions of N-CAM are present in the 65,000-dalton polypeptide but are more sensitive to protease when they are part of this fragment.

Comparison of N-CAM from Different Sources—In our previous studies (5), N-CAM was isolated from supernatants of cultures of 10-day retinal tissue. Rabbit antibodies against this material specifically inhibited nerve cell aggregation and primarily recognized a M\(_r\) = 140,000 polypeptide obtained from a Nonidet P-40 extract of 10-day retinal cells that had been obtained by trypslnization and cultured in suspension (Fig. 10, lane a). The M\(_r\) = 140,000 component was also precipitated from this cell extract by each of the nine monoclonal antibodies prepared against the higher molecular weight N-CAM obtained in our present studies (three are shown in Fig. 10, lanes b to d). In addition to the M\(_r\) = 140,000 component, both the rabbit and monoclonal antibodies precipitated diffuse, high molecular weight material that varied in amount from preparation to preparation. These results indicate that the M\(_r\) = 140,000 component identified as N-CAM in the earlier studies shares antigenic determinants with the higher molecular weight molecule obtained from fresh tissue by immunoaffinity chromatography.
The material immunoprecipitated by the monoclonal antibodies from extracts of fresh 14-day brain consistently differed from that obtained from fresh 10-day retinal tissue with respect to its migration on SDS-PAGE (compare lanes e to g with i to k in Fig. 10). N-CAM from both sources appeared as a diffuse band, the retinal N-CAM migrating further and in addition containing a larger amount of a $M_T = 140,000$ component with the same electrophoretic mobility as the material immunoprecipitated from extracts of cultured retinal cells. As with the brain N-CAM (Fig. 2B, lane 4), treatment of the retinal tissue N-CAM with neuraminidase produced a molecule with a molecular weight in SDS of 140,000 (Fig. 10, lane h).

**Fig. 8.** Generation and fractionation of autolysis products of N-CAM. A, a 1 mg/ml solution of N-CAM was incubated at 37 °C in 10 mM ammonium bicarbonate, 10 mM sodium azide (pH 8.3), except as indicated. Aliquots (15 μl) were removed at intervals and resolved by SDS-PAGE. Lane 1, no incubation; lane 2, 2 h; lane 3, 6 h; lane 4, 24 h; lane 5, 48 h; lane 6, 96 h; lane 7, 96 h at 25 °C; lane 8, 96 h at 4 °C; and lane 9, 96 h in buffer plus 0.5 M NaCl. B, N-CAM was incubated as described above, then separated on the anti-(N-CAM) immunoaffinity column into two fractions, unbound and bound, as described under "Materials and Methods." Aliquots of each sample were resolved by SDS-PAGE on a 15% gel. Lane 1, 30 μg of unfractionated, autolyzed N-CAM; lane 2, 15 μg of bound fraction; and lane 3, 15 μg of unbound fraction.

**Fig. 9.** S. aureus V8 protease digestion of N-CAM and the 65,000-dalton autolysis product of N-CAM. N-CAM and the 65,000-dalton autolysis product of N-CAM were resolved by SDS-PAGE on a 7.5% gel, cut from the gel, and processed for re-electrophoresis on a 15% gel as described (25) with and without proteolytic digestion with 0.5 μg of S. aureus V8 protease. Lane 1, 65,000-dalton polypeptide without proteolysis; lane 2, N-CAM with proteolysis; lane 3, 65,000-dalton polypeptide with proteolysis; and lane 4, enzyme alone. Undigested N-CAM does not enter a 15% gel as seen in Fig. 7B, lane 1. Arrows indicate the positions of proteolytic fragments common to N-CAM and the 65,000-dalton polypeptide.

**Fig. 10.** N-CAM from different sources. Lactoperoxidase-radiiodinated N-CAM was isolated from the indicated sources as described under "Materials and Methods," resolved by SDS-PAGE, and the samples visualized by autoradiography. The antibodies used to isolate N-CAM were rabbit antibody (RAb) prepared against N-CAM from retinal tissue culture supernatants (5) and nine different mouse monoclonal anti-(N-CAM)s prepared against N-CAM from brain tissue. The material immunoprecipitated by anti-(N-CAM) from three representative clones (anti-(N-CAM) Nos. 1, 2, and 3) is shown. Lanes a to d, material immunoprecipitated from cultured retinal cells by the rabbit antibody and by monoclonal anti-(N-CAM) Nos. 1, 2, and 3; lanes e to h, material immunoprecipitated from retinal tissue by monoclonal anti-(N-CAM)s Nos. 1, 2, and 3 (lane h was the same sample as lane e except that the material was treated with neuraminidase after immunoprecipitation); and lanes i to k, material immunoprecipitated from brain tissue by monoclonal anti-(N-CAM) Nos. 1, 2, and 3. Lanes a to d and e to k were run on separate gels. NANAase, N-acetylneuraminidase.

**DISCUSSION**

We have purified N-CAM, a glycoprotein involved in the calcium-independent adhesion of nerve cells, and have carried out a partial chemical characterization of this molecule. N-CAM was found to be a relatively abundant cell surface molecule, accounting for about 1% of the total membrane protein in the brain of chick embryos. The abundance of N-CAM in brain tissue combined with the efficiency of immunoaffinity purification (78% yield) allowed the routine isolation of greater than 15 mg of highly purified N-CAM from the brains of 1200 embryonic chicks and thus make practical a detailed structural analysis of this molecule. In addition, the speed and mild conditions of the isolation should aid in preserving the chemical and biological activities of N-CAM. For example, recent experiments using material obtained by the methods described here have shown that N-CAM in solution or reconstituted into lipid vesicles binds specifically to retinal cells and inhibits their adhesion (18).

The N-CAM purified in our earlier work (5) was obtained from retinal tissue culture supernatants and contained three components with molecular weights on SDS-PAGE of 140,000, 120,000, and 65,000. A $M_T = 140,000$ component was also immunoprecipitated by anti-(N-CAM) from detergent extracts of cultured retinal cells. Because these polypeptides share antigenic determinants with the higher molecular weight N-CAM described here and also neutralized the adhesion blocking activity of anti-(retinal cell) Fab' fragments, it is likely that these lower molecular weight species represent N-CAM that had been partially degraded, probably including the loss of sialic acid and proteolytic cleavage.

The N-CAM isolated from 14-day brain tissue and 10-day retinal tissue differs in its electrophoretic mobility. The observations that the mobility of N-CAM on SDS-PAGE varies...
with the amount of sialic acid associated with the protein and that neuraminidase can convert both retinal and brain N-CAM to Mr 100,000 material, suggest that this difference is at least in part due to sialic acid content. The metabolic control and biological significance of these different forms of N-CAM are currently under investigation.

In the present study, we have found three properties of N-CAM that are unusual and that could be crucial to its function: its self-association in solution, its carbohydrate content and structure, and the apparent autolysis of the molecule to yield a polypeptide that is relatively free of sialic acid.

Sedimentation equilibrium and gel filtration studies suggest that N-CAM in solution is large and polydisperse in size. The high intrinsic viscosity of N-CAM solutions indicates that the molecule is not a typical globular protein. The shape of N-CAM may contribute to its apparent high molecular weight on gel filtration, but should not significantly distort the estimate of molecular weight by analytical ultracentrifugation which is less dependent on molecular asymmetry. Removal of sialic acid from N-CAM, reduction and alkylation of half-cystinyl residues, and exposure to 6 M guanidine hydrochloride did not reduce the heterogeneity or apparent degree of association of N-CAM in solution as analyzed either in the ultracentrifuge or by gel filtration. The self-association of N-CAM in solution could result from nonspecific aggregation caused by hydrophobic interactions among regions normally in contact with the lipid bilayer. It is possible, however, that the aggregation of N-CAM in solution reflects the same binding activity involved in the formation of cell-cell bonds. In accord with this hypothesis, there is some evidence suggesting that the cell surface receptor for N-CAM in solution or reconstituted into lipid vesicles, is itself N-CAM (18).

N-CAM was also heterogeneous in its migration in polyacrylamide gels during electrophoresis and isoelectric focusing. Unlike the heterogeneity in molecular weight of N-CAM in solution, however, the heterogeneity in charge and gel electrophoretic mobility appeared to be due primarily to sialic acid because specific removal of this sugar from N-CAM caused a marked decrease in the degree of both types of heterogeneity. Some evidence for homogeneity of the polypeptide portion of N-CAM was provided both by the similarity of proteolytic fragments from N-CAM fractions of different mobilities on SDS-PAGE, and by the fact that a predominant sequence was found at both the NH2 and carboxyl termini of N-CAM. N-CAM prepared from adult tissue was also heterogeneous in charge and electrophoretic mobility due to its sialic acid content although within a different mobility range than embryonic N-CAM. Embryonic N-CAM contains 13 mol of sialic acid/100 mol of amino acids, a value 3 times that found in fetuin (50), but only one-third that found in bovine submaxillary mucin (51). The sialic acid present in N-CAM appears to be N-acetylneuraminic acid. Although we have not yet determined the linkage of the sialic acid or the nature of the oligosaccharide units, it is striking that despite the high sialic acid content, N-CAM contains only 1.4 mol of galactose, 1.0 mol of fucose, 2.2 mol of mannose, 2.7 mol of N-acetylglucosamine, and less than 0.1 mol of N-acetylgalactosamine/100 mol of amino acids. In all glycopeptides isolated from glycoproteins of higher organisms, sialic acid has been found distal to either galactose or N-acetylgalactosamine (52). The low level of these carbohydrates in N-CAM indicates that the carbohydrate composition of N-CAM cannot be accommodated by the usual patterns, and suggests that the sialic acid in N-CAM is a multimer or is linked directly to amino acids. Polysialic acid has been found in trisialogangliosides, on the cell envelope of Escherichia coli (49), and in a glycoprotein from trout eggs (53).

Another unusual, and possibly related, feature of the sialic acid in N-CAM is its relative resistance to release by neuraminidase treatment or acid hydrolysis. Prior treatment with base did not enhance the rate of sialic acid release, suggesting that there are no ester or lactone linkages between sialic acid molecules as seen in polysialic acid from E. coli (49). The combined data suggest that the sialic acid in N-CAM is in an unusual linkage or protected by protein folding or both.

The large amount and unusual properties of sialic acid in N-CAM raise the question of its role in N-CAM function. Sialic acid may form part of the active site for the activity of N-CAM as a ligand. Alternatively, the role of sialic acid in N-CAM may be to maintain the conformation, distribution, or orientation of the molecule on the cell surface. For example, the active site of N-CAM may be held away from the membrane by sialic acid, or the charged sugar might act as a barrier preventing intramolecular ligation or aggregation of N-CAM on the cell surface of a single cell.

A third striking property of N-CAM is its ability to undergo a spontaneous, autolytic conversion to lower molecular weight material. The low level of this activity, however, requires that these results be interpreted cautiously. Preliminary studies indicate that the activity is temperature- and concentration-dependent, optimal at neutral pH and low salt concentration, sensitive to denaturation, and that it leads to the appearance of new NH2-terminal residues. It has not been established whether the inhibition of autolysis by high salt concentrations is due to the inability of N-CAM to function as an enzyme or as a substrate under these conditions. Inhibitors of microbial growth such as azide, penicillin, and streptomycin do not abolish the activity. The activity thus appears to be proteolytic, enzymatic, and intrinsic to the N-CAM preparations.

Attempts to characterize this activity more precisely with defined proteolytic substrates indicated that N-CAM is positive in the milk-clotting assay (64) and in the cleavage of N-benzoyl-L-tyrosine-p-nitrophenyl ester, a chymotrypsin-specific substrate. In these assays, however, N-CAM preparations exhibited an apparent specific activity no more than 1/1000 that of chymotrypsin. It is therefore possible that this proteolytic activity is due to trace contamination of N-CAM. On the other hand, N-CAM may be an enzyme specific for an as yet undetermined substrate and this activity could be of considerable biological significance, for example in the making or breaking of cell-cell or cell-substrate bonds.

While the biological significance of this activity remains unclear, the degradation products of N-CAM are of intrinsic interest. The data suggest that these products are derived from two distinct regions in N-CAM: 1) a 65,000-dalton polypeptide, which contains relatively little sialic acid but possesses the antigenic site(s) recognized by the monoclonal antibody and most or all of the determinants recognized by anti-retinal cell Fab' fragments that block adhesion, and 2) a sialic acid-rich region that does not contain these antigenic sites. Preliminary studies involving partial digestion of N-CAM with other proteases are consistent with this hypothesis.

The distinctly different regions observed in N-CAM, its possible self-degradation and self-aggregation, and its unusual sialic acid content suggest that continued studies of the chemical and structural properties of N-CAM will be of central importance to understanding its biological function. The purification scheme and the availability of several monoclonal
antibodies described here should provide adequate amounts of highly purified N-CAM and anti-(N-CAM) for these studies, as well as for investigations of this molecule’s binding activities, developmental expression, and physiological significance.

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