Isolation and Partial Characterization of the Internal Structural Proteins from Murine Intracisternal A Particles†

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ABSTRACT: Intracisternal A-type particles were prepared from two types of plasma cell tumors in BALB/c mice and from cultured neuroblastoma cells of A/Jax origin. The particles were treated with the detergent sodium dodecyl sulfate to dissolve the outer membrane and yield a preparation of inner shells. The structural proteins from the inner shells were dissociated in sodium dodecyl sulfate by disulfide exchange and separated by gel chromatography in the presence of the detergent. The isolated fractions were analyzed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate or a mixture of phenol, urea, and acetic acid. The main polypeptide which was common for all the intracisternal A particles studied here had a molecular weight of 73,000 daltons as determined by both gel chromatography and electrophoresis with sodium dodecyl sulfate. The minor components differed in proportions in the particles from the three different tumors. Electrophoresis in the phenol-containing system showed homogeneity of the main protein in RPC-20 and neuroblastoma intracisternal A particles but revealed heterogeneity of the MOPC-104E main component and in the lower molecular weight components from particles of all three sources. Immunodiffusion showed reactions of apparent identity between the inner shell proteins regardless of size and origin. Electrophoretic comparison of the CNBr-cleavage products from the main proteins and the 46,000 molecular weight proteins from MOPC-104E and RPC-20 origin showed that all the proteins share a significant fraction of the peptides. Chemical analysis failed to detect sialic acid in the intracisternal A particles. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of inner shell components isolated from neuroblastoma cells that had been double labeled with [^{14}C]-amino acids and [^{3}H]glucosamine showed that a fraction of the tritium label migrated with the ^{14}C-labeled structural proteins. Thin-layer chromatography of hydrolysates of lipid-extracted intracisternal A particles showed that most of the tritium label was in glucosamine and a small fraction in galactosamine.

Intracisternal A particles are present in various murine neoplastic tissues (for references, see Kuff *et al.*, 1968, 1972), in oocytes and preimplantation stages of mice (Calarco and Szollasi, 1973), in thymus of newborn mice (Aoki *et al.*, 1970), and in adult tissues (Wivel and Smith, 1971). Particles of similar appearance have been also observed in tumors of several other rodents (Novikoff and Biempica, 1966; Tumilowicz and Cholon, 1971; Nadel *et al.*, 1967) and recently in cultured human tumor cells (Dalton and Stewart, 1972). The particles are localized within the cisternae and are formed by budding at the rough endoplasmic reticulum (Dalton *et al.*, 1961), in a manner that superficially resembles the budding of oncogenic viruses from plasma membranes (Bernhard, 1960) and the budding of virus-like H particles from the rough endoplasmic reticulum in transformed hamster cells (Thomas *et al.*, 1967; Cesarini and de Micco, 1972). The intracisternal A particles consist of two concentric electron dense shells surrounding a zone that is electron lucent. These particles have been isolated from several murine neoplastic tissues and found to exhibit common antigenic specificity and to contain a major structural protein of an apparent molecular weight near 70,000 (Kuff *et al.*, 1968, 1972). The outer shell components can be selectively solubilized and separated from the inner shells by treatment with sodium dodecyl sulfate (Wivel *et al.*, 1973) or by treatment with guanidine thiocyanate (D. J. Marciani and E. L. Kuff, unpublished observation); the outer shell contains numerous polypeptides, the most abundant with a molecular weight near 80,000. The inner shell is formed by the major structural protein and smaller fractions of other components.

Recently, Wilson and Kuff (1972) have shown a DNA polymerase activity to be associated with the intracisternal A particles. Although this enzyme transcribes poly(rA) in the presence of oligo(dT) or poly(dT) primers, the activity differs in a number of respects from that found in murine leukemia virus. No antigenic relationship between intracisternal A particles and recognized oncogenic RNA viruses of the leukemia-sarcoma or mammary tumor types has been detected (Kuff *et al.*, 1972; Tanaka *et al.*, 1972); furthermore, attempts to show infectivity have been inconclusive.

The widespread occurrence of intracisternal A particles in normal and neoplastic tissues raises the question of their nature and biological role. A systematic study of the particle proteins could help to clarify the relationship between intracisternal A particles from different species, and provide a critical probe for homologies between the A particles and known oncogenic viruses. Beyond this is the more general question of the possible relationship between the A-particle protein and the structural components of the endoplasmic reticulum. In the present communication we describe the isolation of the structural polypeptides from inner shells of murine intracisternal A particles of different origins, and compare some of their biochemical and immunological properties.

Materials and Methods

Tissues. Mouse plasma cell tumors MOPC-104E and RPC-20 were maintained by subcutaneous transplantation in BALB/c mice. Neuroblastoma cell line F1007, derived from tumor

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C1300 of A/Jax mice, was obtained from Flow Laboratories, Bethesda, Md., and cultured in Eagle's minimum essential medium (Eagle, 1959) containing 10% fetal calf serum. The cell cultures were maintained at 37° in an atmosphere of 95% air and 5% CO_2. Intracisternal A particles were isolated from these tissues following the method of Kuff *et al.* (1968).

Preparation of Radioactive A Particles. Neuroblastoma cells, at a density of about 5×10^5 cells per T75 flask, were grown for 20 hr in a modified Eagle's medium containing 5 μCi/ml of 6-[^{3}H]glucosamine (10 Ci/mmol, Amersham/Searle), and 0.25 μCi/ml of each of the following uniformly labeled [^{14}C]-amino acids: lysine, 2.5 Ci/mol, and leucine, 3.15 Ci/mol, from Schwarz/Mann; valine, 3.18 Ci/mol, and phenylalanine, 6.45 Ci/mol, from Amersham/Searle. The concentrations of these four amino acids were one-tenth those in the usual Eagle's basal medium. Ten per cent fetal calf serum was added as usual. At the end of the incorporation period, the cells were washed twice with Hank's buffered saline solution, once with NET (0.1 M NaCl–1 mM Na_2EDTA–0.05 M Tris-HCl, pH 7.4), and then scraped in 3 ml of NET/flask. Cells from ten flasks were pooled and collected by low-speed centrifugation. The labeled intracisternal A particles were isolated by the following procedure. Cells were lysed in NET by addition of Triton X-100 to 1.3% and the nuclei were removed by low-speed centrifugation. The cytoplasmic extract was made 11 mM in Na_2EDTA and sheared through a 25-gauge hypodermic needle. One milliliter of the extract was loaded on a 3-ml linear gradient, 17.5–30% (w/w) sucrose in D_2O buffered with 0.01 M Tris-HCl–1 mM Na_2EDTA (pH 7.6), which had been formed over a 1-ml cushion of 46.2% (w/w) sucrose in D_2O. The A particles were collected at the interface between the gradient and the cushion.

Gel Chromatography and Sample Preparation. Intracisternal A particles (5–10 mg of protein) were resuspended in 2.5 ml of 0.02 M sodium phosphate buffer (pH 7.2), containing 1% sodium dodecyl sulfate (Schwarz/Mann), and incubated 5–10 min at room temperature, with occasional shearing through a 23-gauge hypodermic needle to disrupt aggregates. The suspension was then layered over a discontinuous gradient formed by 1 ml of 10% sucrose and 1.5 ml of 20% sucrose, both containing 0.02 M sodium phosphate (pH 7.2) and 0.1% sodium dodecyl sulfate. The tube was centrifuged for 60 min at 35,000 rpm (300,000*g*) in the Spinco SW 50.1 rotor at 15°. The gelatinous pellet of sodium dodecyl sulfate resistant inner shells (Wivel *et al.*, 1973) was resuspended in 1.5 ml of 0.1 M dithiodipropionic acid–2 mM Na_2EDTA–2% sodium dodecyl sulfate and adjusted to pH 8.7 with NaOH. The disulfide-exchange reaction was started by addition of 1 μl of 2-mercaptoethanol and followed until the nitroprusside test for sulfhydryl groups (Chinard and Hellerman, 1954) was negative as a result of oxidation by air. Separation of the disulfide-exchanged proteins from the other products was achieved by either gel chromatography on Bio-Gel P-2 (Bio-Rad) or by dialysis. In either case, the proteins were recovered in 0.01 M sodium phosphate buffer (pH 6.8)–0.1% sodium dodecyl sulfate and lyophilized. The dry product was dissolved in 1 to 2 ml of water and applied to a Bio-Gel A-1.5 M (Bio-Rad Labs) column operated with upward flow, using 0.1 M sodium phosphate–0.2% sodium dodecyl sulfate (pH 6.8) as eluent. Collected fractions were monitored at 260 and 280 nm, and the protein concentrations were determined with ninhydrin after alkaline hydrolysis (Hirs, 1967). Each fraction was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis to determine the precise elution positions of the individual polypeptides. Fractions containing the desired proteins were pooled and concentrated by dialysis against Aquacide II (Calbiochem). In some instances cross-contaminated fractions were subjected to a second gel chromatography on Bio-Gel A-5m (Bio-Rad Labs) under the conditions above described. For estimation of molecular weights the Bio-Gel A-1.5m column was calibrated with reduced and alkylated proteins of known molecular weights following the method of Fish *et al.* (1970).

Thin-layer gel (tlg) chromatography on Sephadex G-200 (Pharmacia) or Bio-Gel P-300 (Bio-Rad Labs) in presence of 6 M Gdn·HCl[1] was performed as described by Klaus *et al.* (1972) using a Pharmacia tlg apparatus. A-particle polypeptides were prepared by the same procedure used for the standards.

Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of sodium dodecyl sulfate and molecular weight determinations were carried out as described by Dunker and Rueckert (1969). Reduction of disulfide bonds was achieved by heating the samples in 1% 2-mercaptoethanol–1% sodium dodecyl sulfate at 90° for 1–2 min just prior to electrophoresis. Sucrose (6%) and traces of Pyronin dye were added to the samples and layered over the gels. Electrophoresis in the presence of phenol, urea, and acetic acid was carried out by a modification of the system described by Cotman and Mahler (1967); 6 or 8% acrylamide gels containing 5 M urea were equilibrated 4–5 days in a mixture of phenol–acetic acid–5 M urea–2-mercaptoethanol (45:25:30:2, w/v/w/v), and electrophoresis was performed using 10% acetic acid as tray solution. Samples were dissolved by incubation with one volume of 0.01 N NaOH–2% 2-mercaptoethanol for 40 min, followed by addition of four volumes of phenol–acetic acid–urea mixture and incubation at room temperature for 15–20 hr. Staining was done by a modification of the procedure of Fairbanks *et al.* (1971). The gels were fixed and stained overnight in 25% isopropyl alcohol–10% acetic acid–0.05% Coomassie Blue (Colab), and destained with three changes of 10% isopropyl alcohol–10% acetic acid and stored in 5% methanol–7% acetic acid. Stained gels were scanned at 570 or 600 nm in a Gilford spectrophotometer modified for gel scanning. Periodic acid–Schiff reaction was carried out as described by Fairbanks *et al.* (1971).

For radioactivity measurements, sodium dodecyl sulfate polyacrylamide gels (0.6 × 10 cm) were fractionated at 1-mm intervals with a Gilson high-precision acrylamide gel fractionator. The extruded gel particles were dried overnight at 50°, then 50 μl of water was added to wet the gel, followed by 0.6 ml of NCS solubilizer (Amersham/Searle). The capped vials were incubated at 50° for 6 hr and allowed to stand at room temperature for 20 hr. At the end of this period, 10 ml of toluene–Liquifluor (New England Nuclear) was added and radioactivity was measured in a Beckman liquid scintillation counter.

Amino Acid Analysis. The main protein isolated from MOPC-104E or RPC-20 intracisternal A particles was precipitated from phosphate–sodium dodecyl sulfate buffer by addition of an equal volume of cold 20% (CCl_3COOH) solution. After 15 min the protein was collected by centrifugation at 4500*g* for 20 min and the pellet (80 μg) was resuspended in 6% CCl_3COOH and recovered as before. The insoluble protein was resuspended in 0.2 ml of water and the residual CCl_3COOH was extracted twice with 2.5 ml of ether. The protein after being dried *in vacuo* was hydrolyzed for 18 hr with 6 N HCl, and the amino acid content was de-

[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

termined in a Durrum amino acid analyzer by Biochemical Data Corp. The values of amino acid residues were corrected for destruction of serine.

Tryptophan was determined by the method of Spies and Chambers (1949) using lysozyme as a standard for tryptophan. The structural proteins from the particles were precipitated with 10% CCl_3COOH and washed with acetone. The insoluble proteins and the standards were digested with Pronase for 24 hr (Spies, 1967), and tryptophan was determined following the original procedure.

CNBr Cleavage of Structural Proteins. Structural proteins reduced with 1% 2-mercaptoethanol were precipitated by addition of CCl_3COOH to a final concentration of 15%, collected by centrifugation, washed with acetone, and dried *in vacuo*. This material (70 μg) was resuspended in 0.1 ml of 80% formic acid containing 1 mM dithiothreitol, flushed with nitrogen, and let stand at room temperature for 20 hr (Bryce and Crichton, 1971). At the end of this period 0.1 ml of 90% formic acid containing 2 μmol of CNBr was added to the protein samples, and the reaction was allowed to proceed for 120–140 hr. The amount of CNBr represents an excess of about 300 times the number of methionine residues present in the sample, as calculated from the amino acid analysis data. At the end of the cleavage period the reaction mixture was diluted ten times with water, frozen, and lyophilized. The lyophilization step was repeated three times using 10% formic acid to remove the residual CNBr. The products from the CNBr cleavage were analyzed by polyacrylamide gel electrophoresis in phenol–urea–acetic acid and sodium dodecyl sulfate urea systems.

Spectroscopic Methods. Absorption spectra of the purified proteins in the ultraviolet and visible region were recorded in a Acta III Beckman spectrophotometer using 0.1 M sodium phosphate buffer (pH 6.8) using 0.1% sodium dodecyl sulfate as solvent. Emission and excitation fluorescence spectra were recorded using an Aminco-Bowman spectrophotofluorometer. The solvent was the same mentioned above. In some instances 1% 2-mercaptoethanol was present in order to break possible inter- or intramolecular disulfide bonds. The protein concentrations were measured with the ninhydrin method (Hirs, 1967).

Immunodiffusion Assay. Immunodiffusion in sodium dodecyl sulfate containing agarose gels was performed essentially as previously described using rabbit antiserum 98/11 (Kuff *et al.*, 1972); however, because of the absence of sulfhydryl groups in the sample, alkylating agent was omitted from the gels. The plates were washed for 48 hr with several changes of phosphate–saline buffer, stained for 15–20 min with 0.05% Coomassie Blue in 25% isopropyl alcohol–10% acetic acid, and destained with 7% methanol–5% acetic acid.

Carbohydrate Studies. Sialic acid was determined by the method of Aminoff (1961). Thin-layer chromatography of radioactively labeled amino sugars was carried out as described by Moczar and Moczar (1970). The dried chromatographic strips were cut in 2-mm sections, each of which was transferred to a scintillation vial, wet with 0.1 ml of water, followed by 0.5 ml of NCS solubilizer, and 10 ml of toluene–Liquifluor system. Radioactivity was measured in a Beckman liquid scintillation spectrometer.

Results

In the present study, the "inner shell' fraction is defined operationally as that portion of the A particle which resists solubilization by sodium dodecyl sulfate or guanidine thiocyanate in the absence of disulfide bond reduction. It has been shown that this fraction indeed consists of particulate elements whose size and appearance under the electron microscope are consistent with this concept (Wivel *et al.*, 1973). It is recognized that some components originally localized in the inner shell of the inact particles could have been removed if the bond(s) responsible for their association with the inner shell were disrupted by sodium dodecyl sulfate.

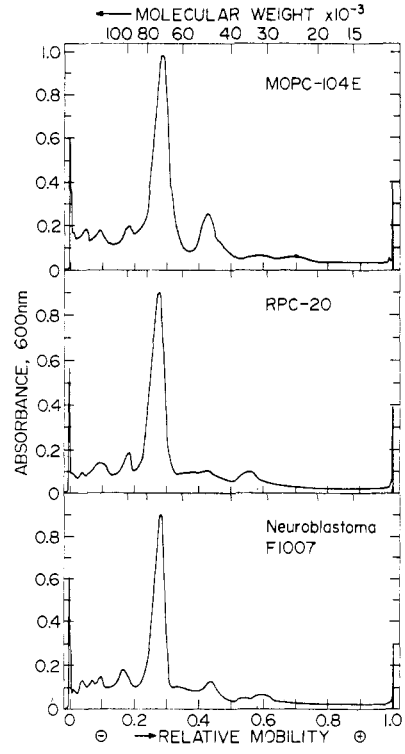

FIGURE 1: Scan of sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns of inner shells from murine intracisternal A particles. Between 20 and 25 μg of inner shell protein treated as described in Methods was applied to each 6% polyacrylamide gel. Electrophoresis was carried out wih 6 mA/gel for 5 hr; at the end of this period the front of the Pyronin dye marker was indicated by a notch in the gel. Gels were stained and scanned densitometrically as described in the text. The mobilities are expressed as relative mobilities with respect to the dye front.

Preparation and Analysis of Inner Shells. Inner shells from intracisternal A particles were disrupted into individual polypeptides by a disulfide-exchange reaction carried out in the presence of sodium dodecyl sulfate. Analysis of the reaction products by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 1) showed a common main polypeptide of mol wt 73,000. However, preparations from the three different cell types consistently showed differences in the distribution of minor components. Intracisternal A particles from MOPC-104E and neuroblastoma F1007 contained a significant amount of protein with a molecular weight around 46,000, which seems to be present at much lower levels in particles from RPC-20 (Figure 1). In addition, polypeptides of molecular weights around 100,000 and 30,000 are present in all three types of intracisternal A particles. Some preparations contained high molecular aggregates; upon treatment with sodium dodecyl sulfate and 2-mercaptoethanol (pH 10) at 100° for 5 min, the aggregates reverted only in part to the normal components of the particles. In all the preparations the 80,000 molecular weight outer envelope component (Wivel *et al.*, 1973) was absent.

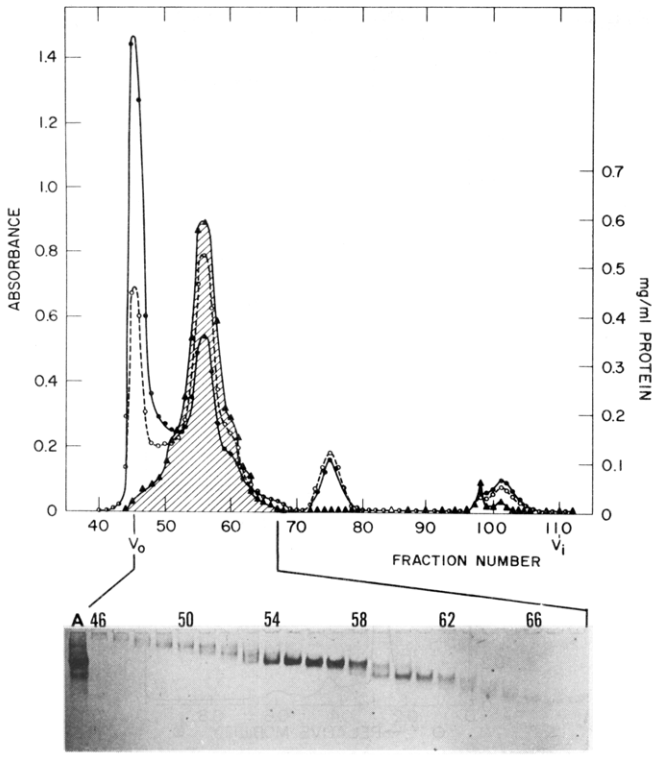

FIGURE 2: Gel chromatography of disulfide-exchanged inner shells from MOPC-104 intracisternal A particles. About 5 mg of total protein was applied to a 1.5 × 97 cm column of Bio-Gel A-1.5m (8% agarose), 200–400 mesh. The eluent was 0.1 M sodium phosphate buffer–0.2% sodium dodecyl sulfate (pH 6.8) and the flow rate was 5 ml/hr. Fractions of 1.5 ml were collected and monitored spectrophometrically at 260 nm (●—●) and 280 nm (○- - -○). Protein concentrations were determined with ninhydrin (▲—▲), and effluent fractions in the protein region (shaded area) were analyzed by sodium dodecyl sulfate PAGE. The numbers over the gel pattern serve to identify the fractions. (A) Total inner shell proteins from MOPC-104E intracisternal A particles.

Gel Chromatography. The elution profile obtained by sodium dodecyl sulfate gel chromatography of the dissociated inner shells from MOPC-104E tumors is shown in Figure 2. Similar profiles were obtained for inner shells from A particles isolated from RPC-20 tumors and neuroblastoma F1007. The peak emerging at the void volume position (V_0) and showing a higher absorption at 260 nm than at 280 nm represents low molecular weight RNA, 4–6 S. The following peak with a higher absorption at 280 nm corresponds to the protein region, as revealed by ninhydrin determination. Smaller peaks (fractions 72–79 and 97–105) with equal absorption at 260 and 280 nm and ninhydrin-negative reaction represent components of unknown nature. The resolution of individual proteins could not be clearly appreciated because of close range of molecular weights and the high proportion of the main component as compared to the other proteins. However, scanning of the fractions with sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 2) allowed precise estimates of elution positions for the different polypeptides. From this information the molecular weights for several proteins from different particles were obtained (Table I). A molecular weight of approximately 72,000 was obtained for the major protein of particles from the three sources.

In gel chromatography the only parameter that is being evaluated is the hydrodynamic volume (Fish *et al.*, 1970). Therefore, the accuracy of the molecular weight determinations depends on the similarity of hydrodynamic shapes of all

TABLE I: Apparent Molecular Weights of Proteins from Inner Shells of Murine Intracisternal A Particles from Three Different Tumor Sources as Determined by Polyacrylamide Gel Electrophoresis (PAGE) and Gel Chromatography (Gel C) in the Presence of Sodium Dodecyl Sulfate[a] (Mol Wt × 10^{-3}).

MOPC-104E		RPC-20		F1007	
PAGE	Gel C	PAGE	Gel C	PAGE	Gel C
100		100		100	
73	72	73	72.5	73	72
46.5	43	46.5	45	46.5	48.5
31	32	30	32	31	29

[a] Molecular weights were determined as described in the text. Markers used in sodium dodecyl sulfate PAGE were lysozyme, α-chymotrypsinogen, pepsin, ovalbumin, and bovine serum albumin. Markers used for gel chromatography were lysozyme, α-chymotrypsinogen, ovalbumin, and bovine serum albumin. The void volume (V_0) and included volume (V_i) were determined with Blue Dextran and Dnp-glycine, respectively.

the sodium dodecyl sulfate–protein complexes being investigated. In an effort to obtain confirmation of the molecular weights in a sodium dodecyl sulfate free system, Gdn·HCl was tested as a dissociating agent (Tanford, 1968). Suspensions of intracisternal A particles or sodium dodecyl sulfate free inner shells became optically clear when treated with 2-mercaptoethanol and alkylated in presence of 6–7.5 M Gdn·HCl. However, tlg filtration with 6 M Gdn·HCl on Sephadex G-200 (exclusion limit 90,000) or on Bio-Gel P-300 (exclusion limit ~110,000 in 6 M Gdn·HCl) revealed aggregates that moved with the velocity of the excluded marker (Blue Dextran). Similarly, the purified main component previously separated from MOPC-104E A particles in the presence of sodium dodecyl sulfate was found to migrate as an aggregate in Gdn·HCl. These findings indicate that intracisternal A-particle polypeptides exist in associated forms in Gdn·HCl.

Polyacrylamide Gel Electrophoresis (PAGE). The molecular weights of chromatographically isolated inner shell proteins were estimated by sodium dodecyl sulfate PAGE. The accuracy of the method depends on the condition noted by Reynolds and Tanford (1970), that all the sodium dodecyl sulfate–protein complexes involved in the determination (*i.e.*, both unknowns and reference standards) should have the same charge density and hydrodynamic shape. Similar charge density implies that their absolute free mobilities, as determined from a Ferguson plot (Ferguson, 1964), must be essentially identical (Neville, 1971). This has been experimentally verified for the main A-particle protein (Figure 3) and the 46,000 molecular weight component from MOPC-104E (not illustrated). It has not yet been possible to evaluate the degree of deviation from rodlike shape for the sodium dodecyl sulfate–protein complexes from intracisternal A particles. Owing to the lack of information from sodium dodecyl sulfate free systems, the molecular weights given in this paper must be regarded as "apparent" until further study with independent methods would allow a cross-examination of these results (Table I).

Figure 4 shows sodium dodecyl sulfate gel electrophoretic patterns of the isolated polypeptides of inner shells from the three different tumors. The main structural protein is the

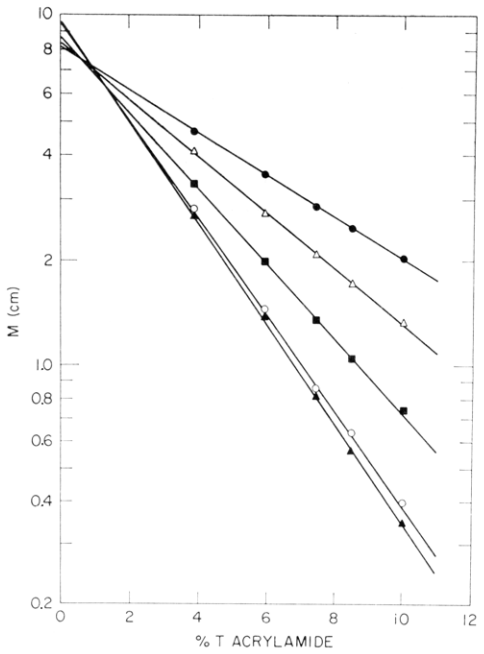

FIGURE 3: Ferguson plot or relationship between the electrophoretic mobility (M) and the total gel monomer concentration ($\%\ T$). The free mobilities were estimated by extrapolation of the plots to $\%\ T = 0$. All the sodium dodecyl sulfate–protein complexes studied showed nearly identical free mobilities. Preparation of proteins, electrophoresis, and staining were carried out as described in Methods. The mobilities were expressed in centimeters migrated during the electrophoresis period of 3.5 hr; lysozyme, 14,300 (●); α-chymotrypsinogen, 25,700 (△); ovalbumin, 43,000 (■); bovine serum albumin, 68,000 (○); main structural protein from MOPC-104E intracisternal A-particles, 73,000 (▲).

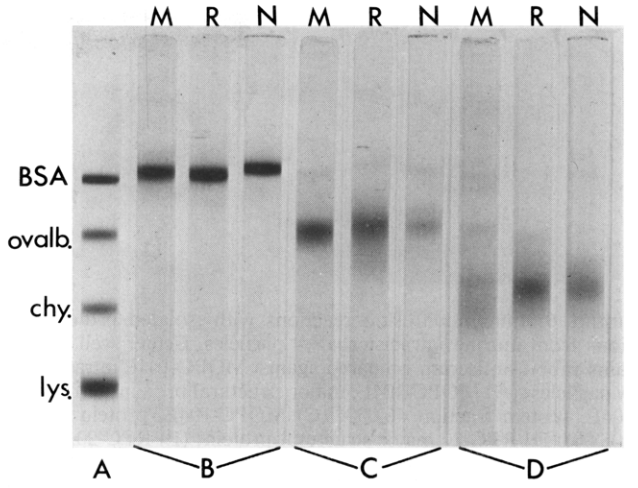

FIGURE 4: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of isolated protein fractions from intracisternal A particles; 4–7 μg of each protein in 40 μl of a solution containing 1% SDS–1% 2-mercaptoethanol–6% sucrose (pH 8) was layered over 6% acrylamide gels. Electrophoresis was carried out as described earlier until the Pyronin dye marker reached 7 cm. Gels were fixed and stained as described in the text: (A) bovine serum albumin (BSA), ovalbumin (ovalb.), α-chymotrypsinogen (chy.), and lysozyme (lys.); (B) main structural protein, mol wt 73,000, MOPC-104E (M), RPC-20 (R), and neuroblastoma F1007 (N); (C) fraction containing polypeptides with molecular weight around 46,000; (D) fraction containing low molecular weight components, around 30,000.

only component with well-defined limits. The minor components show different degrees of spreading, suggesting heterogeneity of the fractions. The molecular weights given for these components probably represent those of the most abundant specie present in each fraction.

Heterogeneity of the polypeptides isolated and characterized in presence of sodium dodecyl sulfate was further analyzed by electrophoresis in a phenol–urea–acetic acid system (Cotman and Mahler, 1967). The main proteins from RPC-20 and neuroblastoma F1007 intracisternal A particles were found to be homogeneous. However, in A particles from MOPC-104E, the main protein appears as two bands, the minor component presenting a slightly higher electrophoretic mobility (Figure 5). All the other protein fractions were found to be grossly heterogeneous in this system. Analysis of the whole inner shell preparations revealed all of the different fast-moving proteins found in the isolated fractions (Figure 5), and also components with lower electrophoretic mobilities than the main protein. These slower moving bands must represent the 100,000 molecular weight component as well as aggregates formed during the preparation of inner shells.

Spectroscopic Studies. Isolated structural proteins from intracisternal A particles showed the absorption spectra pattern characteristic for proteins containing the aromatic amino acids tyrosine and tryptophan. There were no absorption peaks in the near-ultraviolet or in the visible regions that could suggest other chromophores. Fluorescence spectroscopic studies confirmed these results. The emission and excitation spectra were typical of tyrosine and tryptophan residues and did not reveal any other characteristics. From the fluorescence studies and following the method of Shelton and Rogers (1971) the tryptophan content of the main structural proteins from MOPC-104E and RPC-20 origin was estimated in 2.9% of the protein weight.

Immunochemical Studies. Isolated inner shell proteins from the three tumors gave reactions of apparent identity on immunodiffusion with rabbit antiserum 98/11 (Figure 6). The immunoprecipitates were well-defined lines. Chromatographic

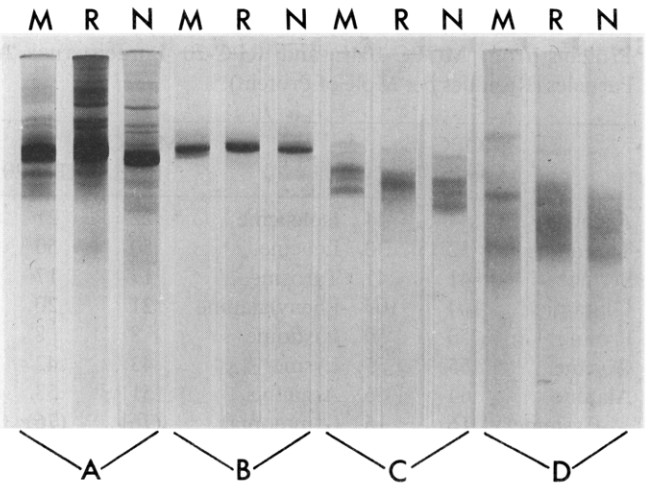

FIGURE 5: Polyacrylamide gel electrophoresis of inner shells and isolated proteins in a phenol–urea–acetic acid system. About 20 μg of inner shell protein and 4–5 μg of isolated fractions were treated as described in the text and layered over 6% acrylamide gels equilibrated with the phenol–urea–acetic acid solvent containing 2% mercaptoethanol. Electrophoresis was carried out at room temperature with 2.5 mA/gel (15 V/cm) until a cytochrome *c* marker reached 5 cm: (A) inner shells of intracisternal A particles from MOPC-104E (M), RPC-20 (R), and neuroblastoma F1007 (N); (B) major structural protein; (C) protein fraction 46,000; (D) protein fraction 30,000.

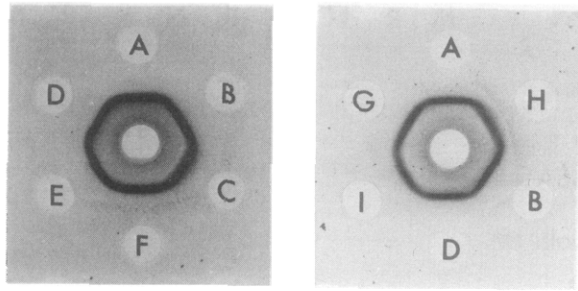

FIGURE 6: Immunodiffusion reactions with isolated protein fractions from murine intracisternal A particles. Center well (c) contains rabbit antiserum prepared against MOPC-104E intracisternal A particles: (A) MOPC-104E, major structural protein; (B) MOPC-104E, protein fraction 46,000; (C) MOPC-104E, protein fraction 30,000; (D) RPC-20 major structural protein; (E) RPC-20, protein fraction 46,000; (F) RPC-20, protein fraction 30,000; (G) F1007, major structural protein; (H) F1007, protein fraction 46,000; and (I) F1007, protein fraction 30,000. The antigen wells each contained 3 μg of protein. Agar gels were fixed and stained as described in the text.

fractions containing lower molecular weight components show in some cases a band with a electrophoretic mobility similar to that of the major structural protein (Figures 4 and 5). The degree of contamination, as evaluated from densitometric measurements of stained gels, was not higher than 5–7%. Since the total protein added to the antigen well was 3 μg in all cases, the applied samples of the lower molecular weight fractions contained, at most, 0.15–0.2 μg of the major protein. However, calibration experiments carried out with pure major structural protein showed that stable immunodiffusion lines were not formed at antigen concentrations below 0.5 μg. As the patterns illustrated in Figure 6 are typical for balanced systems (Clausen, 1969), it is unlikely that they resulted from contamination with the major protein.

No apparent relationship between intracisternal A particles and murine leukemia virus or mammary tumor virus was detected by complement fixation and immunodiffusion (Kuff *et al.*, 1972). Much more sensitive radioimmunoassays (Scolnick *et al.*, 1972) of purified intracisternal A-particle proteins have also failed to detect any gs antigen of murine leukemia–sarcoma virus type (Dr. David Livingston, private communication). The sensitivity of the method was 1 ng of gs antigen and levels of A-particle proteins up to 1500 times the amount needed to register a positive reaction were used.

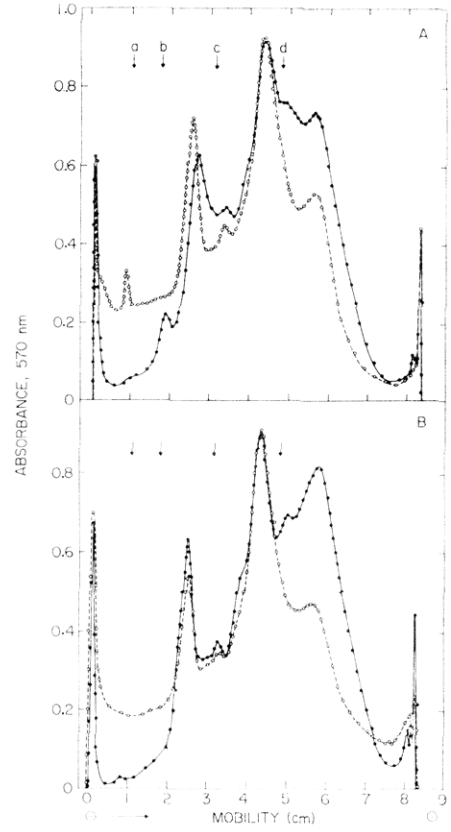

FIGURE 7: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of CNBr cleavage products from inner shell structural proteins of intracisternal A particles. About 35 μg of total protein was applied to 10% acrylamide gels, containing 0.1% sodium dodecyl sulfate–4 M urea (pH 7.2). Gels were fixed and stained as described in the text and scanned at 570 nm. (A) CNBr peptides from MOPC-104E A particles and (B) from RPC-20 A particles. Comparison of the products from the main structural protein, mol wt 73,000 (○- - - -○), and from the mol wt 46,000 protein (●—●). The letters indicate the position of the following protein standards: (a) bovine serum albumin, (b) ovalbumin, (c) α-chymotrypsinogen, and (d) lysozyme, in a parallel gel.

TABLE II: Amino Acid Composition of the Main Structural Proteins from MOPC-104E and RPC-20 Intracisternal A Particles (Residues per Mole of Protein).[a]

	MOPC-104E	RPC-20		MOPC-104E	RPC-20
Aspartic	44	44	Isoleucine	22	21
Threonine	32	30	Leucine	50	50
Serine[b]	41	41	Tyrosine	17	17
Glutamic	104	105	Phenylalanine	21	20
Proline	50	50	Histidine	8	8
Glycine	55	55	Lysine	43	42
Alanine	64	66	Arginine	51	53
½-Cysteine[c]	16	16	(Ammonia)	(76)	(76)
Valine	27	27	Tryptophan[d]	10	10
Methionine	8	8			
			Total residues	663	663
			Molecular weight	73,660	73,653

[a] The expressed values are average at four determinations. [b] Serine values corrected for 10% degradation. [c] Cystine was determined as carboxymethylcysteine. [d] Tryptophan was determined by the colorimetric method of Spies and Chambers (1949).

Amino Acid Composition. The complete amino acid composition of the main structural protein (mol wt 73,000) from intracisternal A particles from MOPC-104E and RPC-20 origin are shown in Table II. The ratios of acidic to basic amino acids (Glu-Asp:Lys-His-Arg) are 1.46 and 1.44 for MOPC-104E and RPC-20, respectively, indicating that the structural proteins are not basic. The per cent polarity of the proteins was calculated following the approach of Capaldi and Vanderkooi (1972). The polarities (per cent) were found to be 48.96 and 48.79 for MOPC-104E and RPC-20, respectively, values that fall between the normal range of polarity (per cent) of most water-soluble proteins, 46–52%. The amino acid analysis did not revealed any unusual residues or any amino sugars.

CNBr Cleavage of Structural Proteins. CNBr cleavage of the structural proteins of mol wt 73,000 and 45,000 from MOPC-

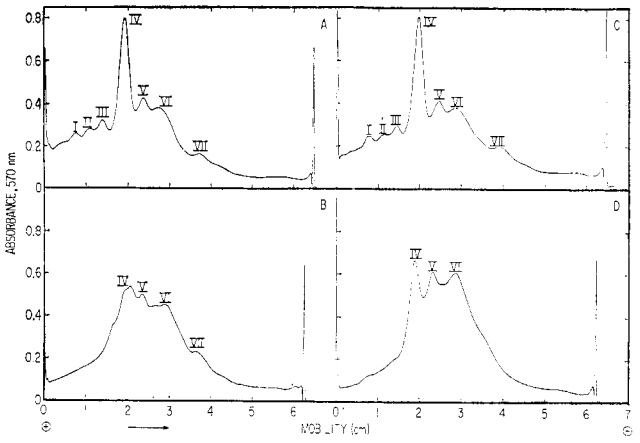

FIGURE 8: Polyacrylamide gel electrophoresis in phenol–urea–acetic acid system of the CNBr cleavage products from inner shell structural proteins of intracisternal A particles. About 30–35 μg of protein was applied to 8% acrylamide gels equilibrated with the phenol–urea–acetic acid solvent containing 2% 2-mercaptoethanol. Electrophoresis was carried out until the Pyronine dye marker reached the bottom of the gel. Staining was carried out as described in the text. Stained bands have been denominated I–VII for comparison purposes: (A) main structural protein, mol wt 73,000, MOPC-104E origin; (B) protein mol wt 46,000, MOPC-104E origin; (C) main structural protein, mol wt 73,000, RPC-20 origin; (D) protein mol wt 46,000, RPC-20 origin.

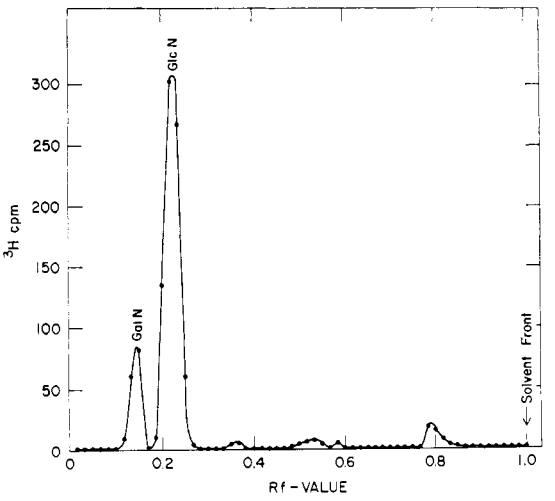

FIGURE 9: Thin-layer chromatographic identification of ^{3}H-labeled amino sugars in acid hydrolysates of a lipid-extracted fraction containing intracisternal A particles from neuroblastoma F1007. The cells were grown in the presence of [^{3}H]glucosamine and [^{14}C]-amino acids mixture. Samples of galactosamine (GalN, R_F 0.145) and glucosamine (GlcN, R_F 0.220) were cochromatographed on a polycarbonate sheet with the particle hydrolysate. The solvent used was ethanol–25% aqueous ammonia–water (85:0.5:14.5, v/v), and the standards were located by reaction with Violet Tetrazolium. Radioactivity was measured as described in the text.

104E and RPC-20 origin was carried out in order to obtain smaller peptides that could facilitate a direct comparison between the structures of the different proteins. The products from the cleavage presented solubility and associative properties very similar to the original proteins.

The peptides of the main structural proteins from MOPC-104E and RPC-20 origin were sound to be practically identical by electrophoretic analysis in sodium dodecyl sulfate–urea 10% acrylamide gels (Figure 7A,B) and in phenol–urea–acetic acid 8% acrylamide gels (Figure 8A,C). In sodium dodecyl sulfate polyacrylamide gel electrophoresis the most prominent peptides showed apparent molecular weights of 32,700, 16,700 and approximately 10,000. The electrophoretic patterns in the phenol–urea–acetic acid system were more complex and seven bands were identified. On the bases of the amino acid composition of the main structural protein, a maximum of nine peptides could be expected from CNBr cleavage.

Electrophoresis in sodium dodecyl sulfate–urea system of the products from the 46,000 molecular weight components revealed all the peptides observed in the main structural proteins (Figure 7A,B). However some differences were observed: the 10,000 molecular weight components became more prominent and a new peptide of mol wt 13,500 was present in the cleavage products. The RPC-20 protein showed a higher proportion of the 10,000 molecular weight component. In phenol–urea–acetic acid PAGE, the CNBr peptides derived from the 46,000 molecular weight protein again corresponded in mobility to several of the peptides derived from the 73,000 molecular weight proteins (Figure 8). In this system, the pattern of peptides from the MOPC-104E 46,000 molecular weight protein was somewhat more complex than that presented by the RPC-20 sample (Figure 8B, D). This difference may be related to variations in microheterogeneity of the original proteins (Figure 5C).

Carbohydrate Studies. Determination of sialic acid was negative under conditions that would have detected 1 residue/100 molecules of main structural protein. Gels containing electrophoretically separated inner shell proteins (up to 100 μg/gel) were negative by periodic acid–Schiff reaction (Fairbanks *et al.*, 1971) sensitive to 0.5 μg of carbohydrate (Glossmann and Neville, 1971), confirming previous results (Kuff *et al.*, 1972).

In vivo incorporation of [^{3}H]glucosamine was studied as another indicator of possible presence of carbohydrate. A crude A-particle fraction was isolated from neuroblastoma cells that had been labeled with [^{3}H]glucosamine and [^{14}C]-amino acids for 20 hr. This fraction contained approximately 0.9 and 2.0% of the total incorporated ^{3}H and ^{14}C in the cell, respectively. To test the identity of the incorporated ^{3}H label, a hydrolysate of the lipid-free fraction was subjected to thin-layer chromatography analysis. Most of the [^{14}C]amino acids released by the partial hydrolysis of the proteins migrated with the solvent front (not shown) whereas the ^{3}H label migrated in two zones (Figure 9). One zone representing approximately 80% of the total applied ^{3}H radioactivity had an R_F of 0.220, corresponding to glucosamine, and the other an R_F of 0.145, corresponding to galactosamine.

Sodium dodecyl sulfate resistant inner shells were then prepared from the remainder of the crude A-particle fraction and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 10). Approximately 20% of the ^{3}H label migrated with the main structural protein. However, most of the labeled amino sugar was found in other portions of the gel, particularly toward the origin, where relatively little labeled protein was recovered. Similar results were obtained for phenol–urea–acetic acid electrophoresis.

Discussion

The structural proteins from inner shells of intracisternal A particles from three different murine neoplastic tissues have been isolated and characterized. After cleavage of intermolecular disulfide bonds, the inner shells were dissociated into single polypeptides by sodium dodecyl sulfate or phenol–

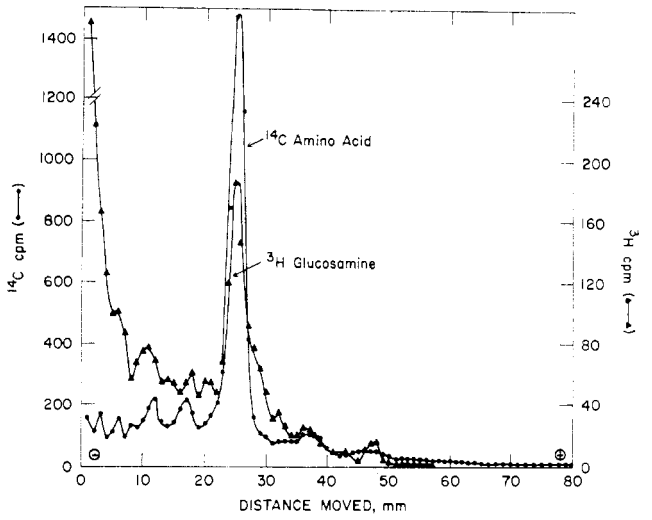

FIGURE 10: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of intracisternal A particles from neuroblastoma F1007 labeled with [^{3}H]glucosamine, [^{14}C]amino acid. The radioactive particles were isolated by the procedure summarized in the text. Inner shells prepared by treatment with sodium dodecyl sulfate were then reduced with 1% of 2-mercaptoethanol in 1% sodium dodecyl sulfate and the solution layered over a 6% acrylamide gel. Electrophoresis was carried out with 7 mA/gel until the Pyronin dye marker reached 9 cm. The fractionation of the gel and the measurements of radioactivity were carried out as described in the text: [^{14}C]amino acids (●—●), [^{3}H]glucosamine (▲—▲). The first point for [^{3}H]glucosamine is 1400 cpm.

acetic acid–urea systems, but resisted the action of Gdn·HCl, urea, and nonionic detergents. The peculiar properties of the A-particle polypeptides in Gdn·HCl resemble those observed for envelope glycoproteins of several avian and mammalian leukemia–sarcoma viruses (Fleissner, 1971; Nowinski *et al.*, 1972), and also for the sialoglycoproteins from erythrocyte membrane (Maddy and Kelly, 1971; Steck, 1972). One of the erythrocyte sialoglycoproteins is known to contain a terminal peptide region rich in hydrophobic amino acids (Marchesi *et al.*, 1972). A similar situation has been observed in the case of cytochrome b_5 (Spatz and Strittmatter, 1971) which contains an extremely hydrophobic section that contributes to its polymerization in aqueous solutions, except when in the presence of sodium dodecyl sulfate. The overall amino acid analysis of the major A-particle structural protein has not shown an unusual abundance of hydrophobic residues that might explain the limited solubility properties. By analogy to the cases mentioned above, the A-particle polypeptide may present a distribution of amino acid residues which force the protein to adopt a conformation in which the hydrophobic regions are exposed to the solvent. These exposed areas could result in hydrophobic interactions between individual proteins and be responsible for the formation of aggregates in denaturating agents like Gdn·HCl. The same hydrophobic areas might serve as association centers during the assembly of the particle at lipoprotein membranes *in vivo*. The solubility properties of the A-particle proteins are not characteristic of the internal structural proteins of oncogenic RNA viruses. For example, Gdn·HCl has been used to isolate proteins from avian and mammalian leukemia–sarcoma viruses (Fleissner, 1971; Nowinski *et al.*, 1972). Also nonionic detergents have been used in the isolation of proteins from Rous sarcoma virus (Hung *et al.*, 1971) and less efficiently in the separation of some proteins from mammary tumor virus (Nowinski *et al.*, 1971).

The major structural protein (mol wt 73,000) has been found to be fairly homogeneous by electrophoretic analysis in two different systems, whereas the fractions containing the smaller polypeptides isolated in the presence of sodium dodecyl sulfate showed extensive heterogeneity when analyzed by polyacrylamide electrophoresis in phenol–urea–acetic acid system. In contrast to the continuous sodium dodecyl sulfate electrophoresis, this system seems to resolve proteins in a manner dependent on both size and charge (Demus and Mehl, 1970), the effective size being a function of the length of the polypeptide and the particular conformation adopted when it is exposed to the essentially nonaqueous solvent. Accordingly, the observed heterogeneity of the proteins from intracisternal A particles could reflect small differences in the polypeptide chains, arising either from variations in the number of amino acid residues or as a result of modifications such as acetylation or phosphorylation. There is also a possibility that small quantities of nonprotein moieties such as lipid or carbohydrate could be bound to the proteins in a heterogeneous distribution. This would be consistent with the results of the glucosamine incorporation experiment which suggested that some glucosamine-containing material could be associated with the structural proteins.

In spite of the observed heterogeneity, all the isolated protein fractions from intracisternal A particles gave reactions of apparent identity using an antiserum prepared against MOPC-104E particles. In a sodium dodecyl sulfate free system such a pattern of identity would indicate identical antigens or identical determinant groups with respect to the antiserum used (Clausen, 1969). However, in the presence of sodium dodecyl sulfate, otherwise distinguishing antigenic specificities can be modified, so that nonidentical proteins which share common antigenic groups can give reactions of apparent identity (Fukui *et al.*, 1971). Thus the immunodiffusion results suggest that inner shell proteins of different size contain some common antigenic determinants, without, however, specifying that they are antigenically identical. Electrophoretic comparison of the CNBr-cleavage products of the main structural proteins and the 46,000 molecular weight components show that proteins of both sizes share a significant fraction of the peptides, providing a structural basis for the antigenic relatedness.

The inner shell proteins of the intracisternal A particles resemble some mitochondria membrane proteins which have extremely similar amino acid composition and peptide maps in spite of differences in molecular weight (Yang and Criddle, 1970), as well as tonofilament proteins that show immunological identity over a wide size range (Tezuka and Freeberg, 1972). These analogies open the possibility that the formation of A particles in transformed cells result from abnormal modification of some cellular structural components or inappropriate production of a fetal protein. However it is also possible that intracisternal A particles may be viral in nature as suggested by their morphology, budding process, presence of a characteristic DNA polymerase activity and evidence of a particle associated high molecular weight RNA (Yang and Wivel, 1973). Recently Vogt and Eisenman (1973) have presented evidence for a large polypeptide precursor in avian myeloblastosis virus (mol wt 76,000) which is cleaved during a maturation process. Intracisternal A particles can be viruses (oncogenic?) in which the proteolytic step is unoperative and as a consequence the particle produced is a defective one. The mode of transmission will be only vertical, owing to the lack of infectivity.

It may be possible to establish the nature of this particle by

a more detailed comparative study of intracisternal A-particle polypeptides and membrane associated proteins from normal and transformed cells as well as proteins from known oncogenic viruses. Furthermore, a knowledge of the biochemical and physicochemical properties of these structural polypeptides might allow an understanding of the assembly of cellular membranes and membrane-associated viruses.

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