

Conversion of Embryonic Form to Adult Forms of N-CAM In Vitro: Results from *De Novo* Synthesis of Adult Forms

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ABSTRACT During normal development, the neural cell adhesion molecule N-CAM changes at the cell-surface from a sialic acid-rich embryonic, or E form, to several adult, or A forms that have less sialic acid (E-to-A conversion). To investigate the cellular and molecular mechanisms that underlie these changes, we have established conditions under which E-to-A conversion occurs in cultured explants of central nervous system tissues. Mouse cerebellum, chick spinal cord, and chick retina that express the E form of N-CAM were dissected and cultured on collagen gels. After 3–6 d in culture, increased proportions of A forms were synthesized, as revealed by specific immunoprecipitation and immunoblotting. The rate of E-to-A conversion and the proportions of the different A forms synthesized in vitro were similar to those observed for the tissues in vivo at comparable times. In addition, the explants incorporated radioactive precursors of amino sugars into N-CAM, and the electrophoretic mobilities of the E and A forms of N-CAM were altered by treatment with neuraminidase in a way comparable to that found for N-CAM obtained directly from tissue. These results suggest that the post translational processing in vitro was similar to that in vivo.

Logistic studies on cell division and death in the explants suggested that E-to-A conversion resulted mainly from a specific increase in synthesis of A forms in individual cells rather than as a consequence of differential birth or death within distinct cell populations. The data were consistent with the possibility that the increase in synthesis of A forms occurred either in cells that had previously synthesized E forms or in a distinct population of cells that already synthesized A forms. Cells dissociated from embryonic central nervous system tissues and cultured in vitro were also found to undergo E-to-A conversion at the same rate as the explant cultures, which suggests that if intercellular signals were responsible for initiation of the change in synthetic pattern, they had already occurred in vivo before the time of culture.

In pulse-chase experiments, the E form of N-CAM that was synthesized during the first day after explantation persisted as E form for several days, at times when newly synthesized N-CAM was predominantly in A forms. These results indicate that in cultured neural tissue, the E form of N-CAM is not processed into A forms but is gradually degraded and replaced by newly synthesized A forms. This is consistent with the idea that E-to-A conversion results at least in part from intracellular regulation of the activity of a sialyl transferase with turnover of N-CAM at the cell surface.

The formation of tissue patterns during embryogenesis results in part from regulation of the arrangement of specific intercellular contacts. It has been suggested (1, 2) that the regulation of neural pattern results from a dynamic interplay between the fundamental processes of cell growth, movement, and death and the controlled expression of a small number

of cell adhesion molecules, each with different specificities. This proposal stresses the key role played by changes in the expression of these cell adhesion molecules and by chemical modifications that alter their binding efficacy, changes collectively termed local cell surface modulations (3).

One cell adhesion molecule that undergoes changes in both

expression and structure during development is the neural cell adhesion molecule N-CAM,¹ a large cell-surface sialoglycoprotein (4–7) that mediates the Ca²⁺-independent aggregation of neurons (5, 8), the initial binding between neurons and myotubes in vitro (9, 10), and the collection of neurite processes into fascicles (11). Recent work has established that the neural antigens D2, BSP-2, NS-4, and 224-1A6-A1 are identical to N-CAM (12–17). Dynamic changes in the expression of N-CAM occur during the early development of several mesodermal and ectodermal tissues (18–20).

During the perinatal period, N-CAM undergoes structural changes in its carbohydrate that result in altered binding properties. N-CAM preparations isolated from embryos (E form) contain a large amount of sialic acid (6, 7), present as polysialic acid (21, 22) attached at three binding sites in the central region of the polypeptide chain (7, 23). In contrast, N-CAM species isolated from adult neural tissue (A forms) contain only one-third as much polysialic acid, but their polypeptides are otherwise identical to those of the E form of N-CAM (24, 25). This alteration in N-CAM, termed E-to-A conversion (1, 26), is reflected in striking differences in electrophoretic mobility. Both E and A forms of N-CAM consist of closely related polypeptide chains of M_r 160,000 and 130,000. E forms migrate on SDS polyacrylamide gels as a broad, diffuse band ranging in molecular weight from 180,000 to 250,000 (6), and A forms migrate as discrete bands of M_r 180,000 and 140,000 in the chick, with an additional component of M_r 120,000 in the mouse (24, 25, 27). The number of carbohydrate attachment sites is the same in E and A forms of N-CAM (23), which supports the idea that the transition from E to A forms probably involves changes in the posttranslational attachment or removal of sialic acid. This alteration is keyed to the overall course of development, occurring at different rates and to various extents in different brain regions (20, 26). Similar changes in N-CAM expression have also been observed in vitro (28–30), but the molecular and cellular bases for these changes have not been investigated.

The potential functional significance of E-to-A conversion is suggested by two additional observations. First, the binding rate of A forms of N-CAM reconstituted into synthetic vesicles is four times higher than that of the E form (31). This suggests that in tissue, E-to-A conversion may result in tighter adhesions that could serve to stabilize connections. Second, the failure to form specific connections between parallel fibers and Purkinje cells in the cerebellum of the mouse neurological mutant staggerer has been correlated with a delay in E-to-A conversion (26). Although this delay is unlikely to be the primary cause of the pleiotropic disorders seen in staggerer mice, it is a reasonable hypothesis that a causal relationship exists between the failures in synaptogenesis and E-to-A conversion.

Because of the potential functional significance of E-to-A conversion during neurogenesis, we have begun experiments to determine the signals that initiate this process and the cellular and molecular mechanisms that underlie it. In the present paper we describe the development and use of in vitro systems that permit exploration of these issues. The key findings are that E-to-A conversion in vitro occurs on sched-

ules similar to those seen in vivo and that, in vitro, this conversion is a result of turnover of the E form with synthetic replacement by A forms.

MATERIALS AND METHODS

Tissues: Cerebella from 17 embryonic day to 8 day postnatal NCS mice (Laboratory Animal Research Center, The Rockefeller University) were freed from meninges, cut into fragments smaller than 1 mm³, and cultured in a square grid pattern of 0.5-cm periodicity. Spinal cords from 6–8-d chick embryos (White Leghorn) were freed of the meninges, cut along the central canal, and further divided into eight long fragments per cord. Spinal cords from older embryos were cut to yield pieces of size similar to that described above. The separation between each spinal cord explant in the culture dish was ~1 cm. Neural retinas from 5–7-d chick embryos were cultured flat, with the ganglion cell layer facing the substratum. For cultures of dissociated cells, tissue was washed in Eagle's minimum essential medium containing suspension salts (SME), cut into small pieces, and incubated at 35°C in SMEM with 10 mg/ml trypsin (Worthington Biochemical Corp., Freehold, NJ) and 100 µg/ml DNase (Worthington Biochemical Corp.) for 30 min. Fetal calf serum was added to a final concentration of 20% (vol/vol) and the tissue was triturated with a Pasteur pipette. Cells were washed with SMEM containing 100 µg/ml DNase and resuspended in medium. Clumps were removed by a brief low speed centrifugation and cells were plated at concentrations ranging from 2 × 10⁶ to 2 × 10⁷ cells per 35-mm dish.

Substratum and Media: Explants were grown in 35- and 60-mm plastic tissue culture dishes (Costar, Cambridge, MA or Corning Glass Works, Corning Medical and Scientific, Corning, NY) on a collagen gel (32). Typical amounts of total tissue were two cerebella, or four spinal cords or four retinas per 60-mm dish and about one-third these amounts per 35-mm dish. Dissociated cells were grown in 35-mm dishes covered with either polylysine or a collagen gel.

For chick tissue, the medium consisted of Dulbecco's modified Eagle's medium containing 10% (vol/vol) heat-inactivated fetal calf serum. For mouse explants, Dulbecco's modified Eagle's medium was supplemented with 10% heat-inactivated horse serum and 2.5% chick embryo extract. All media constituents were obtained from Gibco Laboratories (Grand Island, NY). Cultures were grown at 34°C in a 10% CO₂ incubator, and fed every 2–3 d with 1 ml (for 35-mm dishes) or 2 ml (for 60-mm dishes) of medium.

Immunoblot Analysis: After they were rinsed with SMEM, tissue fragments were collected into ice-cold SMEM containing 1% (vol/vol) Trasylol (Mobay Chemical Corp., New York) and pelleted. The supernatant was decanted, the pellet volume was estimated and 10 vol of 0.5% Nonidet P-40/10 mM Tris-HCl, pH 7.5/1% (vol/vol) Trasylol was added. The mixture was repeatedly agitated or, if necessary, homogenized with a micro-glass homogenizer until the tissue was solubilized; it was then centrifuged for 10 min in a Beckman Microfuge (Beckman Instruments Inc., Palo Alto, CA). The supernatant was collected, its volume was measured, and one-half of this volume of three-times concentrated SDS sample buffer was added. The sample was heated for 5 min at 100°C and then stored at –70°C. Aliquots of these extracts were separated by electrophoresis on 8.5% SDS gels (33), transferred to nitrocellulose, and immunoblotted with 4 µg/ml anti-N-CAM antibodies as described (34). N-CAM antigens were detected by binding of ¹²⁵I-protein-A and subsequent autoradiography.

Iodination of Cell-Surface N-CAM: Dispersed cultures of mouse cerebellar cells that had been incubated for 1 or 6 d were rinsed twice with Dulbecco's phosphate-buffered saline (PBS) and then iodinated with lactoperoxidase (Calbiochem-Behring Corp., La Jolla, CA) (35). The cells were rinsed with PBS containing 10 mM KI, harvested with a rubber policeman, washed twice with PBS, and then extracted with hot SDS and processed for specific immunoprecipitation with antibodies to N-CAM as described below.

Metabolic Labeling: For metabolic labeling with radioactive amino acids, sera and embryo extracts were dialyzed and added to minimal essential medium that lacked the appropriate amino acid. For sugar labeling experiments, low glucose (200 µg/ml) Dulbecco's modified Eagle's medium with dialyzed supplements was used. Tissues were washed two or three times with the appropriate medium for a total of 2 h, and then fed with medium containing radiolabel. Activities were 0.5 mCi/ml for [³H]leucine, 0.25 mCi/ml for [³⁵S]methionine, and 0.125 mCi/ml for [³H]glucosamine. All labeled compounds were obtained from New England Nuclear (Boston, MA).

[³H]Thymidine Labeling and DNA Determinations: Cultures were grown in standard media to which 3 µCi [³H]thymidine (³H]TdR) (Schwartz-Mann, Orangeburg, NY) was added. After 6 d in culture, the explants were fixed overnight with 4% formaldehyde and 0.5% glutaraldehyde in Hanks' basic salt solution (Gibco Laboratories) and 0.1 M Na-cacodylate, pH 7.2, and

¹Abbreviations used in this paper: A forms, adult forms; ara-C, cytosine arabinoside; E form, embryonic form; [³H]TdR, [³H]thymidine; N-CAM, neural cell adhesion molecule; SMEM, Eagle's minimum essential medium containing suspension salts.

embedded in JB-4 (Polysciences, Inc., Warrington, PA). 2- μ m sections were attached to glass slides, coated with NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), and developed after ~3 wk exposure at 4°C.

For determination of the DNA content of the explants, sister cultures of mouse cerebella from postnatal day 1, containing equal numbers of explants cut as uniformly as possible, were cultured for 1 or 6 d. The explants were collected and extracted with 1 N HClO₄ at 4°C for 72 h to hydrolyze RNA. The DNA was detected colorimetrically with indole (36).

Immunoprecipitation Analysis: To determine the proportions of the rates of synthesis of E and A forms, mouse cerebellar explants were labeled for 6 h, during which time the incorporation of label into total protein and immunoprecipitated N-CAM was linear. The rate of total protein synthesis determined by incorporation of [³H]leucine into trichloroacetic acid-precipitable material was 4.1×10^2 cpm/explant per h on day 1, and 3.3×10^2 cpm/explant per h on day 6. In other experiments, tissue fragments that had been labeled for 16–24 h with [³H]leucine (1.0 mCi), [³⁵S]methionine (0.5 mCi), or [³H]glucosamine (250 μ Ci) were collected, then solubilized by the addition of 0.2 ml boiling SDS buffer (1% [wt/vol] SDS, 0.15 M NaCl, 25 mM EDTA, 20 mM Na/HPO₄, pH 7.4), mixed vigorously, and stored at -70°C until used. The samples were then thawed, 4 vol of 1.25% (wt/vol) Nonidet P-40, 0.15 M NaCl, 25 mM EDTA, 20 mM Na/HPO₄, pH 7.4, were added, and the sample was centrifuged at 100,000 g for 30 min. The extracts were incubated with nonimmune serum or specific antibodies to N-CAM and then with protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The immune precipitates were washed, solubilized, and separated on 8.5% SDS polyacrylamide gels (33) as described (37). The gels were stained with Coomassie Brilliant Blue, destained, and then impregnated with sodium salicylate (38). Radiolabeled proteins were visualized by fluorography at -70°C with Kodak SB-5 X-ray film.

Photodensitometry: To determine the amounts of E and A forms of N-CAM, densitometric profiles of autoradiographs were obtained with a Quick Scan (Helena Laboratories, Beaumont, TX) scanner. We estimated the areas under each of the peaks by cutting and weighing the traces. However, because the discrete A form peaks co-migrated with the heterogeneously migrating E form of N-CAM, the dimensions of the A form peaks to be cut were chosen by an empirically determined procedure. It was assumed that the A form peaks present in mixtures of A and E forms would be symmetrical and have the same half-width and mobility as in samples that contained only A forms. In the case of chick spinal cord, it was further assumed that the 180-kD and 140-kD peaks had the same half-widths. It is important to note that this convention adopted for distinguishing between E and A forms is necessarily arbitrary, inasmuch as the precise relationship between sialic acid concentration and electrophoretic mobility is not known.

RESULTS

We cultured tissues from the central nervous systems of mouse and chick to study developmental changes in the expression of N-CAM *in vitro*. Because we were primarily interested in the patterns of N-CAM synthesis, we chose the ages of donor animals to correspond to the onset of rapid E-to-A conversion observed *in vivo* in these tissues (20). The appropriate ages were found to be 1–2 postnatal days for mouse cerebella, 5–7 embryonic days for chick retinas, and both 7–8 and 13–14 embryonic days for chick spinal cord. The explants were cultured for 1–7 d; microscopic examination of the tissues revealed good overall health as judged by neuritic outgrowth and transparency of the explants.

Immunoblots of N-CAM Present in the Explants

The forms of N-CAM present in the explants at various times were compared with the forms in developing tissue at comparable times by immunoblotting using specific antibodies to N-CAM (Fig. 1). In mouse cerebellum at postnatal day 2, N-CAM is present primarily as heterogeneously migrating material with $M_r > 180,000$. During the next 6 d of embryonic development *in vivo*, discretely migrating components of M_r 180,000, 140,000, and smaller amounts of M_r 120,000 appear, as previously reported (26). When the forms of N-CAM in the cultured explants were analyzed, similar results were

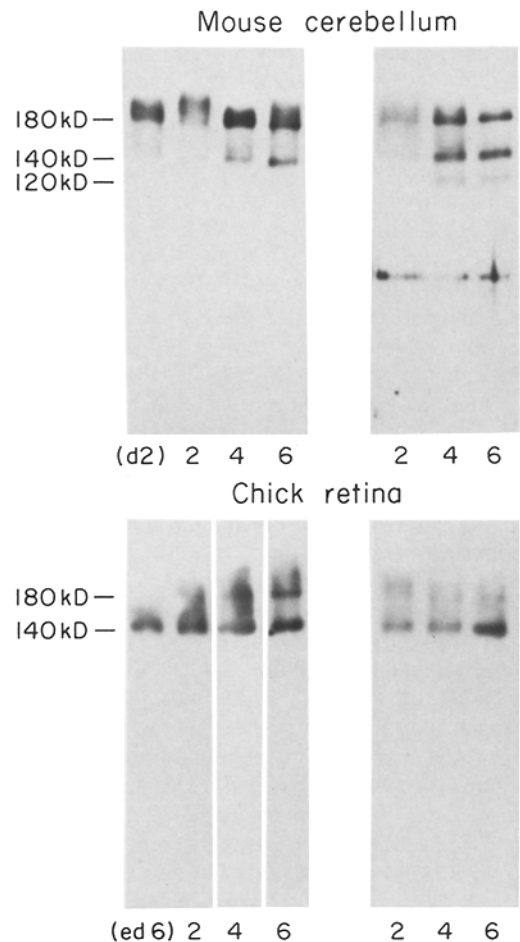


FIGURE 1 N-CAM expression in central nervous system explants. The figure shows a comparison of the expression of N-CAM in explants of mouse cerebellum and chick retina cultured *in vitro* (right panels) with expression *in vivo* (left panels) at comparable times. Explants of mouse cerebellum from postnatal day 2 (d2) mice and from 6-d embryonic chicks (ed6) were maintained for 2–6 d *in vitro*. At the indicated times, intact tissue or explants were harvested, extracted, and fractionated by SDS PAGE. The forms of N-CAM present were analyzed by immunoblotting using rabbit antibodies to purified mouse or chicken N-CAM, followed by ¹²⁵I-labeled protein A. Radioactivity was visualized by autoradiography. The indicated molecular weights were determined by comparison with standards run in parallel.

obtained. Appreciable amounts of 180- and 140-kD forms of N-CAM were apparent after 4 d in culture, and by 6 d, most of the N-CAM was present in these forms (Fig. 1). E-to-A conversion in cerebellar explants *in vitro* was complete ~2 d earlier than *in vivo*.

In developing retina, the proportion of heterogeneously migrating material decreased between embryonic day 10 and 12 (Fig. 1) as previously described (20). In cultured explants of retina, a progressive decrease in the proportion of heterogeneously migrating material was also observed. After 6 d *in vitro*, most of the N-CAM was present as the 140-kD form, with a lower proportion of the 180-kD form than was seen *in vivo* at the corresponding time. It is interesting that most of the N-CAM present in tissue at early ages (see embryonic day 6 in Fig. 1) migrated as a discrete band of M_r 140,000.

These results indicate that during culture *in vitro*, N-CAM present in the explants changed from predominantly the E

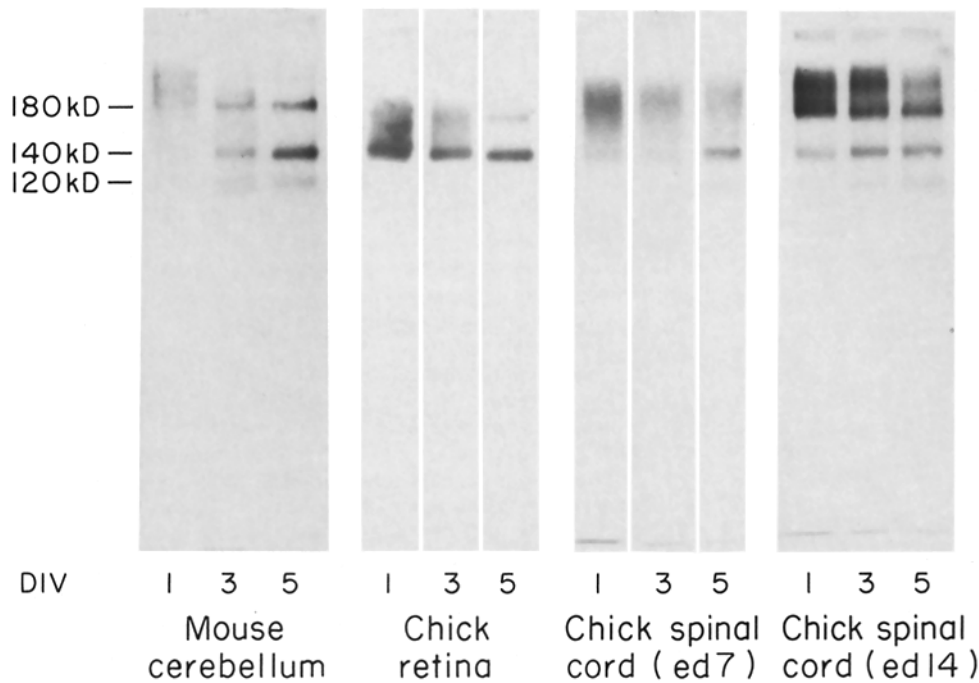


FIGURE 2 Synthesis of N-CAM by central nervous system explants. Explants of cerebellum from mice at postnatal day 2, of retina from chickens at embryonic day 7, and chicken spinal cords from embryonic days 7 (ed7) and 14 (ed14) were maintained in culture for 1–5 d. Cultures were labeled with [³H]leucine, extracted, and specifically immunoprecipitated with antibodies to N-CAM. The immunoprecipitates were fractionated by SDS gel electrophoresis and the forms of N-CAM visualized by fluorography.

form to the A forms, but they do not indicate precisely when the change in synthesis occurred. To examine this question, we turned to metabolic labeling studies.

Immunoprecipitation Analysis of N-CAM Synthesized by the Explants

To determine the forms of N-CAM synthesized by the explants, cultures of mouse cerebellum, chick retina, and chick spinal cord were incubated with [³H]leucine at various times after explantation, and the labeled N-CAM was immunoprecipitated and resolved by SDS gel electrophoresis (Fig. 2). The fluorographs were analyzed by densitometric scanning (Table I). The results show that the explants culture initially synthesized the E form of N-CAM and that, later, increasing proportions of A forms were synthesized. Mouse cerebellum exhibited the most complete conversion. After several days in culture, prominent 140- and 180-kD bands and a weaker 120-kD band were detected.

N-CAM synthesized by explants of chick retina after 6 d in culture consisted of material in a prominent 140-kD band and much smaller amounts of 180-kD material. Spinal cord explants from 7-d chick embryos also showed an increase in the relative amount of 140-kD material after 6 d in culture. This change was accompanied by a decrease of polydisperse material in the range of 140–180 kD but no apparent concomitant increase in the amount of 180-kD material. However, we observed an increase in the relative amount of 180-kD material after 5 d in vitro when explants of spinal cord were taken from 14-d chick embryos (Fig. 2). These results correlate well with observations on the conversion of N-CAM from chick spinal cord in vivo, where material migrating at 140-kD appears earlier than the 180-kD material (not shown).

To test whether the change in forms of N-CAM was artifactually induced by tissue isolation or by the culture conditions, we examined the synthesis of E and A forms in tissues explanted from younger embryos that, if undisturbed, would continue to synthesize the E form of N-CAM in vivo for several days. In explants of cerebella from 17-d embryos, the

TABLE I. Synthesis of A Forms of N-CAM In Vitro

Tissue	Days in vitro		
	1	3	6
	% of total N-CAM*		
Mouse cerebellar explants	20 ± 9 (4)	43 (1)	61 ± 9 (4)
Chick retinal explants	43.4 ± 1.7 (3)	44.2 (1)	58.7 ± 1.6 (3)
Chick spinal cord 7-d embryo explants	16 ± 6 (3)	22 (1)	32 ± 12 (3)
Chick spinal cord 14-d embryo explants	38.8 ± 2.5 (2)	38.5 ± 2.1 (2)	50.3 ± 0.6 (2)
Dissociated mouse cerebellum	31.5 ± 0.4 (2)	ND	63.5 ± 0.7 (2)
Dissociated chick retina	26 (1)	ND	54 (1)

This table shows the percentage of newly synthesized N-CAM in A forms at various times in culture. The values were obtained by densitometric scanning of autoradiograms. The values shown (±SD) represent averages; the number of experiments is shown in parentheses. ND, not determined. Mouse cerebellar explants were incubated for 6 h with 1 mCi [³H]leucine. * A forms.

synthesis of A forms in vitro was found to be delayed by at least 3 d (not shown). This indicates that the in vitro conversion was not a simple consequence of the explantation procedure or isolation from its normal context and suggests that it was occurring in response to normal developmental cues.

To assess a possible requirement for tissue integrity in E-to-A conversion in vitro, cerebella from 1-d postnatal mice and retinas from 6-d embryonic chick were dissociated into dispersed cells, cultured as monolayers, and labeled with [³H]leucine. After 1 and 5 d in vitro, the pattern of N-CAM labeling closely resembled that observed in explants of the corresponding age in vitro (Fig. 3, A and C). Similar results were obtained with spinal cord from 7-d embryonic chick (not shown).

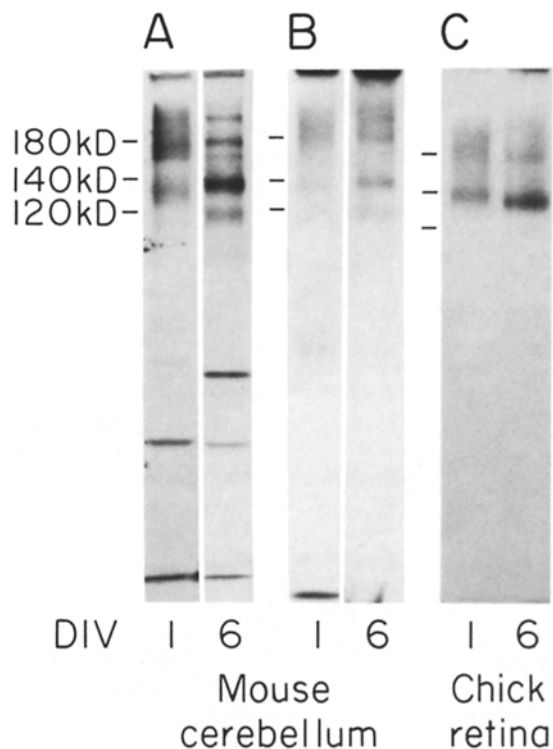


FIGURE 3 E-to-A conversion in dissociated cells. Cerebella from 1-d postnatal mice and retinas from 6-d chick embryos were dispersed and cultured for 1 or 6 d in vitro (DIV). The cultures were labeled with [³H]leucine (A and C) or the cell surface proteins were labeled with ¹²⁵I using lactoperoxidase (B). The cells were extracted, N-CAM was specifically immunoprecipitated, resolved by SDS gel electrophoresis, and visualized by fluorography (A and C) or autoradiography (B).

In evaluating the functional significance of the observed E-to-A conversion in vitro, it was important to determine whether the changes occurred in N-CAM located at the cell surface. N-CAM on the surface of cultured mouse cerebellar cells was labeled with ¹²⁵I using lactoperoxidase, extracted, and analyzed by SDS gel electrophoresis. The results (Fig. 3B) show that cell surface N-CAM was predominantly in the E form after 1 d in culture and that increased proportions of A forms had appeared after 6 d.

These results indicate that the shift in synthesis from the E form to the A forms of N-CAM occurred during the period of culture and that this was reflected in N-CAM at the cell surface.

Processing of N-CAM Synthesized In Vitro

It was important to determine the extent to which the synthesis and posttranslational modification of N-CAM in the explant cultures were similar to these processes in the intact tissues during normal development. Several experiments designed to examine the synthesis and glycosylation of the N-CAM polypeptide chains suggested that these processes were in fact occurring normally in the explant cultures.

Because posttranslational glycosylation alters the electrophoretic mobility of N-CAM, it was possible to monitor these events by pulse-chase experiments. Explants of mouse cerebellum that had been cultured for 1 or 6 d were pulsed with [³H]leucine for 20 min and then chased with excess cold leucine for 0, 20, 60, and 120 min (Fig. 4). In explants cultured

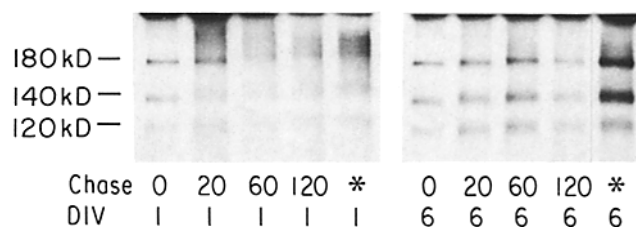


FIGURE 4 Posttranslational modification of N-CAM synthesized in vitro. Explants from postnatal day 1 mice were cultured for 1 or 6 d in vitro (DIV) and labeled with [³H]leucine for 20 min, briefly washed, and incubated in growth medium containing 10 times the normal amount of unlabeled leucine for the indicated times (chase) before harvesting. The extracted material was immunoprecipitated with specific antibodies, and the forms of N-CAM were analyzed by fluorography of SDS gels. For comparison, explants cultured for 1 or 6 d were labeled for 16 h (*). The figure shows the high molecular weight region of the gels.

for 1 d, the N-CAM labeled during the short pulse was initially detected as discrete bands of M_r 160,000 and 130,000, which were processed into polydisperse higher molecular weight material during the chase period. This change in mobility results from the addition of large amounts of sialic acid (6, 7). In contrast, the M_r 160,000 and 130,000 forms of N-CAM synthesized by explants that had been cultured for 6 d were not processed into polydisperse material but into discrete bands that had mobilities corresponding to M_r 180,000 and 140,000. Inasmuch as the addition of neutral and amino sugars occurs rapidly, this slight alteration of mobility, which was consistently observed, probably resulted from the addition of relatively small amounts of sialic acid. The addition of sugars to the N-CAM synthesized in vitro was also examined by metabolic labeling with a specific precursor to *N*-acetylglucosamine and by testing the effect of neuraminidase treatment on the electrophoretic mobility of N-CAM. Identical patterns of labeling were observed with both [³H]glucosamine and [³H]leucine, indicating that amino sugars were incorporated into both E and A forms of N-CAM synthesized by mouse cerebellar explants in vitro (not shown). In addition, treatment of both E and A forms with neuraminidase resulted in alterations of their electrophoretic mobilities similar to those described for N-CAM isolated directly from tissue (6, 7). Taken together, the results suggest that the posttranslational glycosylation of N-CAM was occurring normally in vitro.

Cellular Basis of E-to-A Conversion

In the attempt to interpret these results, a key question was whether the changes observed were a direct consequence of changes in the synthetic patterns in individual cells, or were a trivial logistic consequence of the selective birth or death of cells within distinct populations. In the absence of techniques for distinguishing E and A forms of N-CAM on individual cells, this issue was addressed through population studies of the whole cultures. The analyses focused on mouse cerebellar cultures because they exhibited the highest degree of conversion among the tissues studied.

To estimate the proportion of cells in the explants that expressed N-CAM, frozen sections from mouse cerebellar explants cultured for 1 or 6 d were stained with antibodies to N-CAM. In each case, almost all of the cells were stained, at a roughly similar level of intensity (not shown).

CELL DIVISION AND CELL DEATH IN THE CULTURED EXPLANTS: Sister cultures were grown in the presence of [³H]TdR to determine the percentages of cells that had divided in vitro. 2- μ m sections of plastic embedded cultures were processed for autoradiography. Cell counts in random sections indicated that 20% of the cells had incorporated [³H]TdR during the culture period (Table II).

It was possible that E-to-A conversion occurred by the accumulation of A forms of N-CAM synthesized only in these new cells. In this case, elimination of the dividing cells would abolish the conversion. To test this possibility, cultures were grown for 6 d in the presence of cytosine arabinoside (ara-C) and the synthesized N-CAM was analyzed. Only 5% of the cells incorporated [³H]TdR in the presence of ara-C (Table II). Nonetheless, the pattern of N-CAM synthesis was similar in the control and ara-C-treated cultures, except for the 120 kD band (Fig. 5), which appeared to be reduced in the ara-C-treated cultures. These results suggest that the accumulation of the 180- and 140-kD A forms of N-CAM cannot be due solely to the appearance of a new population of cells.

We estimated cell death during the period of explant culture to be ~50% by measuring the total amounts of DNA on days 1 and 6 in sister cultures (Table II). Microscopic examination of toluidine blue-stained plastic sections of the cultured explants (deposited on the substratum in random orientations) indicated that at least part of the cell death is associated with a necrotic core that typically develops on the substratum side of cultured explants (not shown).

TABLE II. Cell Division and Death during Culture of Mouse Cerebellar Explants

	1 d	6 d	% of original
DNA content* (μ g/explant)			
Exp. 1	2.3 \pm 0.4	1.2 \pm 0.3	52
Exp. 2	3.1 \pm 0.5	1.6 \pm 0.2	52
% Labeled cells [†]			
- ara-C	—	19.3 \pm 4.0%	
+ ara-C	—	4.7 \pm 2.5%	

* The DNA content of 50 pooled explants was determined after 1 or 6 d in vitro as described in Materials and Methods. The values shown (\pm SD) are the average of triplicate determinations.

[†] To determine the proportion of cells that divided in culture, explants were cultured for 6 d in the presence (+) or absence (-) of 10⁻⁵ M ara-C in medium containing [³H]TdR, and processed for autoradiography. The percentage of labeled cells was determined after counting ~500 cells in random sections. The values shown are the averages from four experiments (\pm SD).

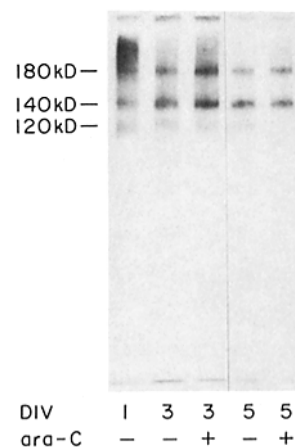


FIGURE 5 Effects of ara-C on N-CAM synthesis. Cerebellar explants from postnatal day 1 mice were grown in the presence (+) or absence (-) of the mitotic inhibitor ara-C during the indicated periods in vitro (DIV), then labeled with [³H]leucine. The forms of N-CAM that were synthesized were visualized by fluorography after specific immunoprecipitation and SDS gel electrophoresis.

MODELS OF CELL BIRTH AND DEATH IN E-TO-A CONVERSION: To evaluate whether selective cell birth or death alone might account for the increase in the proportion of the A forms of N-CAM synthesized during culture, we considered three possible models. In each of these models, the explants were assumed to consist of two distinct subpopulations, one synthesizing only the E form of N-CAM (E-cells) and the other synthesizing only the A forms (A-cells). In the first model, cell birth and death were assumed to be random. In the second model, birth was assumed to occur randomly, whereas death was assumed to occur only among E-cells. In the third model, it was assumed that birth occurs only among A-cells, whereas deaths are randomly distributed between E- and A-cells. We used the experimentally determined values of birth and death and assumed that the initial rates of N-CAM synthesis were identical in the two populations. We then calculated the changes in cell population and the final ratio of synthetic rates of N-CAM in the E- and A-cells that would be necessary to yield the experimentally determined proportions of E and A forms of N-CAM that were synthesized by cerebellar explants after 6 d in vitro.

In all three cases, we found that simple changes in the populations of E- and A-cells could not result in a sufficient increase in total A form synthesis, without a selective increase in synthesis by individual A-cells (Table III). A fourth model (not shown) did not require selective changes in the rates of synthesis of E or A forms. We considered this model unlikely inasmuch as it required both that cell death occur only among E-cells and that cell birth occur only among A-cells. We conclude that E-to-A conversion in vitro is due either to a switch of synthesis in individual cells from E to predominantly

TABLE III. Cellular Basis of E-to-A Conversion

Model	Initial composition	Population changes	Resulting composition	Required ratio of A-to-E form synthesis
I	E 80 A 20	Randomly distributed births and deaths	E: 42 A: 10	6.2
II	E 80 A 20	Random deaths All births among A	E: 34 A: 18	3.0
III	E 80 A 20	Random births All deaths among E	E: 30 A: 22	2.1

This table summarizes the changes in cell populations and the required ratios of A forms to E form synthesis predicted by different models of E-to-A conversion. In all cases, the initial composition of the population (80% E-cells, 20% A-cells) was derived from the proportions of E and A forms of N-CAM synthesized by mouse cerebellar explants during the first day in culture (Table I), under the assumption that the rate of synthesis of N-CAM is the same in all cells. The proportion of E- and A-cells after 6 d in culture (Resulting composition) was calculated using the measured amounts of dividing cells (19%, Table II) and cell death (48%, Table II) combined with the assumptions of each model. For example, in model II, the total number of cells on day 6 must be 52 (52% of original 100). At most, 10 of these cells (19% of the remaining) are new and, in this model, all of these are assumed to be A-cells. Therefore, 58 cells died, 46 (80%) of which were E form and 12 (20%) were A form, yielding a final population of 34 E-cells and 18 A-cells. For this population to synthesize the experimentally determined properties of E and A forms of N-CAM (39% E form and 61% A forms [Table I]), the ratio of A form to E form synthesis must increase to 3.0.

A forms or to a selective increase in the synthetic rate in a distinct population that is already synthesizing A forms. These results directed our attention back to the possibilities that in each cell, the existing E form is converted to A forms, or that A forms are synthesized de novo.

De Novo Synthesis of A Forms

We used pulse-chase experiments to investigate the candidate mechanisms that might be involved in E-to-A conversion in vitro. After explantation, cultures were labeled for 16 h with radioactive amino acids and then chased with excess unlabeled amino acids for 0, 2, and 4 d. The forms of N-CAM present were analyzed by immune precipitation, SDS PAGE, and either fluorography or, in the case of double-labeling experiments, by slicing and counting the gel for both isotopes.

If A forms accumulated by modification of pre-existing E forms, then significant amounts of heterogeneously migrating material labeled during the first day of culture would be processed into discretely migrating A forms during the chase period. We observed, however, that the E form of N-CAM that was synthesized during the first day after explantation by mouse cerebellar explants (Fig. 6A) or by chick spinal cord explants (Fig. 6B) persisted as E form for several days, although in greatly reduced amounts. At this time, newly synthesized N-CAM was predominantly in the A forms (Fig. 6).

To minimize sample-to-sample variation, the pulse-chase experiment was also performed by double-labeling a single explant culture. This protocol confirmed that after 6 d in culture, the labeled E forms synthesized on day 1 were still present as E forms, although they were reduced in amount. The N-CAM synthesized in the same explants at the end of

the 6-d culture period was largely in the A forms (not shown). These results indicate that, in vitro, the E forms of N-CAM are not processed into A forms but are gradually degraded and replaced by newly synthesized A forms.

DISCUSSION

The major purpose of this study was to devise rapid in vitro assay systems for investigating the cellular and molecular mechanisms that underlie the conversion of E form to A forms of N-CAM and for testing the effects of different cellular signals on this conversion. We focused initially on explant cultures because they maintain the cell density and intercellular contacts of intact tissue, which might help to preserve the developmental cues that initiate and control E-to-A conversion. We observed that the expression of N-CAM in several central nervous system tissues from mouse and chicken underwent changes that are similar to the developmental alterations previously studied in vivo (20, 24, 26). Metabolic labeling of N-CAM suggested that E-to-A conversion resulted from an increase in the synthesis of A forms during the period of culture in vitro.

Several observations suggested that the process of E-to-A conversion occurred normally in vitro. Studies with lactoperoxidase labeling established that the change from E to A forms was occurring at the cell surface, which was important since N-CAM functions at the surface. The posttranslational modification of N-CAM in vitro, as assessed by short pulse-chase experiments, by incorporation of precursors to amino sugars, and by the effect of neuraminidase treatment, resembled that previously seen in vivo. In addition, the pattern of A forms expressed was characteristic (20) of the tissues used, and the time course of conversion was similar to that in vivo, although some differences were noted. The rate of E-to-A conversion in mouse cerebellum was faster in vitro than in vivo, perhaps reflecting the premature decrease in cell proliferation in vitro. Nevertheless, the change in synthetic pattern was not simply induced by the process of explantation and culture, inasmuch as tissues removed from younger animals showed an appropriate delay in the onset of conversion.

The increase observed in synthesis of A forms relative to the E form of N-CAM could have resulted from different events at the cellular level. The key question was whether this increase occurred in individual cells or was only a consequence of the selective birth of cells that synthesized A forms of N-CAM or of the death of cells synthesizing E forms. Quantitative considerations based on the experimental observations (Table III) suggested that the E-to-A conversion could not result simply from the selective proliferation of cells synthesizing A form N-CAM, without a marked increase in the rate of synthesis of N-CAM in such cells. That cell birth alone is not responsible for E-to-A conversion in vitro is further supported by the fact that the mitotic inhibitor ara-C did not inhibit the conversion of E forms to 180- and 140-kD A forms. This agent did appear to reduce selectively the synthesis of the 120-kD N-CAM component, which raises the interesting possibility that this component is expressed preferentially in a population of proliferating cells. Logistic considerations also ruled out the possibility that the conversion resulted simply from the selective death of cells that synthesized E forms of N-CAM.

The quantitative and qualitative data strongly suggest that E-to-A conversion in vitro requires an increase in the synthesis

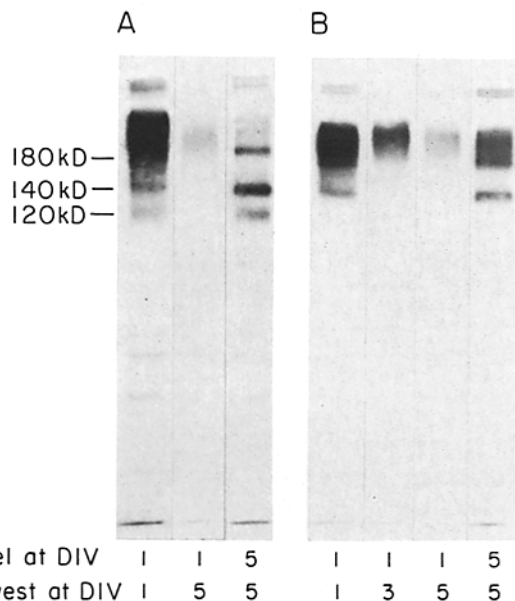


FIGURE 6 Fate of embryonic forms of N-CAM synthesized in vitro. Explants of cerebellum from postnatal day 2 mice (A) and spinal cord from embryonic day 7 chickens (B) were incubated with [³H]leucine during the first day in culture. The cultures were then incubated for several days in the presence of excess unlabeled leucine and analyzed at various times to determine the fate of the E form labeled on day 1. For comparison, A forms of N-CAM that were synthesized by explants labeled after 5 d in vitro (DIV) are also shown. The figure shows fluorographs of SDS gel analyses of specific immunoprecipitates.

of A forms in individual cells. This increase could occur via two types of cellular mechanisms. In the first, cells that originally synthesize the E form could later synthesize A forms. In the second, the increase in the rate of synthesis of A forms would occur only in a distinct population that synthesized A forms, resulting in heterogeneity in the amounts of N-CAM expressed on different cultured cells. Immunofluorescence staining studies did not reveal such heterogeneity, although this technique is not quantitative and therefore did not definitively rule out the possibility. We now favor the interpretation that most cells first express E forms and then later express A forms. Nevertheless, the issue will be conclusively resolved only when reagents become available that can distinguish between E and A forms of N-CAM in individual cells.

E-to-A conversion could result from decreases during development in the levels or activity of transferases that add sialic acid to N-CAM or from increases in the activity of neuraminidases that remove this sugar. The results of the pulse-chase experiments indicated that E forms of N-CAM were not processed *in vitro* into A forms by removal of sialic acid. Instead, E-to-A conversion *in vitro* resulted mainly from *de novo* synthesis of A forms accompanied by the disappearance of the previously synthesized E form. These results imply that there is no important role for an extracellularly acting neuraminidase in the biosynthesis of A forms *in vitro*. It remains possible that an intracellular neuraminidase might remove sialic acid from newly synthesized E forms, converting them into A forms before they appear at the cell surface. In this case, one might expect to observe a short-lived E form N-CAM in brief pulse-labeling experiments. Such a short-lived intermediate was not detected (Fig. 4), although the possibility cannot be completely excluded that removal of sialic acid occurs so rapidly that E form cannot be detected. In view of all the evidence, it appears probable that E-to-A conversion results at least in part from a regulatory decrease in activity or a loss during development of a sialyl transferase specific for N-CAM.

Whereas the metabolic labeling studies established the times during development at which explants of cerebellum, spinal cord, and retina begin synthesizing A forms of N-CAM, they also raised the question of when the cells become committed to this change. The finding that dissociated cells continue to undergo E-to-A conversion at the same rate as the explants may indicate that the cells had already become committed to this change before the time of dissociation. If, for example, cells stopped synthesizing an N-CAM-specific transferase early in development, turnover of the transferase would later result in E-to-A conversion. The present work suggests that further insight into the control of E-to-A conversion will probably come from studies on the expression of transferases that add sialic acid to N-CAM during development.

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