

Anchorage of Collagen-tailed Acetylcholinesterase to the Extracellular Matrix Is Mediated by Heparan Sulfate Proteoglycans

ENRIQUE BRANDAN, MAFALDA MALDONADO, JORGE GARRIDO, and NIBALDO C. INESTROSA

Laboratory of Neurophysiology, Group of Molecular Neurobiology, Department of Cell Biology, Catholic University of Chile, Santiago, Chile. Dr. Garrido's permanent address is the Laboratory of Histology, Catholic University of Chile, Santiago, Chile.

ABSTRACT Heparan sulfate and heparin, two sulfated glycosaminoglycans (GAGs), extracted collagen-tailed acetylcholinesterase (AChE) from the extracellular matrix (ECM) of the electric organ of *Discopyge tschudii*. The effect of heparan sulfate and heparin was abolished by protamine; other GAGs could not extract the esterase. The solubilization of the asymmetric AChE apparently occurs through the formation of a soluble AChE-GAG complex of 30S. Heparitinase treatment but not chondroitinase ABC treatment of the ECM released asymmetric AChE forms. This provides direct evidence for the in vivo interaction between asymmetric AChE and heparan sulfate residues of the ECM. Biochemical analysis of the electric organ ECM showed that sulfated GAGs bound to proteoglycans account for 5% of the total basal lamina. Approximately 20% of the total GAGs were susceptible to heparitinase or nitrous acid oxidation which degrades specifically heparan sulfates, and ~80% were susceptible to digestion with chondroitinase ABC, which degrades chondroitin-4 and -6 sulfates and dermatan sulfate. Our experiments provide evidence that asymmetric AChE and carbohydrate components of proteoglycans are associated in the ECM; they also indicate that a heparan sulfate proteoglycan is involved in the anchorage of the collagen-tailed AChE to the synaptic basal lamina.

Acetylcholinesterase (AChE)¹ is one of the major functional components of the neuromuscular junction of both muscle and the electric organ, and is responsible at least in part for terminating the actions of the neurotransmitter acetylcholine released from the presynaptic nerve terminals (1). AChE occurs in several molecular forms that can be separated by velocity sedimentation. These molecules have been shown to belong to two distinct structural classes: the asymmetric and the globular forms (2). The asymmetric forms are composed of tetramers of the globular subunits attached to a long, collagen-like tail (3, 4).

We are interested in the study of the interactions involved

¹Abbreviations used in this paper: AChE, acetylcholinesterase; ECM, extracellular matrix; GAGs, glycosaminoglycans.

in the attachment of asymmetric AChE to the synaptic extracellular matrix (ECM). AChE occupies <0.1% of the total area of the basal lamina at the nerve-muscle synapse, which suggests a high degree of specificity in its distribution. The factors involved in the AChE-ECM interaction are unknown. We have previously reported on the association of collagen-tailed AChE with ECM of cultured mouse muscle cells (5) and of the electric organ of *Electrophorus* and Torpedinidae fishes (6). The three most prevalent classes of molecules in the ECM are collagen type IV; glycoproteins, including laminin, entactin, nidogen, and fibronectin; and proteoglycans formed by complex sugars called glycosaminoglycans (GAGs) (7-11). Multiple interactions of ECM components could play a role in the deposition of asymmetric AChE. Accordingly,

the relationship of collagen-tailed AChE with various ECM components has been studied (12, 13), and conflicting evidence has been obtained for fibronectin, laminin, and collagen type V (14, 15). Moreover, because all the above studies have been done in a test tube with isolated macromolecules, the real significance of such results is unknown.

We have previously communicated that heparin, a sulfated GAG, specifically solubilizes the collagen-tailed AChE from homogenates of rat diaphragm muscle enriched in motor endplates (16). These results provide the first evidence for the in situ involvement of GAGs in the anchorage of the asymmetric AChE. Supporting evidence for the above contention comes from the fact that purified *Electrophorus* AChE binds to a heparan sulfate proteoglycan extracted from the murine Engelbreth-Holm-Swarm sarcoma (14). Here we have extended our studies to the ECM of the electric organ of the electric ray by (a) showing the specificity of the solubilizing effect of heparan sulfate and heparin on the collagen-tailed AChE, including the formation of a soluble complex between GAGs and the 16S AChE, (b) describing the solubilization of asymmetric AChE by heparitinase, and (c) characterizing the distribution of GAGs, including the identification of heparan sulfate as one of the carbohydrate components of proteoglycans of the electric organ ECM.

MATERIALS AND METHODS

The following chemicals were used: BW284c51 dibromide, the AChE inhibitor, was obtained from Burroughs Wellcome Co. (Triangle Park, NC). Hyaluronic acid from human umbilical cord, chondroitin sulfate from shark and whale cartilage, heparin from bovine lung, and porcine intestinal mucosa and chondroitinase ABC were obtained from Sigma Chemical Co. (St. Louis, MO). Heparan sulfate and heparitinase were purchased from Miles Laboratories Inc. (Elkhart, IN). Heparin-agarose resin was purchased from Bethesda Research Laboratories (Gaithersburg, MD). [³⁵S]Na₂SO₄ (100 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Other reagents, where not specified, were obtained from commercial sources. *Discopyge tschudii*, a Torpedinidae electric fish (6, 17), was caught in the Pacific Ocean near the port of San Antonio, Central Chile.

Preparation of ECM-like Material

Frozen electric organ (-80°C) was homogenized 1/3 (wt/vol) in 50 mM Tris-HCl, pH 8.4, containing protease inhibitors, 1 mM benzamidine hydrochloride, 20 U/ml aprotinin, 100 mM 6-aminohexanoic acid, and 5 mM EDTA. The homogenate was centrifuged at 15,000 g for 20 min. The precipitate was rehomogenized in 50 mM Tris HCl, pH 8.4, 0.5% Triton X-100, and proteinase inhibitors with constant stirring at 4°C for 10 min. This procedure was repeated four times, and the final insoluble precipitate ECM-like material was used for the experiments described in this paper.

Sedimentation Analysis of AChE

The molecular forms of AChE were separated on sucrose gradients as described previously (18, 19). In general, an extract obtained from frozen electric organ was prepared and clarified by centrifugation at 15,000 g for 15 min. Aliquots (100–200 μl) of the resulting supernatants were loaded on a 5–20% linear sucrose gradient made up in 50 mM Tris-HCl (pH 8.4) buffer with 0.5% Triton X-100. Sedimentation ultracentrifugation was performed in a Beckman LS-65 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) in a SW65 rotor at 215,000 g for 8 or 3 h at 4°C. Sedimentation coefficients for AChE forms were determined by comparison with that for β-galactosidase (16.1S). This enzyme was measured by the method of Craven et al. (20).

Affinity Chromatography of AChE in Heparin-Agarose

The heparin-agarose was poured as 0.2-ml columns in 1-ml pipette tips (0.4 × 2.0 cm) and washed with at least 10 vol of buffer containing 50 mM Tris-HCl (pH 7.4) and 0.2 M NaCl. Chromatography was done at room temperature.

After the enzyme (in 0.2 M NaCl) was applied to the column and washed with at least 10 vol of Tris-NaCl buffer, adsorbed material was eluted with 2 ml of 2 mg/ml heparin in 50 mM Tris-HCl at pH 7.4 containing 0.2 M NaCl.

AChE Assay

AChE activity was measured by the method of Ellman et al. (21). All determinations were done at 37°C in a 1 ml reaction mixture containing 100 mM sodium phosphate buffer (pH 7.0), 0.75 mM acetylthiocholine iodide, and 0.3 mM dithiobis-nitrobenzoic acid. BW284c51 dibromide (10 μM) was used as the AChE-specific inhibitor.

Release of AChE-ECM by GAG-degradating Enzymes

ECM (10 mg wet wt) resuspended in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM calcium acetate, 5 mM benzamidine, 20 U/ml aprotinin, 0.05 M 6-aminohexanoic acid, and 1 mg/ml bovine serum albumin was incubated with different amounts of *Flavobacterium heparinum* heparitin lyase (heparitinase) or chondroitinase ABC during 50 min at 37°C in a final volume of 100 μl. At the end of the incubation, the mixture was diluted with 0.4 ml of the same medium without ECM and enzymes. The AChE activity released by the enzymatic treatments was separated from the ECM by loading the mixture over a cushion of 0.5 ml of 45% sucrose. Then after centrifugation at maximal speed in a Eppendorf centrifuge for 10 min, 0.5 ml were taken from the top of the centrifuge tube, and the AChE activity was determined as described above. Control experiments showed that asymmetric AChE did not sediment at low ionic strength conditions through the sucrose cushion. Aliquots of the incubation medium in the presence or absence of heparitinase (1.5 U) were loaded on 5–20% sucrose density gradients and run as described previously.

Isolation of Sulfated GAGs by Alkaline Treatment

ECM-like material (50 mg dry wt) was homogenized in 0.1 M Tris-HCl pH 8.5 in the presence of 3% Triton X-100 with constant stirring at 4°C, and the homogenate was then centrifuged at 10,000 g for 15 min. The resulting pellet was resuspended in 0.01 M NaOH and 1 M NaBH₄ (22) and hydrolyzed at 45°C for 24 h. After the pH was neutralized with 5 M acetic acid and the solution was centrifuged at 10,000 g for 15 min, the free GAGs were precipitated from the supernatant with cold 70% ethanol. Samples were resuspended and treated with heparitinase, chondroitinase ABC, or nitrous acid as described below.

GAGs Digestion

GAGs digestion was as follows: (a) Treatment with chondroitinase ABC was performed in a buffer consisting of 50 mM Tris-HCl and 100 mM NaCl (pH 7.0). Digestion with 0.05 U/ml was carried out for 3–5 h at 37°C (23). (b) Heparan sulfate was cleaved by two methods: (i) digestion with 0.1 U/ml *Flavobacterium heparinum* heparitin sulfate lyase (heparitinase) in 50 mM Tris-HCl and 5 mM calcium acetate (pH 7.0) for 3–5 h at 39°C, and repeated for 1 h with fresh enzyme (24), or (ii) cleavage by nitrous acid (prepared by mixing equal volumes of 1 M sodium nitrite and 1 N HCl) for 3 h at room temperature (25, 26). Both the enzyme-digested samples and the nitrous acid-treated samples were lyophilized to dryness. GAGs and the products of the above reactions were separated by cellulose acetate electrophoresis or chromatography on Sepharose CL-6B.

Cellulose Acetate Electrophoresis of GAGs

Electrophoresis of individual GAG standards, ECM-GAGs or digested samples, was carried out at 4°C in 0.2 M calcium acetate (pH 7.0) at 5.0 mA for 2 h on cellulose acetate membranes in a Beckman Microzone Instrument. The position of the GAGs was then visualized by staining the electrophoretograms by immersion in an alcian blue solution (0.1% alcian blue in 0.1% acetic acid) for 5 min and destained with 10% acetic acid (27). The identity of the extracted GAGs was determined by comparing the position of the alcian blue staining to that of the known GAGs standards. For quantitative determination of the GAG, the strips were cleared, dried, and scanned on a Canalco densitometer.

Column Chromatography

Sepharose CL-6B gel filtration columns (100 × 1.4 cm) were prepared in and eluted with 0.2 M sodium acetate and 0.2 M sodium sulfate, pH 7.0 (28).

Their flow rates were ~0.1 ml/min. The void (V_0) and total (V_t) volumes of the columns were determined by eluting dextran blue-2,000 (for V_0) and [35 S]- Na_2SO_4 (for V_t). The content of uronic acid was determined by the modified carbazole reaction, with glucuronic acid as the standard (29).

RESULTS

Heparan Sulfate and Heparin Release Asymmetric AChE from the ECM as a 30S Complex

When the ECM-like fraction of *Discopyge* electric organ was resuspended in Tris-Triton buffer with and without heparan sulfate or heparin and the supernatants obtained after centrifugation were assayed for AChE, it was found that most of the residual enzyme activity was released in the sulfated GAGs-containing medium (Fig. 1). Hyaluronic acid and chondroitin sulfate did not solubilize any additional activity from the ECM. On the other hand, protamine, a polycationic agent that has been shown specifically to eliminate other effects of heparin (30), neutralized the solubilizing activity of both heparan sulfate and heparin (Table I). Both results suggest that a specific domain of heparan sulfate and heparin is responsible for the detachment of AChE from its anchoring site in the basal lamina. When the AChE activity released by the GAGs from the ECM was analyzed by sucrose gradient sedimentation, a major peak of asymmetric 16S form was observed (data not shown). Previous studies with rat muscle homogenates (16) and crude membrane fractions of *Discopyge* electric organ (17) have also indicated a preferential solubilization of asymmetric AChE forms, but knowing that a great proportion (20–50%) of the tailed AChE species is localized in intracellular compartments (31–34), it has been conceptually important to establish that the AChE bound to the ECM is also detached by sulfated GAGs.

All the above studies have been done with sucrose gradient

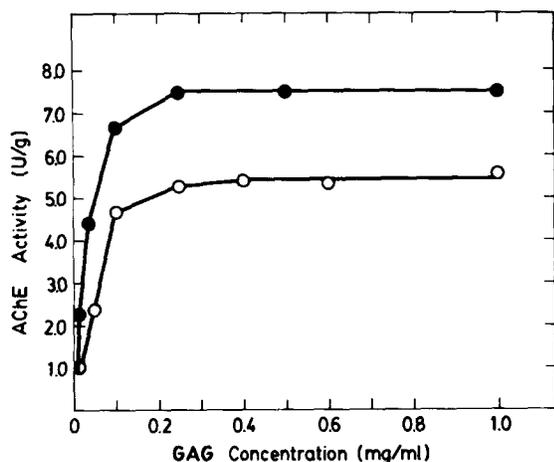


FIGURE 1 Solubilization of AChE activity from the electric organ of *Discopyge tschudii* by heparan sulfate and heparin. The ECM-like material was resuspended in 50 mM Tris-HCl, pH 8.4, plus 0.5% Triton X-100 and separated in aliquots. Heparin and heparan sulfate dissolved in Tris-Triton were added to the different samples to obtain the final concentration shown in the figure (●, heparin; ○, heparan sulfate). After homogenization, the different aliquots were centrifuged at 15,000 g during 20 min, and the AChE activity was determined in the supernatant. Each point represents the average of two different experiments run in triplicate. AChE activity is expressed as units per gram ECM, and 1 U corresponds to 1.0 μmol of substrate hydrolyzed in 1 min at 37°C.

TABLE I. Solubilization of AChE Activity from ECM-like Material of Electric Organ

Substance applied	AChE activity solubilized	Extraction
	$\mu\text{moles/min per g}$	%
Buffer alone	0.99 ± 0.13	0
+ Hyaluronic acid	1.03 ± 0.05	4
+ Chondroitin sulfate	1.09 ± 0.17	10
+ Heparin + protamine	1.03 ± 0.14	4
+ Heparan sulfate + protamine	0.96 ± 0.09	0
+ Heparan sulfate	$5.48 \pm 0.12^*$	453
+ Heparin	$7.30 \pm 0.13^*$	637

The ECM-like material was homogenized in 50 mM Tris-HCl (pH 8.4) and 0.5% Triton X-100 or in the same extraction solution plus: 2.0 mg/ml hyaluronic acid; 5 mg/ml chondroitin sulfate; 0.5 mg/ml of heparin plus 2.0 mg/ml of protamine; 0.5 mg/ml of heparan sulfate plus 2.0 mg/ml of protamine; 0.5 mg/ml of heparin sulfate; and 0.5 mg/ml of heparin. The homogenates were centrifuged at 20,000 g for 20 min. Aliquots of each of the supernatants were added to the assay containing 0.75 mM acetylthiocholine iodide and 0.1 mM iso-octamethyl pyrophosphoramidate. Assays were conducted at 37°C for 10–20 min. Activity values correspond to the mean \pm S.E.M. of five experiments. Percent extraction was calculated taking buffer-extracted activity as the 0% (second column).

* $P < 0.001$, as compared to control without heparin and without heparan sulfate.

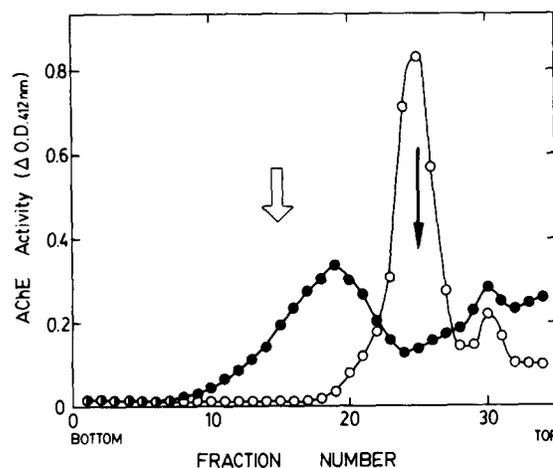


FIGURE 2 Sedimentation profile of GAG-induced AChE aggregates. 200 μl of a supernatant containing heparin prepared from ECM-like material was loaded in 5–20% sucrose gradient in the presence of heparin (2.0 mg/ml) with (○) or without (●) 1.0 M NaCl. Sedimentation was performed for 3 h at 46,000 rpm in a Beckman SW.65 rotor. Gradients were fractionated in 34 fractions and aliquots assayed for AChE activity by the method of Ellman et al. (21). The black arrow indicates the sedimentation position of β -galactosidase (16.1) assayed by the method of Craven et al. (20). The short open arrow indicates the sedimentation 80S of the low ionic strength aggregates obtained after dialysis of asymmetric enzyme extracted with 1.0 M NaCl.

prepared in 1 M NaCl. However, if the GAG-solubilized esterase is sedimented under low ionic strength conditions, the majority of the AChE activity sediments at the bottom of the gradient, suggesting the presence of an aggregate of the 16S AChE. To be certain that this was in fact the case, the GAG-extracted AChE activity was separated by sedimentation analysis with and without 1 M NaCl, but only for 3 h. Fig. 2 shows one of such experiments. In the low salt medium, it was observed that most of the esterase activity gave rise to polydisperse-aggregated components sedimenting around 30S (Fig. 2, closed circles), whereas at high ionic strength conditions, the 16S remained unaggregated (Fig. 2, open circles).

Note that the globular 6.5S form (fraction 30) remained unaggregated in both conditions. The data suggest that sulfated GAGs removed AChE from the ECM by a mechanism that involves the formation of soluble complex between the GAG and the asymmetric 16S esterase.

Binding of ECM-AChE to Sulfated GAG

To prove the direct interaction of *Discopyge* 16S AChE with the sulfated GAG, affinity chromatography in heparin-agarose was done. When a preparation of AChE containing a small amount of asymmetric forms and a higher proportion of globular forms (Fig. 3a) was dialyzed against low salt buffer, passed through a heparin-agarose column, and the eluate analyzed by sucrose gradient sedimentation, all the recovered

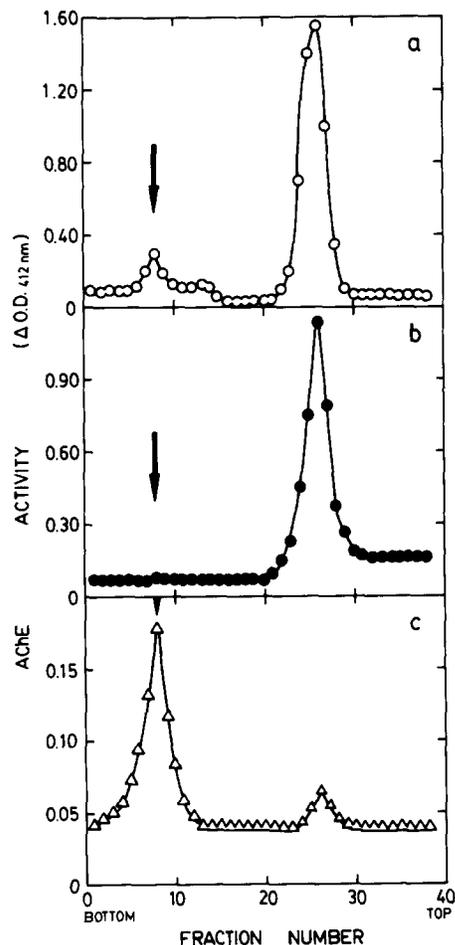


FIGURE 3 Evaluation of the specificity of the heparin-agarose column by sucrose gradient sedimentation of the AChE molecular forms. 1 g of *Discopyge* electric organ was homogenized in 50 mM Tris-HCl, pH 7.4, 1.0 M NaCl and centrifuged at 15,000 g per 20 min. The supernatant was dialyzed against Tris buffer during 4 h, resuspended in the same buffer plus 0.2 M NaCl, and then loaded on the heparin-agarose column equilibrated in 0.2 M NaCl, 5 mM EDTA, 20 U/ml aprotinin, 1 mM benzamidine, 0.1 mg/ml bacitracin, buffered with 50 mM Tris-HCl at pH 7.4. Both the passing through and the AChE activity eluted by heparin were recovered. Aliquots of the sample loaded in the column (a), the passing through (b), and the esterase activity specifically eluted by heparin (c) were sedimented in linear sucrose gradients containing 50 mM Tris-HCl, pH 7.4, 0.5% Triton and 1.0 M NaCl, for 8 h at 46,000 rpm, as described in Fig. 2. The arrows indicate the sedimentation position of β -galactosidase (16.1S).

AChE activity corresponded to globular forms of 6.5S (Fig. 3b). However, the activity specifically eluted by heparin mainly corresponded to the asymmetric 16S AChE form (Fig. 3c). The results support the idea that heparin binds to a specific site of the asymmetric AChE from the electric organ. Similar results have been observed with rat muscle AChE (35).

Heparitinase Solubilizes Asymmetric AChE from the ECM

An independent and more direct criterion, to establish the in situ interaction of AChE and the carbohydrate components of proteoglycans in the electric organ ECM, is the use of enzymes that degrade specific GAGs. In this context, we incubated ECM fractions with and without heparitinase (degrades specifically heparan sulfate) or chondroitinase ABC (degrades chondroitin-4 and -6 sulfates and dermatan sulfate) for 50 min at 37°C. After separation of the particulate ECM through a cushion of 45% sucrose, the AChE activity was determined. As Fig. 4A indicates, increasing amounts of heparitinase, but not chondroitinase ABC, were able to release esterase to the incubation medium. The ECM of *Discopyge* electric organ mainly consists of asymmetric AChE forms (A12 and A8) (17), therefore it is very likely that heparitinase releases these AChE species from the ECM. Fig. 4B clearly shows that in fact the heparan sulfate hydrolase liberated only collagen-tailed AChE forms. The above results provide direct evidence for the in vivo interaction between the asymmetric AChE and heparan sulfate proteoglycans present in the electric organ ECM.

Heparan Sulfate and Chondroitin Sulfate Proteoglycans Are Present in the ECM of the Electric Organ

To evaluate both the amount and the different types of proteoglycans present in the electric organ ECM, the ECM was treated with alkaline borohydride to release the single sulfated GAG chain present in the ECM-proteoglycans. The total amount of GAGs was determined by measuring the uronic acids: ~50 μ g of uronic acid was found per milligram (dry weight) of ECM-like material; consequently, ~5% of the electric organ ECM is considered to represent the carbohydrate component of proteoglycans.

Cellulose acetate electrophoresis of the isolated GAG fraction revealed the presence of two major spots (Fig. 5A). The migration of one of them corresponded to that of the heparan sulfate standard, and we will call this spot the HS peak, whereas the other spot corresponded to chondroitin-6-sulfate (CS peak). When alkaline hydrolyzates of whole ECM were treated with chondroitinase ABC before electrophoresis in the calcium acetate buffer system, the CS peak was no longer present, the other spot (HS peak) decreased ~50%, and the new HS peak was more symmetrical than was the original one (Fig. 5C). If samples were treated with nitrous acid, ~50% of the HS peak was removed (Fig. 5B) and the remaining HS peak seemed to migrate ahead of the original peak and appeared to correspond to the shoulder presented by the HS peak of the ECM sample. Finally, when the ECM-GAGs were first treated with chondroitinase ABC and then with nitrous acid, almost all the GAGs were digested (Fig. 5D). To establish the absolute amount of nitrous acid-sensitive material present

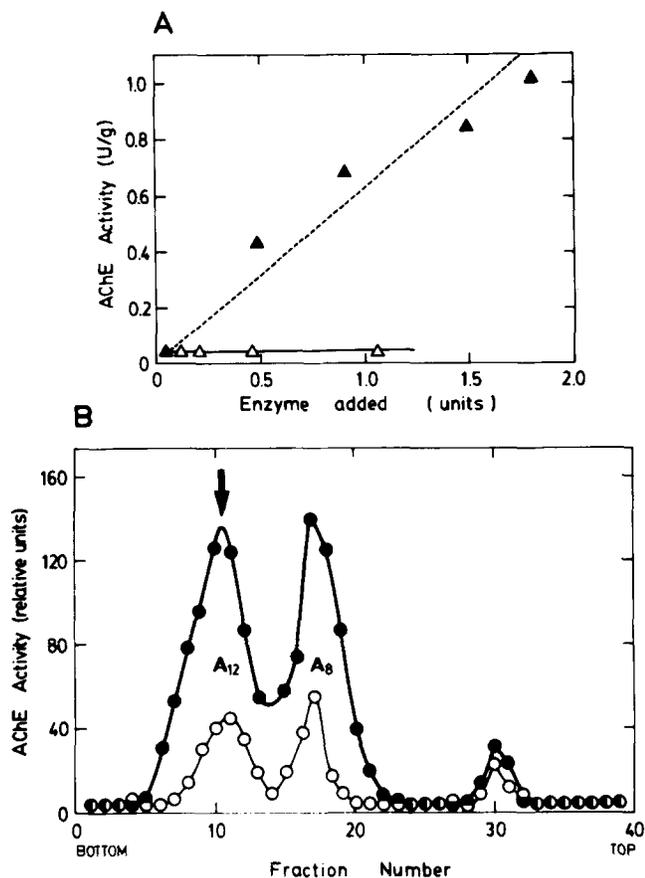


FIGURE 4 Release of asymmetric AChE forms bound to ECM of the electric organ by heparitinase. (A) The ECM was incubated with various amounts of purified heparitinase (\blacktriangle) or chondroitinase ABC (\triangle) for 50 min at 37°C. AChE activity released to the incubation medium was assayed as described in the Materials and Methods section. The values presented correspond to the difference between each treatment and the AChE released in the absence of the enzyme. (B) Sedimentation profile of the AChE activity released in 10 mM Tris-HCl pH 7.4, 100 mM NaCl buffer, with (\bullet) and without (\circ) 1.5 U of purified heparitinase. Only the asymmetric forms A12 and A8 were released by heparitinase. Note that due to the long incubation (50 min) at 37°C, the control sample also presents small amounts of both asymmetric AChE forms. The arrow indicates the sedimentation of β -galactosidase (16.1S).

in the ECM-GAGs, the intensity of the spots with and without nitrous acid treatment were measured in a densitometer for several different samples. Table II shows that 21% of the total GAGs disappeared after nitrous acid oxidation, indicating the presence of heparan sulfate within the ECM-GAGs.

Another way to characterize sulfated GAGs is by using gel filtration columns (26, 28). For these experiments, aliquots of the hydrolyzate samples were chromatographed on Sepharose CL-6B columns. Fig. 6A shows that two main classes of GAG chains with K_{av} of 0.3 and 0.53 were present in the electric organ ECM. Heparitinase treatment of the samples released ~18% of the total uronic acid as low molecular weight material that eluted close to the column total volume; this occurs with a concomitant decrease in the peak of $K_{av} = 0.53$ (Fig. 6B). This result also indicates the presence of heparan sulfate in the ECM of *Discospyge* electric organ, by a criterion independent of the nitrous acid oxidation. Chondroitinase ABC treatment of the sample degraded uronic acid-containing material (75%), and the fragments eluted at a K_{av} of 0.96 close

to the column total volume; this occurred with the concomitant disappearance in the other main peak of ECM-GAGs located at a K_{av} of 0.3 (Fig. 6C). In either case, there was no detectable change in the elution profile of the resistant peaks.

To summarize, we have reliably identified the presence of heparan sulfate in the ECM of the electric organ because (a) a specific spot of ECM-GAGs has the same electrophoretic mobility than does the heparan sulfate standard in the calcium acetate system, (b) 20% of the ECM-GAGs disappear after nitrous acid oxidation, (c) heparitinase treatment of the ECM-GAGs destroys 18% of the total GAGs shown by chromatog-

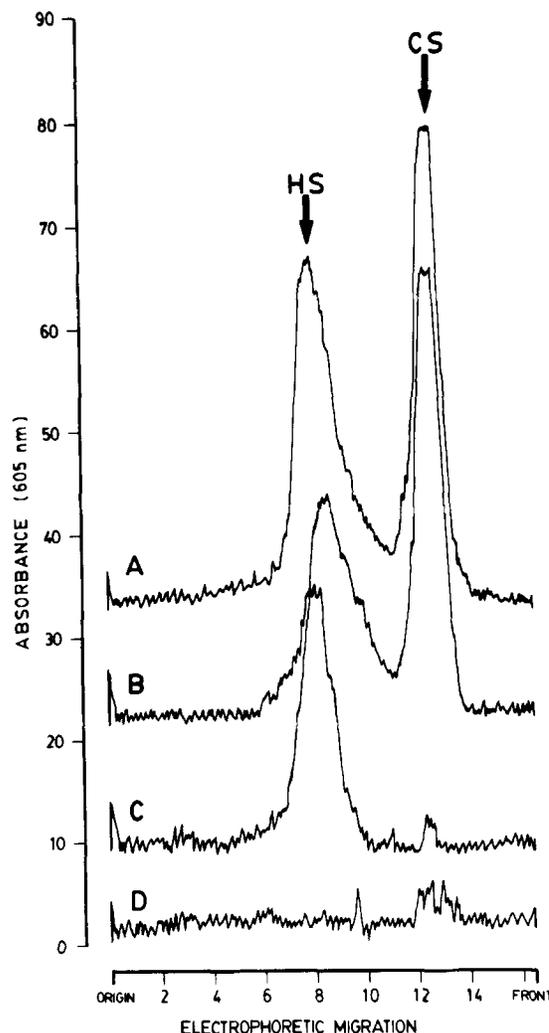


FIGURE 5 Gel scans of GAGs components isolated from the ECM of *Discospyge* electric organ after cellulose acetate electrophoresis. GAGs obtained from ECM-like material by alkaline-borohydride treatment were submitted to electrophoresis in 0.2 M calcium acetate (pH 7.0) at 5 mA per strip for 2 h at 4°C. Samples equivalent to 100 μ g of heparin expressed as uronic acids were visualized by staining with alcian blue. Reference standards were hyaluronic acid (which stays at the origin), and heparan sulfate (HS) and chondroitin-6-sulfate (CS), both indicated in the figure. The gel was cleared and scanned at 605 nm on a Canalco integrating scanner. (A) GAGs from the ECM fraction. (B) Sample after treatment with nitrous acid. (C) Sample digested with chondroitinase ABC. (D) Sample digested with chondroitinase ABC plus nitrous acid treatment. Two peaks were found; one of them co-migrates with heparan sulfate and the other with chondroitin-6-sulfate. Nitrous acid treatment removed ~40–50% of the HS peak, whereas chondroitinase ABC digestion removed 100% of the CS peak and ~50% of the HS peak.

TABLE II. Sensitivity of ECM-GAGs and Heparan Sulfate Standard to Nitrous Acid Treatment

Treatment	ECM-GAGs		HS 100 μ g
	HS peak	CS peak	
Control	50.1 \pm 4.8	49.9 \pm 4.8	100
Nitrous acid	29.3 \pm 1.5	51.2 \pm 4.9	3

The ECM-GAGs sample is equivalent to 100 μ g of heparan expressed as uronic acids. The value for heparan sulfate (HS) and chondroitin sulfate (CS) peaks in the control is the mean \pm S.D. of five different samples. The values for nitrous acid oxidation is the mean \pm S.D. of four different samples. The nitrous acid treatment consisted of incubation of equal volumes of 1.0 M nitrous acid (made fresh as described in Material and Methods) with ECM-GAGs, for 3 h at room temperature. Either the control or the nitrous acid-treated samples were lyophilized to dryness. The samples were separated in cellulose acetate electrophoresis in 0.2 M calcium acetate buffer pH 7.0 at 4°C. For quantitative determination of the GAGs, the strips were cleared, dried, and scanned on a Canalco densitometer.

raphy on Sepharose CL-6B, and (d) the material sensitive to nitrous acid or heparitinase treatment is unaffected by chondroitinase ABC. We have also demonstrated the existence of chondroitin sulfate-type proteoglycans: in fact, of the total sulfated GAGs present in the electric organ ECM, 80% by electrophoretic analyses and 75% by chromatography were sensitive to chondroitinase ABC and insensitive to nitrous acid or heparitinase treatments. Moreover, by its migration in the calcium acetate system, the CS peak seems to be entirely of the 6-sulfate type (50%). The nitrous acid-resistant GAGs of the HS peak, because of its sensitivity to chondroitinase ABC, probably correspond to dermatan sulfate (30%), but further studies are necessary to establish this point.

DISCUSSION

Our findings indicate that the collagen-tailed AChE of the electric organ of *Discopyge* is associated with the ECM via heparan sulfate proteoglycans. Evidence has been presented showing that sulfated GAGs promoted the release of the asymmetric AChE from purified basal lamina fractions. This result could be obtained if heparan sulfate or heparin competed with components of the ECM for binding sites on the collagenous tail of the asymmetric AChE. This model requires the formation of a GAG-enzyme complex and therefore, the existence of a direct binding of the GAG to AChE. Our results are consistent with the above possibility; in fact, we have obtained clear evidence for the existence of a 30S aggregate and for specific binding of the collagen-tailed AChE to a heparin-agarose resin.

Since the sulfated GAG heparin is absent in tissues free of mast cells (36) and is normally an intracellular compound (10), the closely related component, heparan sulfate, may be the molecule involved in the attachment of AChE to the ECM. Since sulfated GAGs are normally associated to a protein core (37), it is very likely that the cell surface heparan sulfate proteoglycans are the factors responsible for the anchorage of AChE. In this context, our results showing that heparitinase, an enzyme that degrades specifically heparan sulfate, releases asymmetric AChE from the ECM and that heparan sulfate is one of the carbohydrate components of the electric organ ECM proteoglycans, are entirely consistent with our earlier suggestion that heparan sulfate proteoglycans are involved in the attachment of the collagen-tailed AChE to the ECM of the neuromuscular junction (16).

Previous studies with muscle cells and electric organ have

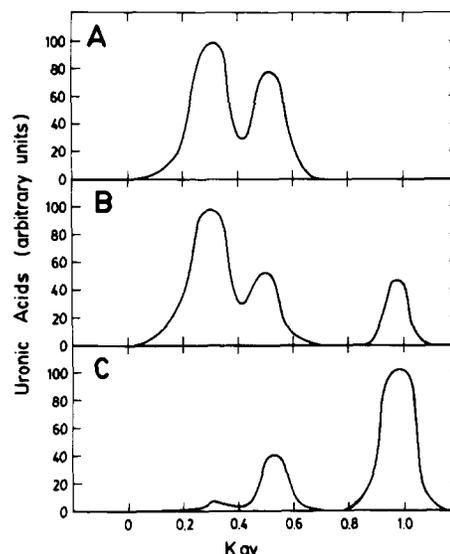


FIGURE 6 Sepharose-CL-6B elution profile of alkaline borohydride-treated ECM from electric organ. ECM-like material was hydrolyzed in the presence of 0.01 M NaOH and 1.0 M NaBH₄ during 24 h at 45°C. Thereafter, the solution was neutralized, and the free GAGs were precipitated with cold 70% ethanol. Aliquots of the GAGs were either directly eluted in a Sepharose CL-6B column (A), eluted after heparitinase treatment (B), or after chondroitinase ABC digestion (C). Treatments with heparitinase and chondroitinase ABC released ~18% and ~75% of the total uronic acid to the total column volume. Heparitinase treatment produces a decrease in the peak of $K_{av} = 0.53$, whereas chondroitinase ABC digestion produces a clear decrease in the peak of $K_{av} = 0.3$.

shown that the asymmetric 16S AChE is associated with the ECM through its collagen-like tail (5, 6, 38). It has also been suggested that the aggregation of the asymmetric AChE at low ionic strength could be related to the immobilization of the molecules at the basal lamina (2). In the electric organ of *Electrophorus*, the aggregation depends on a factor related to a GAG of the chondroitin sulfate type, which formed an 85S complex with AChE (13). However, in mouse myotube cultures (5) as well as in the ECM of *Discopyge* electric organ (this work), no substantial release of AChE was obtained after treatment with chondroitinase ABC. We have shown here that sulfated GAGs release asymmetric AChE not as individual 16S molecules, but as multimolecular aggregates (30S) of such molecules. In this context, Bon et al. (13) have shown that the sedimentation properties of the AChE aggregates depend on the particular aggregating agent used. In fact, when *Electrophorus* AChE interacts with heparin, it forms a complex of 100S, clearly different from the one observed in our experiments. However, when the so-called *Electrophorus* aggregating agent of Bon et al. (13) was added to purified *Torpedo* enzyme, the esterase forms aggregates with a sedimentation coefficient of 30S, suggesting that the size of AChE aggregates depends not only on the aggregating agent, but also on the nature of the enzyme used.

Regarding the mechanism of how the asymmetric AChE form, a molecule assembled intracellularly (33, 34, 39, 40) in the Golgi apparatus (34), is incorporated into an insoluble ECM, one possibility could be the conversion of a soluble secreted macromolecule to a complex array of AChE molecules (i.e., the AChE aggregates). Such a conversion would probably require another ECM component that would cata-

lyze the insolubilization process. One candidate for such a role could be a heparan sulfate proteoglycan present on the cell surface. In this context, it has been shown that a conversion of soluble fibronectin to fibrillar aggregates is catalyzed by heparin, which also enhances the binding of soluble fibronectin to the cell surface (41).

Heparan sulfate proteoglycans are components of basal lamina or ECM, for example, of the glomerulus (27), mammary epithelium (42), and that of a basement membrane producing tumor (43). In all of the above tissues, the heparan sulfates are the main component of the ECM, comprising ~70% of the total proteoglycans present. This does not seem to be the case for the electric organ ECM, in which only 20% of the total sulfated carbohydrate components of proteoglycans correspond to heparan sulfate. Our studies on the chemical composition of the electric organ ECM showed the presence of an important amount (80%) of a chondroitinase ABC-sensitive material, which could be either chondroitin-4-sulfate, chondroitin-6-sulfate, or dermatan sulfate. Most of this sensitive ECM material migrated as chondroitin-6-sulfate (70%); in this context, preliminary evidence from collaborative studies with Dr. C. P. Dietrich and co-workers also indicates the presence of chondroitin-6-sulfate proteoglycans in the ECM of the electric organ. So far, only a small amount of uncharacterized chondroitin sulfate proteoglycans have been observed in the glomerular basement membranes (26, 27). It seems, therefore, that the ECM of the electric organ has a unique biochemical composition in terms of the carbohydrate complement of its proteoglycans.

Several interactions of matrix components could play a role in the deposition of an insoluble ECM (44-46), and the association of the isolated tailed AChE with various ECM components has been studied. Binding occurs with chondroitin sulfate (13), fibronectin (12, 14), laminin (14), collagen type V (14), and heparan sulfate proteoglycan (14). It must be taken into account, however, that Grassi et al. (15) have been unable to find any binding at all of the collagen-tailed AChE with fibronectin, laminin, and collagen type V. The binding to heparan sulfate proteoglycan was not, however, studied. We consider that: (a) heparan sulfate and heparin solubilize asymmetric AChE from the ECM of the electric organ (present work); (b) the asymmetric AChE binds to heparin-agarose ([35]; this work) and to heparan sulfate proteoglycans covalently bound to agarose (14); (c) heparitinase releases the collagen-tailed AChE from the electric organ ECM (present work); (d) the existence of heparan sulfate as a carbohydrate component of the electric organ ECM proteoglycans (this work); and (e) a specific, saturable and reversible binding of asymmetric AChE to a single class of binding sites on cell surfaces rich in heparan sulfate proteoglycans ([47]; Inestrosa, N. C., and Z. W. Hall, unpublished results), it appears very likely that the anchorage of collagen-tailed AChE to the synaptic ECM is mediated by heparan sulfate proteoglycans.

We thank Drs. I. Yamashina (Kyoto University, Sakyo-ku, Japan) and Robert D. Rosenberg (Massachusetts Institute of Technology, Cambridge, MA) for a sample of heparitinase.

This investigation was supported by the Dirección de Investigaciones de la Universidad Católica de Chile (Grant No. 80/84), the Fondo Nacional de Ciencias de Chile (Grant No. 1015/85), and by a grant from the Fundación Gildemeister to Dr. Inestrosa. Additional funding was provided by a Graduate Student Research Grant from the Dirección de Investigaciones de la Universidad Católica de Chile

and a grant from United Nations Development Programme-United Nations Educational, Scientific, and Cultural Organization, both to E. Brandan.

Received for publication 14 December 1984, and in revised form 14 February 1985.

REFERENCES

- Kuffler, S. W., and J. G. Nicholls. 1979. From Neuron to Brain. Sinauer Associates, Inc., Sunderland, MA.
- Massoulié, J., and S. Bon. 1982. The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Annu. Rev. Neurosci.* 5:57-106.
- Bon, S., M. Vigny, and J. Massoulié. 1979. Asymmetric and globular forms of acetylcholinesterase in mammals and birds. *Proc. Natl. Acad. Sci. USA.* 76:2546-2550.
- Mays, C., and T. L. Rosenberry. 1981. Characterization of pepsin-resistant collagen-like tail subunit fragments of 18S and 14S acetylcholinesterase from *Electrophorus electricus*. *Biochemistry.* 20:2810-2817.
- Inestrosa, N. C., L. Silberstein, and Z. W. Hall. 1982. Association of the synaptic form of acetylcholinesterase with extracellular matrix in cultured mouse muscle cells. *Cell.* 29:71-79.
- Inestrosa, N. C., and B. Méndez. 1983. The A12 acetylcholinesterase and polypeptide composition of electric organ basal lamina of *Electrophorus* and some Torpedinidae fishes. *Cell Biochem. Funct.* 1:41-48.
- Hay, E. D. 1981. Extracellular matrix. *J. Cell Biol.* 91:2055-2235.
- Hynes, R. O., and K. M. Yamada. 1982. Fibronectins: multifunctional modular glycoproteins. *J. Cell Biol.* 95:369-377.
- Kleinman, H. K., R. J. Klebe, and G. R. Martin. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88:473-485.
- Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 52:761-799.
- Timpl, R., M. Dziadek, S. Fujiwara, H. Nowack, and S. Wick. 1983. Nidogen: a new, self-aggregating basement membrane protein. *Eur. J. Biochem.* 137:455-465.
- Emmerling, M. R., C. D. Johnson, D. F. Mosher, B. H. Lipton, and J. E. Litten. 1981. Cross-linking and binding of fibronectin with asymmetric acetylcholinesterase. *Biochemistry.* 20:3242-3247.
- Bon, S., J. Cartaud, and J. Massoulié. 1978. The dependence of acetylcholinesterase aggregation at low ionic strength upon a polyanionic component. *Eur. J. Biochem.* 20:1-14.
- Vigny, M., G. R. Martin, and G. R. Grotendorst. 1983. Interactions of asymmetric forms of acetylcholinesterase with basement membrane components. *J. Biol. Chem.* 258:8794-8798.
- Grassi, J., J. Massoulié, and R. Timpl. 1983. Relationship of collagen-tailed acetylcholinesterase with basal lamina components. Absence of binding with laminin, fibronectin and collagen types IV and V and lack of reactivity with different anti-collagen sera. *Eur. J. Biochem.* 133:31-38.
- Torres, J. C., and N. C. Inestrosa. 1983. Heparin solubilizes asymmetric acetylcholinesterase from rat neuromuscular junction. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 154:265-268.
- Méndez, B., J. Garrido, M. Maldonado, F. Jaksic, and N. C. Inestrosa. 1984. The electric organ of *Discopyge tschudii*: its innervated face and the biology of acetylcholinesterase. *Cell. Mol. Neurobiol.* 4:125-142.
- Inestrosa, N. C., B. Méndez, and J. V. Luco. 1979. Acetylcholinesterase like that of skeletal muscle in smooth muscle reinnervated by a motor nerve. *Nature (Lond.)* 280:504-506.
- Inestrosa, N. C., C. G. Reiness, L. F. Reichardt, and Z. W. Hall. 1981. Cellular localization of the molecular forms of acetylcholinesterase in rat pheochromocytoma PC12 cells treated with nerve growth factor. *J. Neurosci.* 1:1260-1267.
- Craven, G. R., E. Steers Jr., and C. B. Anfinsen. 1965. Purification composition and molecular weight of the β -galactosidase of *Escherichia coli* K12. *J. Biol. Chem.* 240:2468-2477.
- Ellman, G. L., K. D. Courtney, V. Andres, and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7:88-95.
- Carlson, D. M. 1968. Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins. *J. Biol. Chem.* 243:616-626.
- Saito, H., T. Yamata, and S. Suzuki. 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *J. Biol. Chem.* 243:1536-1542.
- Rapraeger, A., E. Endo, J. Koda, and M. Bernfield. 1985. The cell surface proteoglycan from mouse mammary epithelial cells bears chondroitin sulfate and heparan sulfate glycosaminoglycans. *J. Biol. Chem.* In press.
- Cifonelli, J. A., and J. King. 1972. Distribution of 2-acetoamido-2-deoxy-D-glucose residues in mammalian heparins. *Carbohydr. Res.* 21:173-186.
- Kanwar, Y. S., V. C. Hascall, and M. G. Farquhar. 1981. Partial characterization of newly synthesized proteoglycans isolated from the glomerular basement membrane. *J. Cell Biol.* 90:527-532.
- Kanwar, Y. S., and M. G. Farquhar. 1979. Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes. *Proc. Natl. Acad. Sci. USA.* 76:4493-4497.
- Yanagishita, M., and V. C. Hascall. 1983. Characterization of low buoyant density dermatan sulfate proteoglycans synthesized by rat ovarian granulosa cells in culture. *J. Biol. Chem.* 258:12847-12856.
- Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4:330-334.
- Ehrlich, J., and S. S. Stivala. 1973. Chemistry and pharmacology of heparin. *J. Pharm. Sci.* 62:517-544.
- Younkin, S. G., C. Rosenstein, P. L. Collins, and T. L. Rosenberry. 1982. Cellular localization of the molecular forms of acetylcholinesterase in rat diaphragm. *J. Biol. Chem.* 257:13630-13637.
- Fernández, H. L., N. C. Inestrosa, and J. R. Stiles. 1984. Subcellular localization of acetylcholinesterase molecular forms in endplate regions of adult mammalian skeletal muscle. *Neurochem. Res.* 9:1211-1230.
- Inestrosa, N. C. 1984. 16S Acetylcholinesterase of the extracellular matrix is assembled within mouse muscle cells. *Biochem. J.* 217:377-381.

34. Rotundo, R. L. 1984. Asymmetric acetylcholinesterase is assembled in the Golgi apparatus. *Proc. Natl. Acad. Sci. USA.* 81:479-483.
35. Brandan, E., and N. C. Inestrosa. 1984. Binding of the asymmetric forms of acetylcholinesterase to heparin. *Biochem. J.* 221:415-422.
36. Straus, A. H., H. B. Nader, and C. P. Dietrich. 1982. Absence of heparin-like compounds in mast-cell-free tissue and animals. *Biochim. Biophys. Acta.* 717:478-485.
37. Höök, M., L. Kjellén, S. Johansson, and J. Robinson. 1984. Cell-surface glycosaminoglycans. *Annu. Rev. Biochem.* 53:847-869.
38. Lwebuga-Mukasa, J. S., S. Lappi, and P. Taylor. 1976. Molecular forms of acetylcholinesterase from *Torpedo californica*: their relationship to synaptic membranes. *Biochemistry.* 15:1425-1434.
39. Fernández, H. L., and J. R. Stiles. 1984. Intra-versus extracellular recovery of 16S acetylcholinesterase following organophosphate inactivation in the rat. *Neurosci. Lett.* 49:117-122.
40. Inestrosa, N. C., W. D. Matthew, C. G. Reiness, Z. W. Hall, and L. F. Reichardt. 1985. Atypical distribution of asymmetric acetylcholinesterase in mutant PC12 pheochromocytoma cells lacking a cell surface heparan sulfate proteoglycan. *J. Neurochem.* 45. In press.
41. Jilek, F., and H. Hörmann. 1979. Fibronectin (cold insoluble globulin). VI. Influence of heparin and hyaluronic acid on the binding to native collagen. *Hoppe-Seyler's Z. Physiol. Chem.* 360:597-603.
42. Gordon, J. R., and M. Bernfield. 1980. The basal lamina of the post-natal mammary epithelium contains glycosaminoglycans in a precise ultrastructural organization. *Dev. Biol.* 74:118-135.
43. Hassell, J. R., P. G. Robey, H. J. Barrach, J. Wilczek, S. I. Rennard, and G. R. Martin. 1980. Isolation of a heparan sulfate containing proteoglycan from basement membrane. *Proc. Natl. Acad. Sci. USA.* 77:4494-4498.
44. Perkins, M. E., T. H. Ji, and R. O. Hynes. 1979. Cross-linking of fibronectin to sulfated proteoglycans at cell surface. *Cell.* 16:941-952.
45. Ruoslahti, E., and E. Engvall. 1980. Complexing of fibronectin, glycosaminoglycans and collagen. *Biochim. Biophys. Acta.* 631:350-358.
46. Kleinmam, H. K., M. L. McGarvey, J. R. Hassell, and G. R. Martin. 1983. Formation of a supramolecular complex is involved in the reconstitution of basement membrane components. *Biochemistry.* 22:4969-4974.
47. Brandan, E., and N. C. Inestrosa. 1985. The synaptic form of acetylcholinesterase interacts with cell surface heparan sulfate proteoglycans. *J. Neurosci. Res.* In press.