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Disruption of Proteoglycans in Neural Tube Fluid by β-D-Xyloside Alters Brain Enlargement in Chick Embryos

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ABSTRACT

Following neurulation, the anterior end of the neural tube undergoes a dramatic increase in size due mainly to the enlarging of the brain cavity. This cavity is filled with so-called neural tube fluid (NTF), whose positive pressure has been shown to play a key role in brain morphogenesis. This fluid contains a water-soluble matrix, rich in chondroitin sulfate (CS), which has been proposed as an osmotic regulator of NTF pressure genesis. The purpose of the present study is to observe the influence of CS on NTF osmolality and its relation to NTF hydrostatic pressure and brain expansion.

NTF was obtained by means of microaspiration from the mesencephalic cavity of chick embryos. The osmolality of NTF between H.H. stages 20 and 29 was measured on the basis of its cryoscopic point. CS synthesis was disrupted by using β-D-xyloside and the induced variations in brain volume were measured by means of morphometry. We also measured the variations in NTF osmolality, hydrostatic pressure, and the concentration of CS and sodium induced by means of β-D-xyloside.

Our data reveal that, at the earliest stages of development analyzed, variations in NTF osmolality show a characteristic pattern that coincides with the developmental changes in the previously described fluid pressure.

Chick embryos treated with β-D-xyloside, a chemical that disrupts CS synthesis, displayed a notable increase in brain volume but no other apparent developmental alterations. Morphometric analysis revealed that this increase was due to hyperenlargement of the brain cavity. β-D-xyloside brings about specific changes in the biochemical composition of NTF, which entails a large increase in CS concentration, mainly in the form of free chains, and in that of sodium. As a result, the fluid's osmolality and brain intraluminal pressure increased, which could account for the increase in size of the brain anlage.

These data support the hypothesis that the intraluminal pressure involved in embryonic brain enlargement is directly dependent on NTF osmolality, and that the concentrations of CS and its associated microions could play a key role in the regulation of this process. Anat. Rec. 252:499–508, 1998.

Key words: neural tube; brain development; chondroitin sulfate; β-D-xyloside; chick embryo

The anterior end of the neural tube, the future brain anlage, undergoes a considerable increase in volume at the earliest stages of development, with a significant difference emerging between the spinal cord and the future encephalon. The mechanisms involved in this important morphogenetic process remain largely unknown to date.

In the last decades, many efforts have been made in order to quantify the rate of brain enlargement at these particular stages of development. In chick embryos, Pa-

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checo et al. (1986) described an initial period of moderate brain growth from H.H. stages 11-18 (Hamburger and Hamilton, 1951), followed by a period of rapid growth between stages 19 and 26. In this final period, and in only 48 h, the embryonic brain undergoes a 30-fold size increase, >70% of this being accounted for by growth of the brain cavity (Desmond and Jacobson, 1977). The explosive growth of the embryonic brain cavity has been directly related to the expanding force generated by the positive pressure of the intraluminal fluid described by Jelinek and Pexieder (1968). Such pressure requires the previous occlusion of the spinal cord lumen, transforming the brain cavity into a physiologically sealed system (Schoenwolf and Desmond, 1984; Desmond et al., 1993). The particular role, based on physical phenomena, that these morphogenetic mechanisms can play in the developmental pattern of various embryonic anlages was summarized by Newman and Comper (1990). In fact, expanding forces based on intraluminal hydraulic mechanisms have been involved in the growth and development of other anlages besides the embryonic brain, such as the otic vesicle (Barbosa et al., 1986) and the eye (Coulombre, 1956). An experimental demonstration that the positive pressure exerted by NTF confined in the brain cavity is a key factor in embryonic brain expansion and morphogenesis was provided by Desmond and Jacobson (1977) and Desmond (1985). They induced an experimental reduction in this pressure in chick embryos, which led to severe dysmorphogenesis and brain collapse.

The composition and function of the fluid contained in the embryonic brain cavity, and their possible influence on early brain development, require more research (Jacobson, 1991). In a previous report (Gato et al., 1993), we suggested that the osmotic pressure of NTF could play a key role in the genesis of this expanding force and demonstrated the presence of a water-soluble matrix rich in the proteoglycan chondroitin sulfate (CS) in this fluid.

Given the special osmotic properties of the proteoglycans (PGs) (Comper and Laurent, 1978), we hypothesized that the osmotic pressure of NTF could be, at least in part, regulated by its CS concentration.

In order to clarify the mechanisms leading to the genesis of pressure and its regulation, we measured the osmolality of NTF in chick embryos from H.H. stages 20 to 29. In order to analyze the relation between the CS concentration in NTF, the osmolality of this fluid and embryonic brain expansion, we disrupted the synthesis of CS in the NTF with β-D xyloside, a chemical that selectively disrupts the synthesis of sulfated PGs (Schwartz et al., 1976).

β-D-xylosides have been widely employed in the study of the roles played by sulfated PGs (particularly CS) at different embryonic development stages in the rat (Morriss-Kay and Crutch, 1982), chicken (Gibson et al., 1978,1979; Kanke et al., 1982; Segen and Gibson, 1982), sea urchin (Kinoshita and Saiga, 1979), and frog (Yost, 1990). β-D-xylosides specifically alter the synthesis of those sulfated PGs whose glycosilation begins with the sequence xylosyl-galactosyl-galactosyl, including chondroitin sulfate and its epimeres dermatan sulfate, heparan sulfate, and heparin (Schwartz et al., 1974; Robinson et al., 1975; Roden, 1980). However, p-nitrophenyl-β-D-xylopyranoside especially disrupts chondroitin and dermatan sulfate synthesis. Several studies have shown its incapability of priming the heparan sulfate and/or heparin synthesis in embryonic tissues (Sobue et al., 1987; Lugemwa and Esko, 1991), or in tissue cultures from different sources (Galligani et al., 1975; Spooncer et al., 1983). The data from the present study indicate that CS is the only sulfated proteoglycan in chick embryo NTF. Therefore, we can assume that the changes in NTF physicochemical properties induced by means of β-D-xyloside are fundamentally due to a disruption in CS synthesis.

MATERIALS AND METHODS

Fertile White Leghorn eggs were incubated at 38°C in a humidified atmosphere to obtain chick embryos at different developmental stages, ranging from H.H. 20 to 29 (Hamburger and Hamilton, 1951). After incubation, a small window was opened in the shell and thereafter the embryos were classified according to stage. For β-D-xyloside treatment, doses of 24 µl of p-nitrophenyl-β-D-xylopyranoside 4 mM (Sigma, St. Louis, MO) were injected with a Hamilton microsyringe into the subgerminal space of H.H. stage 12–13 embryos; the control group was injected with sterile saline solution. Afterward, the opening in the shell was sealed and the eggs were reincubated for 44 h until the embryos reached H.H. stage 23.

Morphometric Analysis

The method used to determine brain volume is very time- consuming; therefore, the number of embryos to be measured was limited to 10 control and 10 β-D-xyloside-treated embryos. After fixation in Bouin's solution for 6 h, embryos were dehydratated in graded ethanol and embedded in paraplast; 8 µm transverse serial sections were cut and stained with haematoxylin-eosin. The volume was calculated from the rostral extent of the brain until the first section in which the otic vesicles appeared, including forebrain, midbrain, and part of the hindbrain. The inner and outer limits of the neuroepithelium were drawn in one of every five sections (section factor = 5) using a Leitz SM Lux microscope equipped with a drawing tube (total magnification 60X), and the corresponding areas integrated with a Videoplan (Kontrol Elektronic GMBH) computerized image-analysis system, via the VIDAS 2.1. stereology program. In each embryo, the volume of the brain, of its cavity, and that of the neuroepithelial wall were obtained by adding the corresponding sectional areas (in μm^2) multiplied by 8 µm (thickness) and by 5 (section factor). According to Desmond and Jacobson (1977) and Pacheco et al. (1986), the measurement of every fifth section differs <10% from the measurement of every section in H.H. stage 23 chick embryos, and this error can be regarded as acceptable. The final volumes are expressed as the arithmetic mean of the values of the 10 embryos +/- standard error. For comparison of the average volumes (total, cavity, and tissue) between experimental and control embryos, two-tailed Student's t-test for independent samples was applied and P values <0.05 were interpreted as indicating statistical significance.

Electron Microscopy

After in ovo treatment, the extraembryonic membranes were excised and both control and treated embryos rinsed in saline solution. The primary brain vesicle regions were sectioned from the embryonic heads with thin tungsten needles, under dissecting microscope control, in a Silgar-coated Petri dish. The brain segments were rinsed again in saline solution, fixed overnight in Karnovsky fixative

containing 2% tannic acid at room temperature, following the method of Singley and Solursh (1980), and then postfixed in 1% osmium tetroxide for 1 h at room temperature. The samples were dehydrated in a graded acetone series and embedded in plastic (Spurr, 1969). Ultrathin sections from the different brain vesicles were cut on an AMC ultra-microtome, MT 6000 XL, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined under a Jeol 1200 EX electron microscope at 80 Kv.

Neural Tube Fluid Acquisition

NTF for osmolality determination and biochemical analysis was obtained as follows. The extraembryonic membranes were removed, the embryos were rinsed twice in sterile saline solution and placed in a dry Petri dish, and the surrounding fluid was aspirated. A micropipette, connected to a microaspirator (Medical System PLI 100), was carefully placed in the middle of the mesencephalic cavity under dissecting microscope control, avoiding contact with the neuroepithelial wall. The NTF of several embryos was slowly aspirated, until a sufficient amount was obtained for osmolality determinations (200 µl for each H.H. stage between 20 and 29) and biochemical analysis (300 µl from both control and treated H.H. stage 23 chick embryos). NTF was aliquoted and frozen at -40°C until it was to be used.

NTF Osmolality Determinations

Measurements of NTF osmolality were made on the basis of its cryoscopic point with an Osmomat 050 (Gonotec). Since a minimum of 50 ul were necessary for accurate assessment, a pool (200 µl) of NTF was made with the NTF obtained from embryos of each H.H. stage between 20 and 28, and another pool (200 µl) from control and β-D-xyloside treated H.H. stage 23 chick embryos. Four different determinations of NTF osmolality were performed in all cases. Mean and standard errors were calculated for each developmental stage and the nonparametric two-tailed Mann-Whitney U test was used to analyse the differences in osmolality among stages. Probability (P) values <0.05 were interpreted as indicating statistical significance.

NTF Pressure Determinations

To measure the intralumen pressure of brain anlage, live embryos were placed in a Petri dish, as described in the section on NTF acquisition, and a micropipette of 30 µm (inner diameter) connected to a microaspirator was carefully placed in the rhombencephalic cavity. Positive pressure of the lumen caused NTF to move into the micropipette. By offsetting this pressure, using the instrument balancing pressure system, we estimated intraluminal pressure as that pressure necessary to make the NTF return to the brain cavity. Only measurements that did not show NTF leakage were valued. A total of 13 measurements for control embryos and 14 for treated ones were made on H.H. 21 stage embryos. Mean and standard errors were calculated, and the two-tailed Student's test for independent samples was applied in order to compare the average pressures of experimental embryos with those of control embryos, probability (P) values <0.05 were interpreted as indicating statistical significance.

Glycosaminoglycans Analysis

Isolation and purification of the NTF's glycosaminoglycans GAGs. The method to isolate and purify the GAGs was based on that proposed by Breen et al. (1976). Aliquots of NTF from control and treated embryos were digested with protease type XIV (Sigma) at 50°C for 6 h. The GAGs were precipitated from the digest by the addition of 5 volumes of 5% potassium acetate in absolute ethanol and centrifugation at 17,500 g at 4°C.

Quantitative analysis of GAGs. The total amount of isolated GAGs was estimated by the uronic acid-carbachol reaction described by Bitter and Muir (1962). Briefly, after vigorous hydrolysis in 0.025 M sodium tetraborate in sulfuric acid, 200 µl of 0.125% carbachol (Fluka) in absolute ethanol were added. The optical density was read at 530 nm and the absorbance values were plotted against those obtained with standard solutions of CS.

Qualitative analysis of GAGs. The composition of GAGs in NTF was determined by monodimensional electrophoresis and specific glycosidase digestion. GAGs isolated from NTF of control and treated embryos, as well as standard solutions of GAGs (Sigma), were applied to cellulose acetate strips (Cellogel 2.5 x 17), and electrophoresed in 0.1 M formic acid-pyrydine buffer, pH 3.1, at 270 v for 1 h according to Beeley (1985). After electrophoresis, the strips were stained with 1% alcian blue 8GX (Gurr) in 0.05 M acetate buffer, pH 5.8, and ethanol (v/v). For enzymatic identification, glycosidases chondroitinase ABC and AC (Sigma) were used, following the digestion procedure of Breen et al. (1976). Chondroitinase ABC specifically digests chondroitin 4- and 6-sulfate, and to some extent, hyaluronic acid, whereas chondroitinase AC only digests chondroitin 4- and 6-sulfate (Yamagata et al., 1968). The activity of the enzymes was previously checked with standard GAGs. Afterward, aliquots of the GAGs isolated from NTF in control and treated embryos were diluted in 50 µl of 0.15 M Tris-HCL buffer containing 0.5 IU of chondroitinase ABC (pH 8), or 0.5 IU of chondroitinase AC (pH 7). The mixtures were incubated at 37°C overnight and gently shaken, and the GAGs were isolated and electrophoresed as described below.

Sodium Determination

Sodium concentration (both bound and free) in NTF from control and treated embryos was measured by flame emission spectroscopy on a Variant Spectra A-80 with a Variant GTA-100 attachment.

Protein Analysis: Western Blot

The total concentration of proteins in NTF was determined by the Bio-Rad protein assay based on the Bradford (1976) dye binding procedure. For Western blot analysis, aliquots of NTF from control and treated embryos were mixed with a sample buffer containing 3.2% SDS, 16% glycerol, 2.8 M β-mercaptoethanol and 0.0016% bromophenol blue. Samples were applied to a 4–20% gradient SDS-PAGE gel (Bio-Rad) according to Laemly (1970), and the proteins obtained were transferred to nitrocellulose sheets, pore size 0.45 µ m (Bio-Rad), according to the method of Towbin et al. (1979). After incubation for 2 h at room temperature in the blocking solution (PBS containing 5% skimmed milk), the nitrocellulose sheets were exposed overnight, at 4°C, to antichondroitin sulfate mono-

clonal antibody (CS-56 from Sigma), which recognizes the CS glycosidic fraction (Avuur and Geiger, 1984), and identified with a peroxidase-labeled mouse IgM PK 4010 Vectastain avidin biotin complex kit (Vector). The peroxidase activity was revealed with diaminobenzidine (0.5 mg/ml in PBS with 0.02% hydrogen peroxide).

RESULTS

Developmental Pattern of NTF Osmolality

NTF osmolality was measured in the period of development (H.H. stages 20–29) in which rapid brain enlargement occurs. Osmolality determinations were not made in previous stages because of the impossibility of obtaining sufficient amounts of NTF.

NTF osmolality variations followed a characteristic pattern during the early development of chick embryos (Fig. 1). NTF osmolality oscillated around 250 mOsm/Kg with no significant changes until H.H. stage 24 ($P > 0.05$, by Mann-Whitney U test). Osmolality then dropped significantly at stage 25 but returned to 250 by stage 27. There were statistically significant differences in values of osmolality between stages 24 and 25 and between stages 25 and 26 ($P = 0{,}029$ by Mann-Whitney U test).

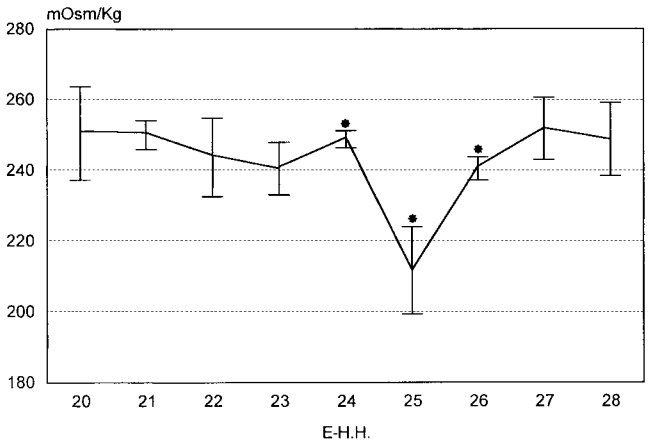


Fig. 1. Graph showing the pattern of NTF osmolality at early stages of chick embryo brain development. The osmolality remains stable until H.H. stage 24; however, a transitory drop can be observed at H.H. stage 25. The evolution of NTF osmolality coincides with that of brain cavity hydrostatic pressure, which has been previously described. The values are mean +/- standard error (n = 4). * $P = 0.0029$ by Mann-Whitney U test.

Effect of β-D-xyloside in Brain Enlargement

After in ovo treatment, the brain vesicles exhibited considerable growth with respect to the rest of the neural tube and began to undergo secondary vesiculation (Fig. 2-A). Histological observation of these embryos (Fig. 2-C) reveals that the brain anlage occupies most of the embryonic head and reveals a large internal cavity.

Embryos treated with β-D-xyloside exhibited morphological development similar to that of the control specimens, although they all, to a greater or lesser degree, displayed a significant increase in head size with respect to the control specimens (Fig. 2-B); this seems to be due to a hyperenlargement of the brain vesicles, whose morphology was not altered to any great extent. No morphological changes were seen in the development of other embryonic anlages with the exception of the eye, which occasionally appeared smaller. The histological sections of these embryos (Fig. 2-D) confirmed the large increase in brain anlage size with regard to the control specimens; this seems to be the result of a global overexpansion of the brain cavity. These β-D-xyloside induced changes are unaccompanied by any histological alterations in the morphology of the brain anlage, which shows normal progress in brain vesiculation; however, some morphological alterations can be appreciated at the border between the diencephalon and the mesencephalon, such as a flattening in the diencephalo-mesencephalic fissure and in the mesencephalic floor (Fig. 2C-D).

Morphometric data (Fig. 3) indicate that the size of the brain anlage of the β-D-xyloside-treated embryos was 37% larger than that of the control ones. Brain cavity volume increased 50% more than that of the control specimens, whereas the increase in the brain neuroepithelial wall was only 11% in the β-D-xyloside-treated embryos. Statistical analysis shows that the total and cavity volumes of the experimental groups were significantly greater than for the controls ($P < 0.001$), whereas differences in tissue volume were not significant ($P > 0.05$). Such data indicate that brain volume increase induced by β-D-xyloside is mainly due to an overexpansion of the brain cavity.

Ultrastructure of Brain Neuroblasts

Our results indicate that β-D-xyloside-induced disruption of CS biosynthesis is accompanied by changes in the ultrastructural appearance of the apical ends of brain neuroepithelial cells. In control embryos, the apical end of the neuroblasts were joined by gap junctions, with numerous and conspicuous intercellular spaces (Fig. 4A). The apical cytoplasm of these cells showed mitochondria and intracellular membrane formations morphologically compatible with endoplasmic reticulum, Golgi cisterns and large (200–300 μm diameter) vesicular formations of a moderately electrondense content. Near the apical surface several small vesicular formations, which resemble the clathrin-coated vesicles involved in endocytosis, could be seen.

The most remarkable ultrastructural changes induced by β-D-xyloside in neuroblasts (Fig. 4B) was a notable increase in apical intracytoplasmic organelles, such as Golgi complexes and, especially, in large vesicular formations gathering in the vicinity of the nucleus and scattered near the apical surface. The ultrastructural appearance of these vesicles does not differ from those described in control embryos, except in their number and greater size.

β-D-xyloside Induces Specific Changes in NTF Biochemical Composition, Osmolality, and Hydrostatic Pressure

Biochemical analysis of the NTF of control embryos (Table 1) revealed that, at H.H. stage 23, its main component is protein (295 ug/100 ul), in which the PGs represent a small percentage (12,5 ug/100 ul). In these embryos, the results of the qualitative analysis of the NTF (Fig.5) identify two different types of GAGs, CS and hyaluronic acid. However, the electrophoretic separation of native NTF (without prior digestion with protease) of embryos of the same stage revealed no migration of any of its components, with the GAGs (identified as positive alcian blue material) remaining at the level at which they were

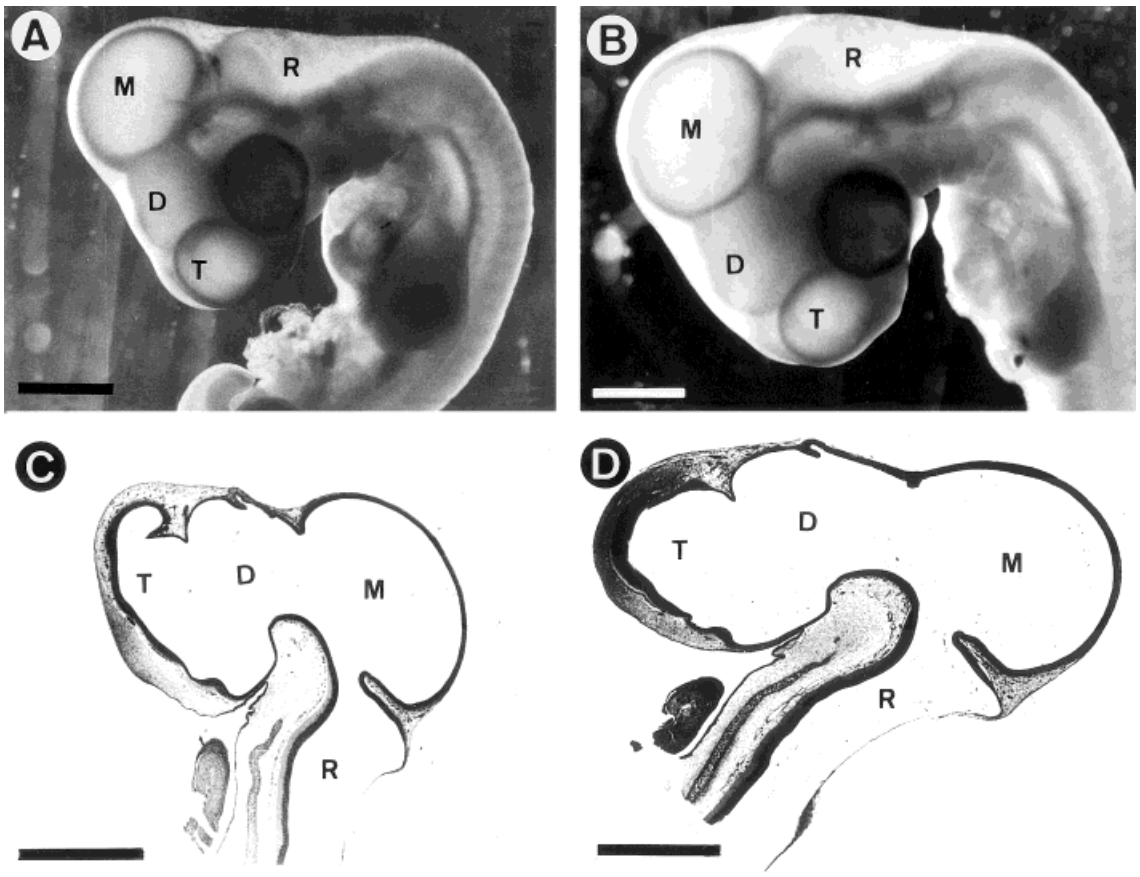


Fig. 2. β-D-xyloside induces overexpansion of the embryonic brain cavity in chick embryos. Although the morphologic pattern of brain vesiculation seems to be preserved, some alterations the diencephalo-mesencephalic borders can be observed. Macroscopic view of H.H. stage 23 control (**A**) and treated (**B**) chick embryos. Sagittal histological section of control (**C**) and treated (**D**) embryos. T, telencephalic vesicles; D, diencephalon; M, mesencephalon; R, rhombencephalon. Scale bars = 1 mm.

deposited (Fig. 6B2). This fact suggests that the GAGs present in the NTF are probably integrated in proteoglycan macromolecular aggregates, as occurs in other extracellular matrices. The results of the Western blot, performed to clarify the nature of NTF chondroitin sulfate, indicate that only a protein fraction, M_r 197 x 10^3 , was present (Fig.7).

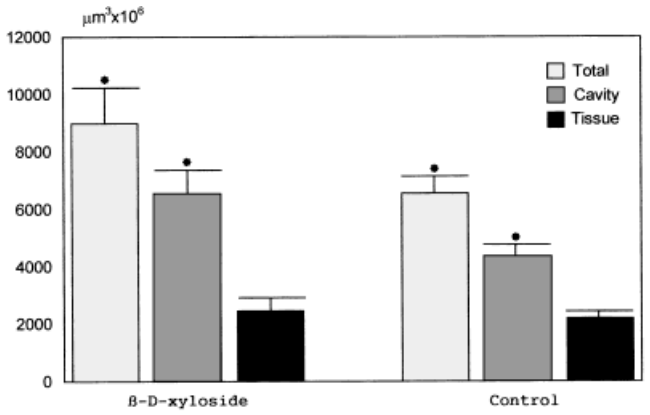
The results of other determinations on NTF composition, such as sodium concentration and osmolality of the control embryos, were of 2,316 ppm and 278 mOsm/Kg, respectively (Table 1).

In the β-D-xyloside treated embryos, the specific changes in the biochemical composition of NTF were only reflected in the PGs and ionic composition, with no alteration in its protein concentration (Table 1). The uronic acid concentration was 200% higher than that of the control embryos (Table 1), and the results of the electrophoretic separation plus digestion with specific glycosidases (Fig. 6A) indicated that this increase is exclusively due to CS, since the amount of hyaluronic acid remained unchanged. In the electrophoresed native NTF of the treated embryos (Fig. 6B), a band of similar mobility to that of CS appeared, which is highly suggestive of the presence of free chains of this proteoglycan. Western blots of the NTF of the treated embryos with antichondroitin sulfate CS-56 antibody showed (Fig. 7) that CS electrophoretic mobility is similar to that of the control embryos, with only a slight reduction in epitope concentration. These data suggest that β-D-xyloside does not bring about substantial variations in the molecular weight of the CS secreted into the brain cavity. Yet our measurements of Na concentration of NTF of β-D-xyloside embryos compared with controls showed nearly a 40% increase in the total sodium concentration (Table 1).

In order to clarify the influence of the increase of CS and sodium in the osmolality of NTF, we measured this parameter in both control and β-D-xyloside-treated embryos. The results show that β-D-xyloside treatment induces an 80% increase in NTF osmolality in comparison with control embryos (Table 1). Furthermore, in order to find out whether this increase in NTF osmolality was related to changes in brain intraluminal pressure during the rapid brain enlargement period, we measured the pressure of control and treated embryos at stage 21 H.H. (after 30 h of β-D-xyloside treatment). As Table 2 shows, the intraluminal pressure in the brain vesicles of treated embryos increased 39% in comparison with controls, and this difference is statistically significant ($P<0.001$).

DISCUSSION

In this report, we test whether the osmolality of NTF is directly related to hydrostatic pressure and whether experi-



	ß-D-xyloside (µm³ x 10⁶)	Control (µm³ x 10⁶)
Total volume	9000 ± 1245.1*	6560 ± 588.5*
Cavity volume	6544 ± 817.4*	4344 ± 395.6*
Tissue volume	2466 ± 448.8	2220 ± 217.7

Fig. 3. Comparison of brain size of control and β-D-xyloside treated H.H. stage 23 embryos shows a significant disruption of the brain expansion process. The total brain anlage volume of the treated embryos is 37% higher than that of the control specimens. This increase is due to brain cavity volume, which is 50% greater than that of control specimens, whereas brain tissue mass increases by only 11%. The values are mean +/− standard error (n = 10).* $P < 0.001$ by Student's t-test.

mentally induced changes in CS concentration of NTF by means of β-D-xyloside would lead to significant alterations in NTF osmolality and hydrostatic pressure, and how much changes affect brain expansion in chick embryos.

One major finding, i.e., a significant drop in the osmolality of the NTF demonstrated by us, is consistent with the drop in intraluminal pressure reported by Jelinek and Pexieder (1970). However, we found that the changes in hydrostatic pressure occur later than the osmolality changes; this could be explained by the time necessary for water interchange between NTF and embryonic tissues. Furthermore, our results show that an experimentally induced increase in the osmolality of NTF by means of β-D-xyloside leads to a significant increase in intraluminal pressure of the brain vesicles and to a hyperenlargement of the brain anlage in chick embryos. This relation could be explained if we bear in mind that the osmotic pressure of NTF implies an increase of water inside the brain cavity, leading to an increase in hydrostatic pressure and/or a neuroepithelial wall distension. This explanation is based on the assumption that the embryonic brain is a physiologically sealed system (Schoenwolff and Desmond, 1984; Desmond et al., 1993). These data support the hypothesis that NTF osmolality could be involved in the control of brain expansion.

In addition, we have already reported the presence of an osmotically active molecule, like CS, inside the brain cavities in chick embryos (Gato et al., 1993), and we have also suggested that the osmotic pressure of NTF could be, at least in part, regulated by its CS concentration. Our study indicates that NTF contains a main protein component, two characterized PGs (CS and hyaluronic acid) and high levels of sodium, all of which could contribute to NTF osmolality; however, the special osmotic properties of CS (Comper and Laurent, 1978) make it a candidate for NTF osmotic pressure regulation. Moreover, our results show that the changes in the CS concentration of NTF, experimentally induced by means of β-D-xyloside (without any other associated change in the hyaluronic acid or the protein component of NTF), lead to significant modifications in NTF osmolality, hydrostatic pressure and brain enlargement.

Proteoglycans have been proposed as fundamental molecules in the regulation of the water content in biological tissues. Certain major biological properties of PGs are the result of their polyanionic nature, which facilitates their interaction with water in biological tissues, hence their special osmotic properties and hydraulic conductivity. CS has the greatest osmotic capacity, determined by its uronic acid content and the nature of the glycosidic union (Comper, 1981; Comper and Zamparo, 1990), and relatively small variations in its concentration produce large increases in its osmotic capacity (Comper and Laurent, 1978).

Our results show that the changes induced in CS concentration of NTF by means of β-D-xyloside, are accompanied by a significant increase in total sodium concentration. β-D-xyloside is a chemical compound that specifically disrupts sulfated proteoglycan biosynthesis, but we have not found any data to justify a direct interference of this compound with ionic transport mechanisms; therefore, the changes in sodium concentration of NTF, induced using β-D-xyloside, seem to be related to the CS variations. These data suggest that there is a functional relation between CS and sodium in NTF, and both molecules could be co-responsible for NTF osmolality regulation. Comper and Laurent (1978) have shown that sulfated PGs bind large amounts of inorganic ion (counterions) by electrostatic interactions so that the osmotic properties of PGs in biological solutions depend to a large extent on the actvity of their associated counterions. Our data show that sodium is a major ionic component of chick embryo NTF, so its ions are probably the counterions associated with CS. Therefore, our data support the hypothesis that the NTF osmolality properties in chick embryos could be attributed to a coordinated regulation in CS and sodium concentrations.

Despite the fact that intraluminal PGs can regulate the sodium concentration in NTF by electrostatic interactions, there is a large sodium concentration in the NTF of chick embryos, which suggests the existence of a sodium transport mechanism across the neuroepithelium toward the brain cavity. We do not have any evidence to support this point and further research is necessary; however, Barbosa et al. (1985) have already reported the possibility of transepithelial transport of ions and water toward the inner cavity of another ectodermal derived anlage, the otic vesicle. In addition, in the neuroepithelial cells of amphibian embryos, the presence of sodium pumps, which actively transport sodium out of the neural cavity, has been described by Hotary and Robinson (1990) and Borgens and Shi (1995). This ionic transport generates a transneural tube potential that seems to be necessary for the structural integrity of the early neuroepithelium and a prerequisite for normal morphogenesis. In amphibian embryos, a transepithelial sodium transport inside the embryo has been described in the epiblast (Metcalf and Borgens, 1994), and it seems to be preserved after neural tube invagination and internalization during neurulation to create the

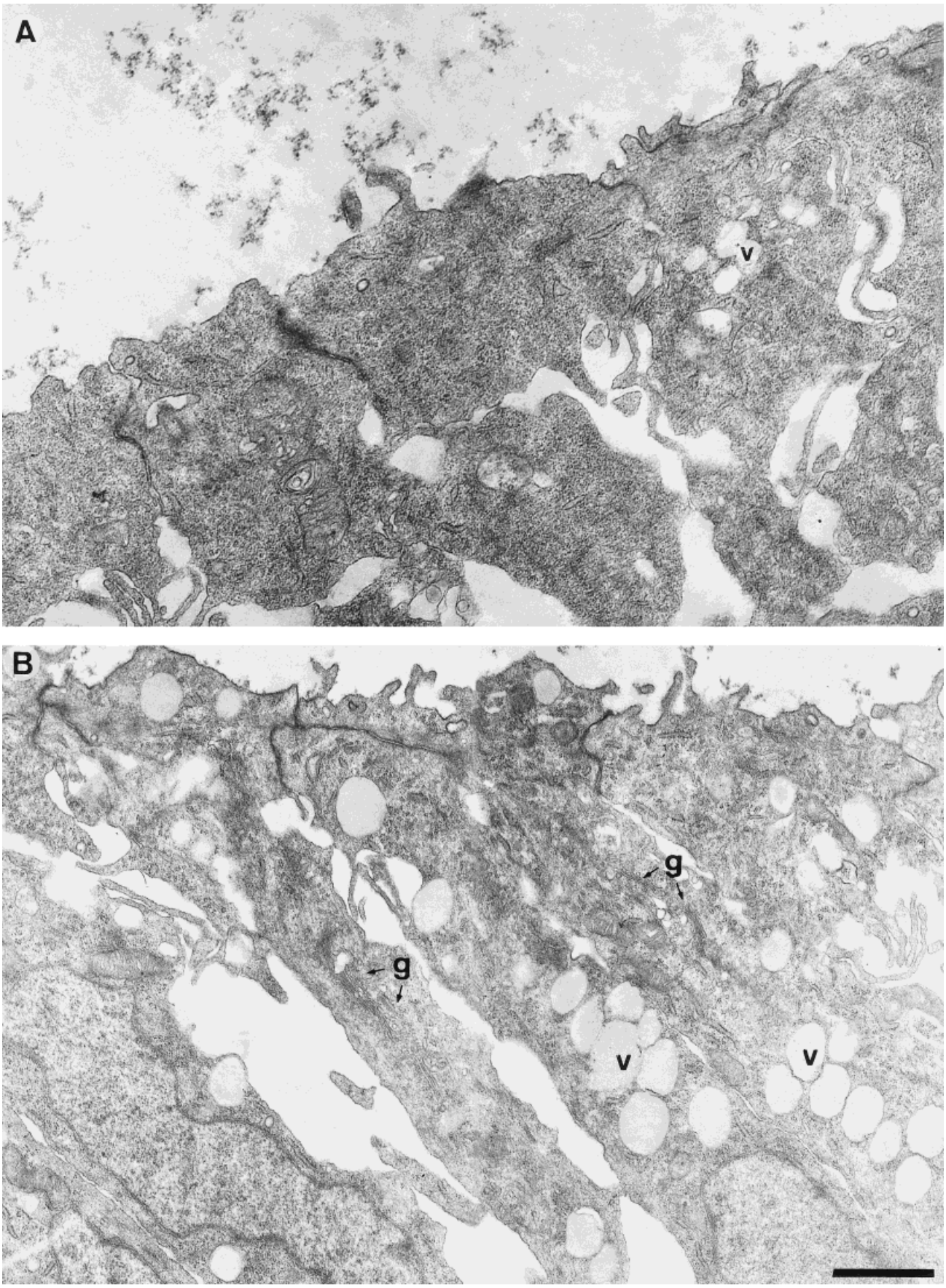


Fig. 4. β-D-xyloside induces ultrastructural changes in neuroepithelial cells. Images are from the apical pole of diencephalic neuroblasts of control (**A**) and treated (**B**) H.H. stage 23 chick embryos and show changes induced by β-D-xyloside, in particular, a surprising increase in apical vesicular formations (V) and Golgi complexes (g). Scale bar = 1 μm in both figures.

TABLE 1. Effects of β-D-Xyloside Treatment on Neural Tube Fluid Composition of H.H. Stage 23 Chick Embryos

Treatment	Proteins µg/100 µl	Uronic acid µg/100 µl	Sodium ppm	Osmolality mOsm/Kg
Control	295	12.5	2316	278
β-D-xyloside	295	37.5	3259	504

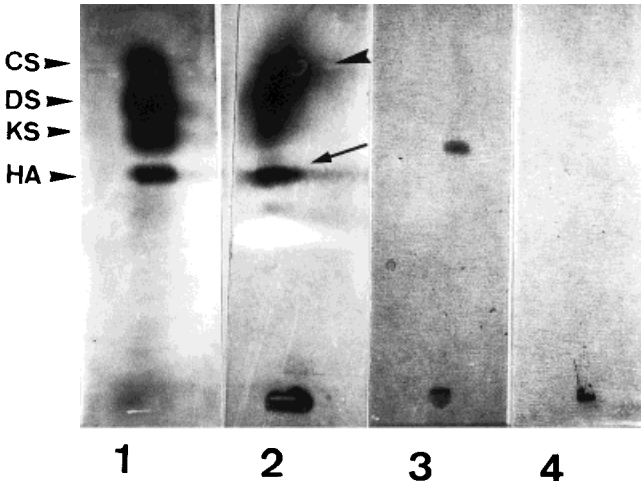


Fig. 5. Two major GAGs, hyaluronic acid and CS, were identified in NTF of H.H. stage 23 chick embryo brains, by electrophoretic separation on cellulose acetate combined with digestion by specific glycosidases. **Lane 1:** Standard GAGs separation pattern (HA: hyaluronic acid, KS: keratan sulfate, DS: dermatan sulfate, CS: chondroitin sulfate). **Lane 2:** Electrophoretic separation of GAGs extracted from NTF of H.H. stage 23 chick embryos. **Lanes 3 and 4:** GAGs extracted from H.H. stage 23 chick embryo after pretreatment with chondroitinase AC and ABC respectively. The identification of the two bands as hyaluronic acid (arrow) and CS (arrowhead), was based on their sensitivity to chondroitinase AC (Lane 3), which specifically digests chondroitin-dermatan sulfate, and to chondroitinase ABC (Lane 4), which digests chondroitin-dermatan sulfate and hyaluronic acid.

transneural tube potential. In the epiblast of avian embryos, a similar sodium transport mechanism has been described (Stern and MacKenzie, 1983); however, the existence of a high NTF concentration of sodium after neurulation, as we have found in our work, suggests that in chick embryos the mechanism of sodium pumping from the brain cavity has not been preserved, or perhaps has been inverted in order to generate an osmotic swelling mechanism.

β-D-xyloside induces ultrastructural changes at the apical end of brain neuroblasts, mainly the increase in the number of Golgi complexes and apical intracytoplasmic vesicles. Golgi complexes have been traditionally associated with glycosylation, fundamental for GAGs chains synthesis; similarly, vesicular formations resembling those described have been related with proteoglycan synthesis and secretion out of the cell (Hay, 1991). Moreover, the presence of numerous vesicules of similar appearance and containing GAGs has been previously described in the neuroblasts' apical cytoplasm by Mak (1978) during the fusion process of neural folds in amphibian embryos. Therefore, if we bear in mind that the cavity of the brain

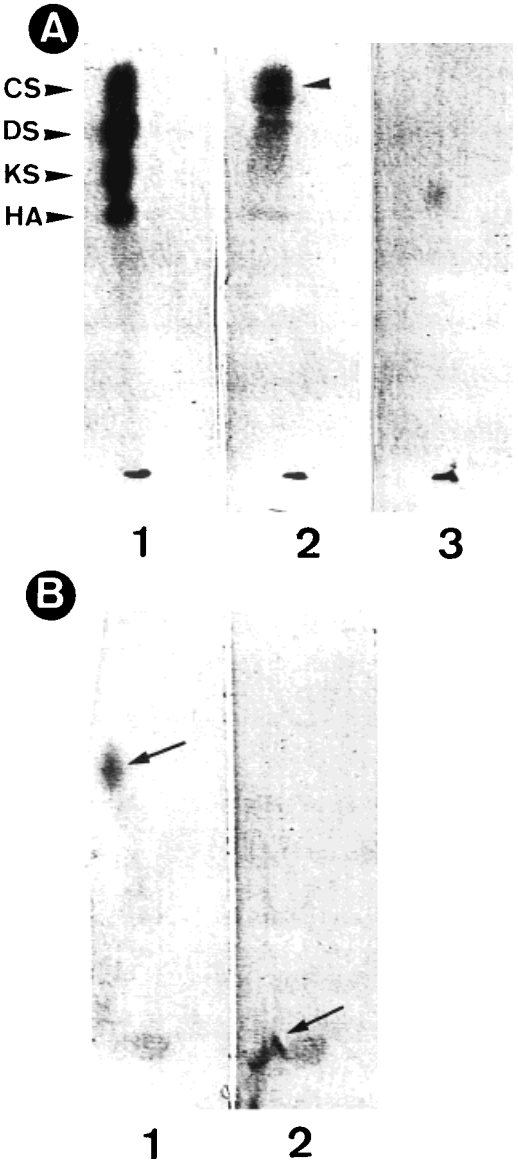


Fig. 6. Changes induced in NTF GAGs composition by means of β-D-xyloside valued by electrophoretic separation on cellulose acetate combined with digestion with specific glycosidases. **A. Lane 1** shows the electrophoretic pattern of standard GAGs (HA: hyaluronic acid, KS: keratan sulfate, DS: dermatan sulfate, CS: chondroitin sulfate). **Lane 2:** Electrophoretic separation of GAGs extracted from NTF of β-D-xyloside treated embryos. **Lane 3:** The same as lane 2 after chondroitinase AC treatment. The electrophoretic separation of GAGs extracted from NTF β-D-xyloside treated embryos shows that most GAGs migrate to a similar level exhibited by the chondroitin and dermatan sulfate standards (Lane 2, arrowhead) and that they are sensitive to chondroitinase AC digestion (Lane 3). These facts suggest that the great increase in NTF uronic acid concentration induced by means of β-D-xyloside is mainly due to chondroitin / dermatan sulfate. **B.** The electrophoretic pattern of GAGs from NTF in their native state (without previous digestion with protease) from β-D-xyloside treated embryos, shows a band of similar mobility to CS (**Lane 1,** arrow), whereas GAGs from control embryos stay at the same level at which they were deposited (**Lane 2,** arrow). These results suggest that in the NTF of control embryos, the CS are arranged to macromolecular aggregates of PG, and that β-D-xyloside primes the synthesis of CS free chains.

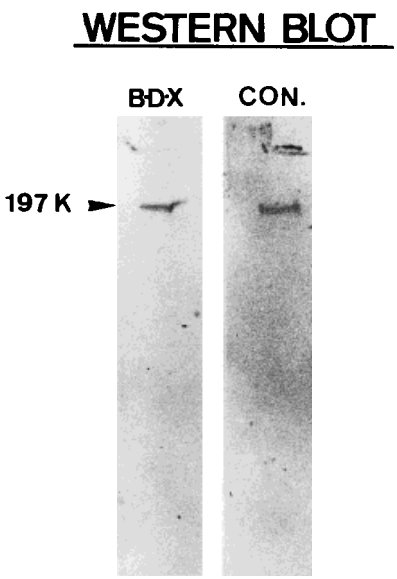


Fig. 7. Western blot analysis of NTF of H.H. stage 23 control (CON) and β-D- xyloside treated (BDX) chick embryos. Monoclonal antibody CS-56 recognizes a single band, M_r 197 x 10^3 , in NTF of control embryos. In β-D- xyloside treated embryos, there are no apparent changes except a slight reduction in epitope concentration.

TABLE 2. Results of the Intraluminal Pressure, in Control and β-D-Xyloside-Treated of H.H. Stage 23 Chick Embryonic Brains[a]

	Control (hPa)	β-D-xyloside (hPa)
Pressure	37.8 ± 1.5 (13)*	52.6 ± 2.8 (14)*

[a]Measurements are mean ± standard error. Number of embryos measured are in parentheses.
*$P < 0.001$, by two-tailed Student's test.

vesicles is delimited by the apical end of neuroblasts, we can assume that the CS present in the embryonic brain cavity could be a product of apical secretion of the neuroepithelial cells.

Synthesis and secretion of PGs are maximally stimulated by the presence of a β-D-xyloside, since this compound avoids the physiological control mechanisms (Galligani et al., 1975). The great increase in CS concentration detected in the NTF of chick embryos treated with β-D-xyloside suggests a capacity of PGs secretion far superior to that occurring in normal conditions, suggesting that it must be a mechanism regulating the secretion of CS. It has been suggested (Desmond, 1989) that the expansive process of the chick embryo brain could be regulated by the secretory state of the neuroepithelium at each embryonic age. Our results suggest that this regulative mechanism could be based on the rate of CS secretion by the brain neuroblasts.

In conclusion, our data indicate that NTF positive pressure, a major morphogenetic mechanism in early brain development, might be generated by an osmotic water-binding phenomenon inside a physiologically sealed brain cavity and regulated by concentrations of substances that are osmotically active in NTF, among which CS and its associated microion, sodium, could play a key role.

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LITERATURE CITED

Avuur Z, Geiger B. Inmunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. Cell 1984;38:811–822.

Barbosa E, Giraldez F, Represa JJ Transepithelial potential and resistance in the otic vesicle of the chick embryo. J.Physiol. 1985; 365,53P.

Barbosa E, Moro JA, Gato A, Represa JJ, Giraldez F. Precipitate matherial by CPC inside the otic vesicle of mouse and chick embryos. Arch Biol VII° Symp.Int. Sciences Morphologiques, 1986.

Beeley JG. Glycoprotein and Proteoglycan Techniques, Vol.16. Amsterdam: Elsevier, 1985.

Bitter T, Muir HM. A modified uronic acid carbazole reaction. Analytical Biochem. 1962;4:330–334.

Borgens R, Shi R. Uncoupling histogenesis from morphogenesis in the vertebrate embryo by collapse of the tube potential. Dev. Dyn. 1995;203:456–467.

Bradford M. A rapid and sensitive method for the cuantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem.1976; 72:248.

Breen M, Weinstein HG, Blach IJ, Borcherding MS, Sittig RA Methods Carbohidr. Chemi., 1976;7:101–115.

Comper WD. Polymer Monographs, Vol.7: Heparin and Related Polysaccharides. New York: Gordon and Breach, 1981.

Comper WD, Laurent TC. Physiological function conective tissue polysacharides. Physiol. Rev. 1978;58:255–315.

Comper WD,Zamparo O. Hydrodynamic properties of connective tissue polysaccharides. Biochem. J. 1990;269:561–564.

Coulombre AJ. The role of intraocular pressure in the development of the chick eye. I. Control of eye size. J. Exp. Zool. 1956;133:221–223.

Desmond ME. Reduced number of brain cells in so-called neural overgrowth. Anat. Rec. 1985;212:195–198.

Desmond ME. Precocious occlusion of the spinal cord in chick embryos results in precocious expansion of the brain. Anat. Rec. 1989;225:32–33.

Desmond ME, Duzy MJ, Federici BD. Second messenger regulation of occlusion of the spinal neurocoel in the chick embryo. Dev. Dyn. 1993;197:291–306.

Desmond ME, Jacobson AG Embryonic brain enlargement requires cerebrospinal fluid pressure. Dev. Biol. 1977;57:188–198.

Galligani L, Hopwood J, Schwartz NB, Dofman A. Stimulation of synthesis of free chondroitin sulfate chains by β-D-xyloside in cultured cells. J. Biol. Chem. 1975;250:5400–5406.

Gato A, Moro JA, Alonso MI, Pastor JF, Represa JJ, Barbosa E. Chondroitin sulfate proteoglycan and embryonic brain enlargement in the chick. Anat. Embryol. 1993;188:101–106.

Gibson KD, Doller HJ, Hoar RM. β -D-xylosides cause abnormalities of growth and development in chick embryos. Nature 1978;273:151–157.

Gibson KD, Segen BJ, Doller HJ Changes in chemical composition of chick embryos treated with a β-D-xyloside and Lathyrogen. Teratology 1979;19:345–356.

Hamburger V, Hamilton HL A series of normal stages in the development of the chick embryo. J. Morphol. 1951;88:49–82.

Hay ED.Cell Biolgy of Extracellular Matrix, 2nd ed. Boston: Harvard Medical School, 1991.

Hotary KB, Robinson KR. The neural tube of the Xenopus embryo maintains a potential difference across itself. Dev. Brain Res. 1990;59:65–73.

Jacobson M. Developmental Neurobiology, 3rd ed. New York: Plenum Press, 1991.

Jelinek R, Pexieder T. The pressure on encephalic fluid in chick embryos between the 2nd and 6th day of incubation. Phisiol. Bhemo. 1961;97–305.

Jelinek R, Pexieder T Pressure of the CSF and the morphogenesis of the CNS. I. Chick embryo. N°2 Folia Morphologica, Vol. XVIII, 1970.

Kanke Y, Doller HJ, Segen BJ, Gibson KD Development of biochemical and morphological changes induced by administration of β-D-xyloside to chick embryos. Teratology 1982;25:81–82.

Kinoshita S,Saiga M. The role of proteoglycan in the development of sea urchins. I. Abnormal development of sea urchin embryos caused by the disturbance of protoglycans synthesis. Exp. Cell Res. 1979;123: 229–236.

Laemly UK. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.

Lugemwa FN, Esko JD Estradiol β-D-xyloside an efficient primer for heparan sulfate biosynthesis. J. Biol. Chem. 1991;266:6674–6677.

Mak LL. Ultrastructural studies of amphibian neural fold fusion. Dev. Biol. 1978;65:435–446.

Metcalf MEM, Borgens RB Weak applied voltages interfere with amphibian morphogenesis and pattern. J. Exp. Zool. 1994; 268:323–338.

Morriss-Kay G, Crutch B. Culture of rat embryos with β-D-xyloside: Evidence of a role for proteoglycans in neurulation. J. Anat. 1982; 134–3:491–506.

Newman SA, Comper WD. Generic physical mechanisms of morphogenesis and pattern formation. Development 1990;110:1–18.

Pacheco MA, Marks RW, Schoenwolf GC, Desmond ME

Quantification of the initial phases of rapid brain enlargement in the chick embryo. Am. J. Anat.1986;175:403–411.

Reynolds T Ch. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. 1963;17:208–212.

Robinson HC, Brett MJ, Tralaggan PJ, Lowther DA, Okayama M. The effect of D-xylose, β-D-xylosides and β-D-galactosides on chondroitin sulfate biositheses in embryonic chicken cartilage. Biochem. J. 1975;148:25–34.

Roden L. Structure and metabolism of conective tissue proteoglycans. In Lennarz W, ed: The Biochemistry of Glycoproteins and Proteoglycans. New York: Plenum Press, 1980: 267–371.

Schwartz NB, Galliani L, Ho PL, Dorfman A. Stimulation of synthesis of free chondroitin sulfate chains by β-D Xylosides in cultured cells. Proc. Nat. Acad. Sci. USA 1974; 71:4047–4041.

Schwartz NB,Ho PL, Dorfman A. Effect of β-D Xylosides on synthesis of cartilage specific proteoglyacn in chondrocyte cultures. Biochem. Biophys. Res. Commun. 1976;71:851–856.

Schoenwolf GC, Desmond ME. Neural tube occlusion precedes rapid brain enlargement. J. Exp. Zool. 1984;230:405–407.

Segen BJ, Gibson KD. Fluid and glycosaminoglycan xcretion by chick embryos treated with a β-D Xyloside. Teratology 1982; 25:89–94.

Singley CT, Solursh M The use of acid tannic for the ultrastructural visualization of hialuronic acid. Histochem. 1982; 65:93–102.

Sobue M, Habuchi H, Ito K, Yonekura H, Oguri K, Sakurai K, Kamoharas S, Ueno Y, Noyory R, Uzuki S. β-D Xylosides and their analogues as artificial initiators of glycosaminoglycan chain synthesis aglycone-retated variation in their effectiveness in vitro and in ovo. Biochem. J. 1987;241:591–601.

Spooncer E, Gallanger JT, Krizsa F, Dexter TM Regulation of haemopoiesis in long-term bone marrow cultures.IV. Beta-D Xylosides. J. Cell. Biol. 1983;96:510–514.

Spurr AR. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Resp. 1969;26:31.

Stern CD, MacKenzie DO Sodium transpot and the control of epiblast polarity in the early chick embryo. J. Embryol. Exp. Morphol. 1983;77:73–98.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 1979;76:4350–4354.

Yamagata T, Saito H, Habuchi O, Suzuki S. Purification and properties of bacterial chondroitinases and chondrosulfatases. J. Biol. Chem. 1968;243:1523–1535.

Yost HJ. Inhibition of proteoglycan synthesis eliminates left-right asymmetry in Xenopus laevis cardiac looping. Development 1990;110: 865–874.