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Evaluation of the roles of intrinsic and extrinsic factors in occlusion of the spinal neurocoel during rapid brain enlargement in the chick embryo

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SUMMARY

The spinal neurocoel normally occludes during the second day of chick embryogenesis as the lateral walls of the spinal cord become apposed closely in the midline. Concomitantly, the brain initiates its rapid and substantial enlargement. Occlusion, although short-lived, might play a major role in brain enlargement. As a result of occlusion, the brain ventricles are sealed off from the external milieu prior to closure of the posterior neuropore, establishing a closed fluid-filled system. The present study focuses on the mechanisms of occlusion of the spinal neurocoel. We tested two postulated intrinsic factors (microtubule-mediated neuroepithelial cell elongation and microfilament-mediated apical neuroepithelial cell constriction) and five extrinsic factors (three mediad pushing forces generated by the somites, perineural extracellular matrix and expanding surface ectoderm; and two stretching forces generated either vertically by pulling of the elongated notochord or longitudinally by elongation of the embryo) in maintaining occlusion. Our results suggest that occlusion is *maintained* by other, untested intrinsic factors and/or by forces generated within a perineural collar, composed of cellular and extracellular materials, intimately associated with the basal aspects of the spinal cord. Cytoskeletal-mediated changes in cell shapes, pushing forces and vertical and longitudinal tensions are not involved. Further studies are needed to examine the intrinsic properties of the neuroepithelium and the factors *initiating* occlusion and reopening.

INTRODUCTION

The developing central nervous system normally undergoes two important morphogenetic events on the second day of chick embryogenesis: the spinal neurocoel becomes occluded and the brain initiates its rapid and substantial enlargement. Although occlusion and the initiation of rapid brain enlargement are correlated temporally, it is unclear whether they are related causally. Future studies will address this possibility. This study focuses on the mechanisms of occlusion of the spinal neurocoel.

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Key words: chick embryo, occlusion, spinal neurocoel, brain.

Occlusion is a widespread developmental phenomenon, occurring in amphibians, birds and mammals, including humans (Freeman, 1972; Desmond & Jacobson, 1977; Löfberg, Ahlfors & Fallstrom, 1980; Desmond, 1982; Kaufman, 1983; Schoenwolf & Desmond, 1984*a*,*b*; Desmond & Schoenwolf, 1985). During occlusion, the apical sides of the lateral walls of the spinal cord are apposed closely, virtually obliterating the neurocoel by reducing it to a narrow slit-like gap. At some levels of the occluded neuraxis, this gap expands as discontinuous pore-like spaces located dorsally, ventrally or centrally within the spinal cord. Occlusion precedes the onset of rapid brain enlargement and is short lived (Schoenwolf & Desmond, 1984*a*,*b*, 1986; Pacheco, Marks, Schoenwolf & Desmond, 1986). It begins as early as Hamburger & Hamilton (1951) stages 9–10, is well established during stages 11–14 and ends during stages 14+ to 17, as reopening of the spinal neurocoel rapidly occurs (Desmond & Schoenwolf, 1985; Schoenwolf & Desmond, 1986). Because occlusion occurs prior to closure of the posterior neuropore, it seals off the brain ventricles from the external milieu and establishes a closed fluid-filled system (Schoenwolf & Desmond, 1984*a*; Desmond & Schoenwolf, 1985). Such a hydraulic system is required for the normal growth, expansion and morphogenesis of the brain (Coulombre & Coulombre, 1958; Jelínek & Pexieder, 1970; Desmond & Jacobson, 1977).

We suggested previously that occlusion might result from several postulated factors acting alone or in concert (Schoenwolf & Desmond, 1984*b*). Such factors were classified as intrinsic or extrinsic. Intrinsic factors originate within the neuroepithelium and might include: elongation of neuroepithelial cells in the lateral walls of the neural groove or tube; apical constriction of neuroepithelial cells in the floor plate; interdigitation of crossluminal neuroepithelial cell protrusions; formation of crossluminal intercellular junctions; and deposition of cell surface coats, especially apically. Ultrastructural studies demonstrated a paucity of interdigitating apical protrusions, crossluminal junctions, and heavy surface coats, suggesting that such factors do not play a major role in occlusion (Kaufman, 1983; Schoenwolf & Desmond, 1984*b*). However, these studies could not rule out roles in occlusion for cell-surface adhesion molecules, increases in neuroepithelial cell cohesive properties, or changes in neuroepithelial cell shapes.

Extrinsic factors originate outside the neuroepithelium and might include: mediad pushing forces (tension generated by the somites, perineural extracellular matrix and expanding surface ectoderm); vertical (dorsoventral) stretching forces (tension generated by pulling of the elongated notochord); and longitudinal (craniocaudal) stretching forces (tension generated by elongation of the embryo and expansion of the blastoderm over the yolk). The fact that occlusion occurs consistently in midsomitic regions, and never cranial to or caudal to levels of the spinal cord flanked by somites, suggests that somites and associated extracellular matrix might function in occlusion (Desmond & Schoenwolf, 1985). However, none of these extrinsic factors has been tested experimentally.

The purpose of this study was to examine the roles of two postulated intrinsic factors (microtubule-mediated cell elongation and microfilament-mediated apical

cell constriction) and five postulated extrinsic factors (three mediad pushing forces and two stretching forces) in the *maintenance* of occlusion (i.e. after occlusion has been initiated and prior to the onset of reopening). Maintenance rather than initiation of occlusion was examined because the onset of occlusion occurs concurrently with neurulation (Schoenwolf & Desmond, 1984*b*) and many of the treatments used to examine occlusion are known to block neurulation (e.g. see Schoenwolf, 1982). Hence, failure of occlusion to occur after treatment could be the result of interference with either neurulation or some factor causing occlusion. In addition, it is clear that forces are present to maintain occlusion as well as to initiate it because occluded areas resist the flow of dye down the neuraxis when brain intraventricular pressure is increased by the injection of fluids (Schoenwolf & Desmond, 1984*a*).

MATERIALS AND METHODS

Fertile White Leghorn chicken eggs were used in all experiments. They were incubated in forced-draft humidified incubators at 38°C until embryos reached stages 11–13 (Hamburger & Hamilton, 1951). This range of stages was used for all experiments, but stage 12 embryos were used chiefly because occlusion is most robust at this stage. Experiments were done on blastoderms developing either *in ovo* or in whole-embryo culture. We describe below how occlusion was assessed during various phases of the experiments, the culture procedure sometimes used and the experimental studies performed.

Assessment of occlusion

Dye injections of the brain ventricles of living embryos

In each experiment, embryos were assayed for occlusion by injecting an aqueous solution of 1 % toluidine blue, 1 % sodium borate into the brain ventricles through the roof of the midbrain as described previously (Schoenwolf & Desmond, 1984*a*, 1986; Desmond & Schoenwolf, 1985). The numbers of embryos injected are listed in Table 1. Briefly, glass micropipettes (tip diameters, 50–80 μm) were attached to a Drummond 10 μl microdispenser modified by replacing its plunger with a micrometer to allow finer injections. The microdispenser was held and positioned with the aid of a Stoelting micromanipulator. Only low pressure injections (i.e. those in which the tip of the micropipette was inserted perpendicularly to the roof of the midbrain, and the micrometer on the microinjection apparatus was turned slowly so that dye leaked from the micropipette gradually) were done as described in detail elsewhere (primarily type 3 injections: Schoenwolf & Desmond, 1986). In many cases, the embryo (including the neuraxis) was transected through the midextent of the suspected occluded area and the roof plate of the spinal cord on the cranial side of the transected region was slit with a tungsten needle (e.g. Fig. 1). These procedures were done to eliminate back pressure that might be present in a nonoccluded but *blind* spinal neurocoel. Embryos were photographed to document the injection results.

Histological examination of the spinal cord

In each experiment, at least three embryos (and often many more) were assayed for occlusion by histological examination of the spinal cord. Embryos were fixed and processed for either paraffin or plastic transverse serial sectioning for light microscopy as described earlier (Desmond & Schoenwolf, 1985). Histological analyses were done in many cases on embryos whose brain ventricles had been injected previously with dye. Routine transmission electron microscopy was also done as outlined earlier (Schoenwolf & Desmond, 1984*b*). The procedure of Vanroelen & Vakaet (1981) was used on the cytochalasin-treated and related control embryos to enhance the preservation of microfilaments. Basically, this method uses tannic acid to prevent osmium

Table 1. *Effects of various procedures on occlusion of the spinal neurocoel as assessed by injection of dye into the brain ventricles through the roof of the midbrain*

	Modified Spratt culture only		Somite extirpation with or without transection	EDTA plus trypsin	Trypsin alone	EDTA alone or EGTA alone	EDTA plus divalent cations	Intraluminal EDTA or EGTA plus dye	*Streptomyces* or testicular hyaluronidase	Collagenase	Cold (4°C)	Cytochalasin D
	Short term (2–6 h)	Long term (18 h)										
Number assayed for occlusion	42	9	23	6	9	30	10	13	13	7	50	42
Number occluded	37	0	23	0	9	2	10	12	13	7	48	40
Per cent occluded	88	0	100	0	100	7	100	92	100	100	96	95

degradation of microfilaments during postfixation and a hypotonic buffer to disperse cytoplasmic components.

Whole-embryo culture

Whole-embryo culture was used extensively, mainly to provide direct access to embryos for microsurgical manipulations or chemical treatments. Egg-agar, whole-embryo culture substrates were made by a modification (Packard & Jacobson, 1976; D. S. Packard, personal communication) of the original Spratt method (Spratt, 1947). The contents of three cold unincubated fertile chicken eggs were homogenized in the cooled container of a Waring blender. The foamy homogenate was dispensed into sterilized 50 ml plastic tubes and centrifuged at 14 900 *g* for 30 min at 5 °C. The supernatant was removed and placed in a sterile glass beaker held in a water bath at 47 °C. A 50 ml aqueous solution of 6 % agar was prepared while the egg homogenate was centrifuging. It was then autoclaved for 8 min (at 120 °C and 103·5 kPa) and placed in the water bath. Once the temperatures of the two ingredients had equilibrated, the agar was added slowly to the egg (being careful not to form bubbles) with continuous swirling in the proportion of 1:3, agar: egg. Approximately 2–3 ml of the mixture was poured in the centre of a warmed Petri dish (60×15 mm), the dish was swirled gently to spread the mixture evenly and excess liquid was poured off so that only a thin coating formed (approximately 1–1·5 mm thick). Plates were placed on a flat surface to cool and then stored in a humidified chamber at 4 °C for as long as 3 weeks. At the end of each experiment, blastoderms were excised from the yolks and rinsed in physiological (0·9 % sodium chloride) saline. Vitelline membranes were removed and blastoderms were carefully positioned dorsal side up on the egg-agar substrate. Excess saline was then removed, flattening blastoderms on the substrate.

Control cultures

To assess the normalcy of development in culture, blastoderms were placed in modified Spratt culture, embryos were staged and photographed, and cultures were incubated at 38 °C, 98 % relative humidity, for either 2–6 h or 18 h.

Microsurgical manipulations of cultured embryos

Microsurgical manipulations were done on cultured embryos. To do this, blastoderms were placed in modified Spratt culture and embryos were staged. Then the lateral portions of the somites, the flanking nephrotomes, lateral plates and adjacent portions of the area pellucida, and the overlying surface ectoderm and underlying endoderm were removed bilaterally with finely sharpened tungsten needles. In some cases, the spinal neuraxis was transected with a tungsten needle following somite extirpation. Photographs were taken to document the nature of each operation and cultures were reincubated at 38 °C, 98 % relative humidity, for 4 h.

Chemical treatments of cultured embryos

Chemical treatments were also done on cultured embryos. To do this, blastoderms were placed in modified Spratt culture, embryos were staged and photographed and 0·5 ml samples of various solutions were added to the dorsal surface of the blastoderm. Embryos were bathed continuously in these samples during the entire length of treatment. In many cases, somites and surrounding tissues were extirpated bilaterally just prior to chemical treatment. Cultures were reincubated at 38 °C, 98 % relative humidity. The specific chemical treatments and lengths of reincubation are listed below.

EDTA (ethylenediaminetetraacetic acid) plus trypsin

An aqueous solution was prepared freshly consisting of 0·4 % EDTA (Fisher Scientific Co., NJ), 0·5 % trypsin ('1-250', Difco Laboratories, Detroit), and 1 M-glycine. In some cases, the neuraxis was teased from surrounding tissues with a tungsten needle after chemical treatment. Embryos were treated for 2 to 3 h.

Trypsin alone

An aqueous solution was prepared freshly consisting of 1 % trypsin. Embryos were treated for 30 min to 3 h.

*EDTA or EGTA (ethyleneglycol-bis-(beta-aminoethylether)-*N,N,N^1,N^1*-tetraacetic acid) alone*

Aqueous solutions were prepared freshly consisting of 0·4, 3, 6 and 12 % EDTA or EGTA and 1 M-glycine. 12 % solutions also contained 1 % toluidine blue and were injected directly into the brain ventricles through the roof of the mesencephalon. Embryos were treated for 2 to 3 h with 0·4 % solutions of EDTA or EGTA; 10 to 20 min with 3–6 %; and 30 min to 1·5 h with 12 %.

EDTA plus divalent cations

Slightly more than equal-molar concentrations (with respect to the chelating agent) of calcium or magnesium were added to EDTA or EGTA solutions. Calcium and magnesium chloride served as the cation sources. Times of treatment are the same for each concentration listed above for EDTA or EGTA alone.

Streptomyces *or testicular hyaluronidase*

Solutions were prepared freshly consisting of 750 i.u. *Streptomyces* hyaluronidase (CalBiochem, La Jolla), or 175 i.u. bovine testicular hyaluronidase (Sigma Chemical Co., St Louis) activity per ml sodium acetate–Tris buffer. The buffer was prepared by mixing 0·02 M-sodium acetate buffer (pH 5·0) with 0·05 M-Tris buffer (pH 7·8) in the ratio of 1:2, sodium acetate:Tris, to obtain a final solution at pH 5·5. The efficacy of hyaluronidase in removing glycosaminoglycan was monitored by examining serial transverse paraffin sections and noting the depletion of alcian-blue-stained extracellular matrix as reported previously (Schoenwolf & Fisher, 1983; Goldstein, Jankiewicz & Desmond, 1986). Embryos were treated for 2 to 4 h.

Collagenase

Solutions were prepared freshly consisting of 1000 or 2000 i.u. collagenase (Type VII, highly purified, Sigma Chemical Co.) activity per ml of 0·05 M-Tris buffer (pH 7·1). Degradation of basement membranes was assessed by transmission electron microscopy in a sample of three embryos. Embryos were treated for 2 to 3 h.

Depolymerization of the cytoskeleton of embryos in ovo

Microtubules

Microtubules were depolymerized by cooling embryos *in ovo* to 4 °C for 24 h. Then depolymerization was assessed with transmission electron microscopy in a sample of six embryos. Embryos were staged at the time of sacrifice. Because development is suspended at 4 °C, embryos at stages 11–13 at the time of sacrifice were also at these stages at the time of cooling.

Microfilaments

Microfilaments were depolymerized by treating embryos *in ovo* with cytochalasin D (5 μg ml^{-1} 0·5 % dimethyl sulphoxide in 0·9 % saline). Shells were windowed with standard techniques, embryos were staged and 20 μl of the cytochalasin solution was first injected sub-blastodermically, then an additional 0·1 ml was dropped directly onto the surface of the blastoderm. Controls received a solution containing 0·5 % dimethyl sulphoxide in saline. Windows were then sealed with tape and eggs were returned to the incubator for an additional 2 or 4 h. Then depolymerization of microfilaments was assessed with transmission electron microscopy in a sample of six embryos.

RESULTS

Reliability of modified Spratt culture

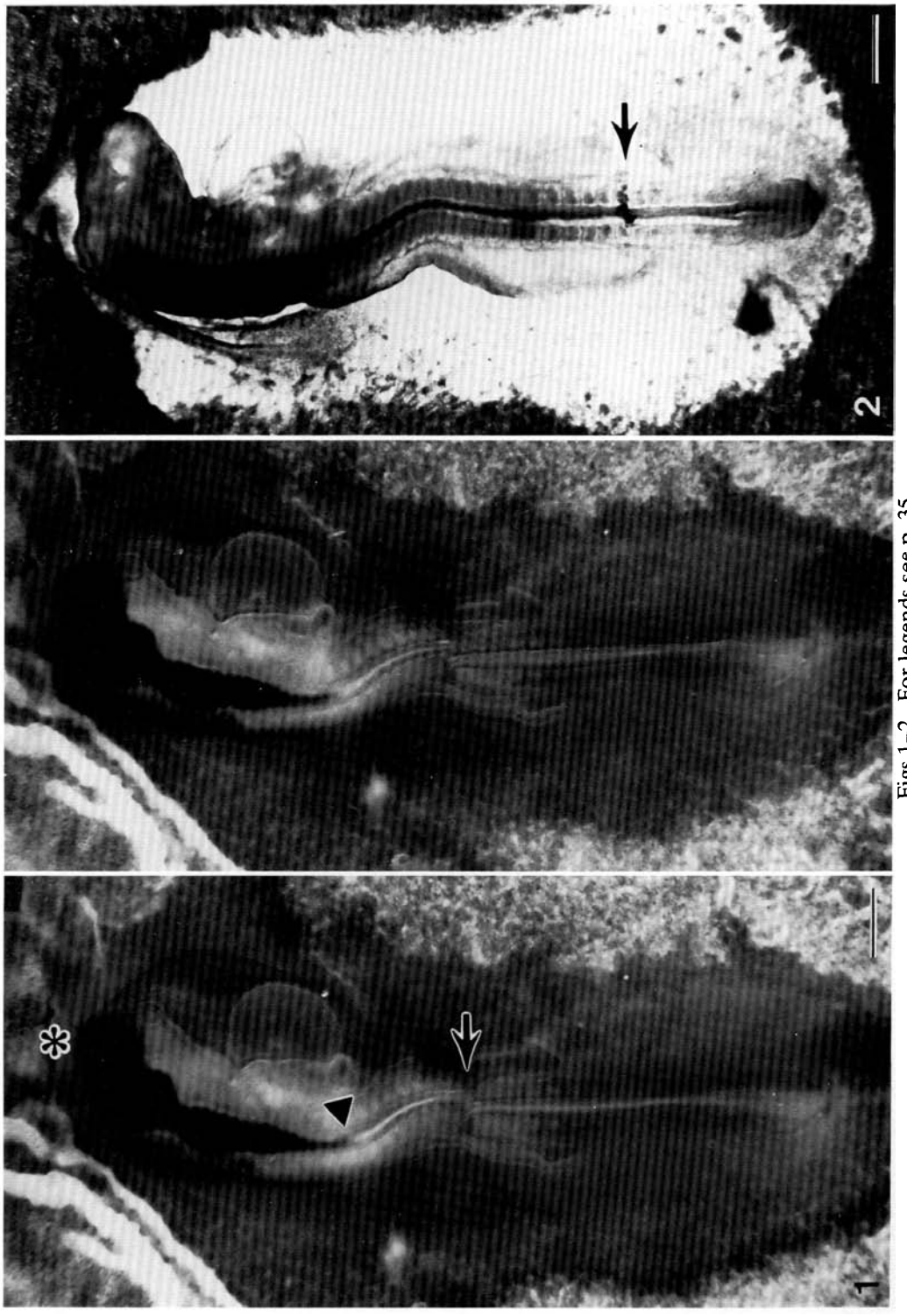
The reliability of modified Spratt culture for studying occlusion of the spinal neurocoel was tested in pilot studies. Blastoderms were cultured and the presence of occlusion was determined 2–6 h or 18 h later. Embryos scored as having occlusion based on dye injection usually exhibited occlusion histologically; those scored as lacking occlusion by dye injection always lacked occlusion histologically. These findings were also true for subsequent experiments. Embryonic development occurred roughly at the same rate on modified Spratt culture as *in ovo* after a brief warm-up period. More importantly for this study, spinal neurocoels were occluded in about 90 % of the embryos injected with dye at stages 11–13+, even though many of the embryos had been cultured for as long as 6 h (Fig. 1; Table 1). (The percentage of neurocoels normally occluded at these stages in control embryos developing *in ovo* varies, ranging from 87 to 100 % as based on dye injections; Desmond & Schoenwolf, 1985.) Furthermore, the spinal neurocoel had reopened in all embryos cultured for 18 h (Fig. 2; Table 1). Such embryos had reached stages 16–18. Reopening normally begins at stages 14+ to 15 in embryos developing *in ovo* and is completed by stages 16–17 (Schoenwolf & Desmond, 1986). Thus, reopening occurred on schedule without any detectable increase in variability in whole-embryo culture, justifying the use of this technique in subsequent experiments.

Microsurgical manipulations

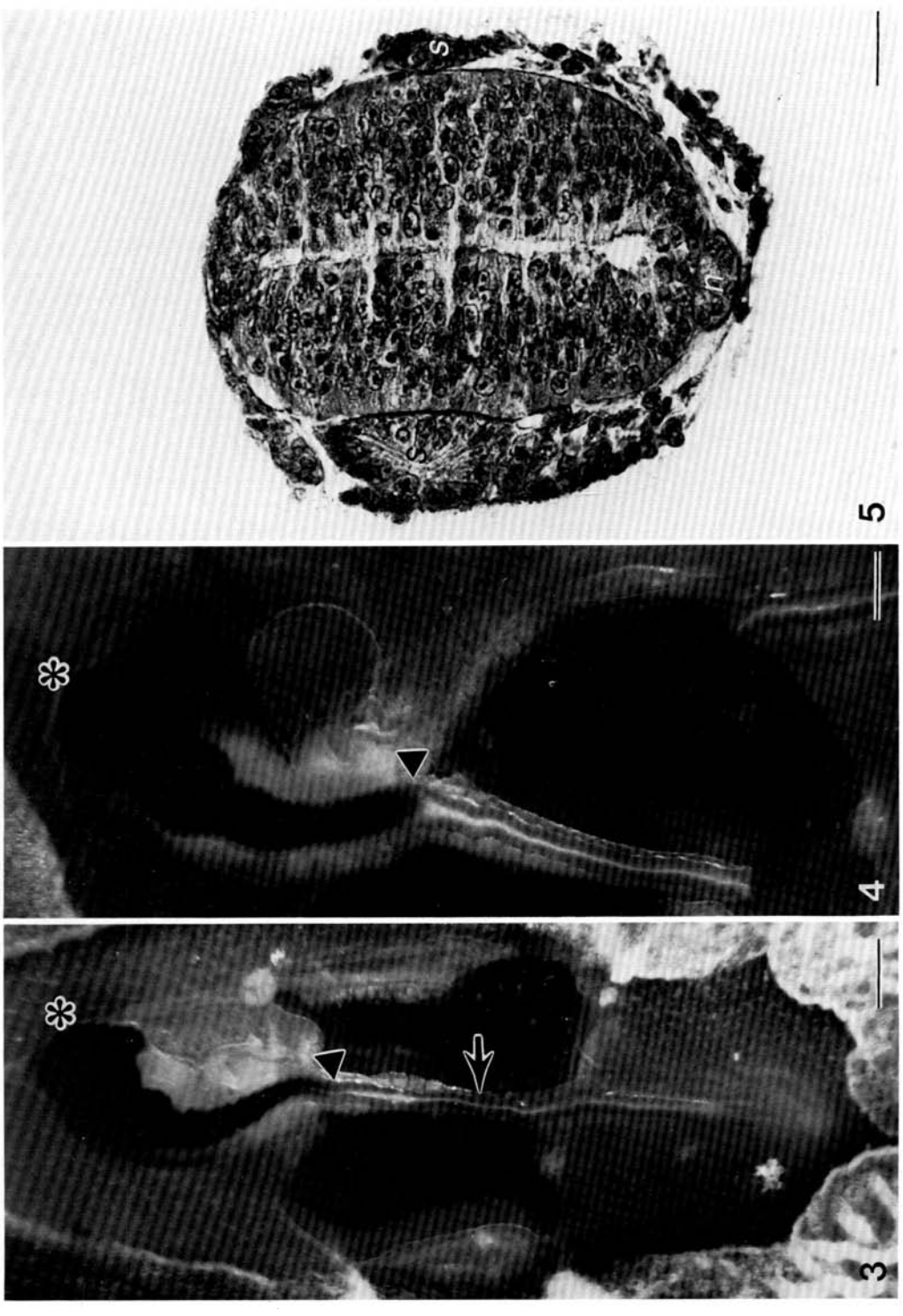
To test the effects of possible mediad pushing forces by lateral tissues on occlusion, lateral portions of the somites and the associated overlying, underlying and lateral tissues were extirpated bilaterally (Fig. 3). Such a radical procedure was carried out to prevent healing. The spinal neurocoel was occluded in all embryos receiving dye injections 4 h later (Table 1). Thus, mediad pushing forces generated by the surface ectoderm, somites or perineural matrix play little or no role in maintaining occlusion. Also, *mediolateral* stretching of the embryo during microsurgery is insufficient to cause reopening artifactually.

The neuraxis was transected in several cases at the time of somite extirpation to relieve tensions generated by the notochord or by craniocaudal elongation of the embryo. Often the cranial portion of the isolated spinal cord rolled up, much like a window shade, suggesting that tensions normally exist but were relieved by transection. Transection had no effect on occlusion – in all embryos injected with dye, the spinal neurocoel was still occluded 4 h after somite extirpation and transection (Fig. 4; Table 1).

Serial transverse sections through microsurgically isolated spinal cords confirmed that the spinal neurocoel was occluded. The notochord, associated perineural extracellular matrix, medial remnants of the somites, neural crest, middorsal surface ectoderm and midventral endoderm remained in close proximity to the spinal cord (Fig. 5).



Figs 1–2. For legends see p. 35



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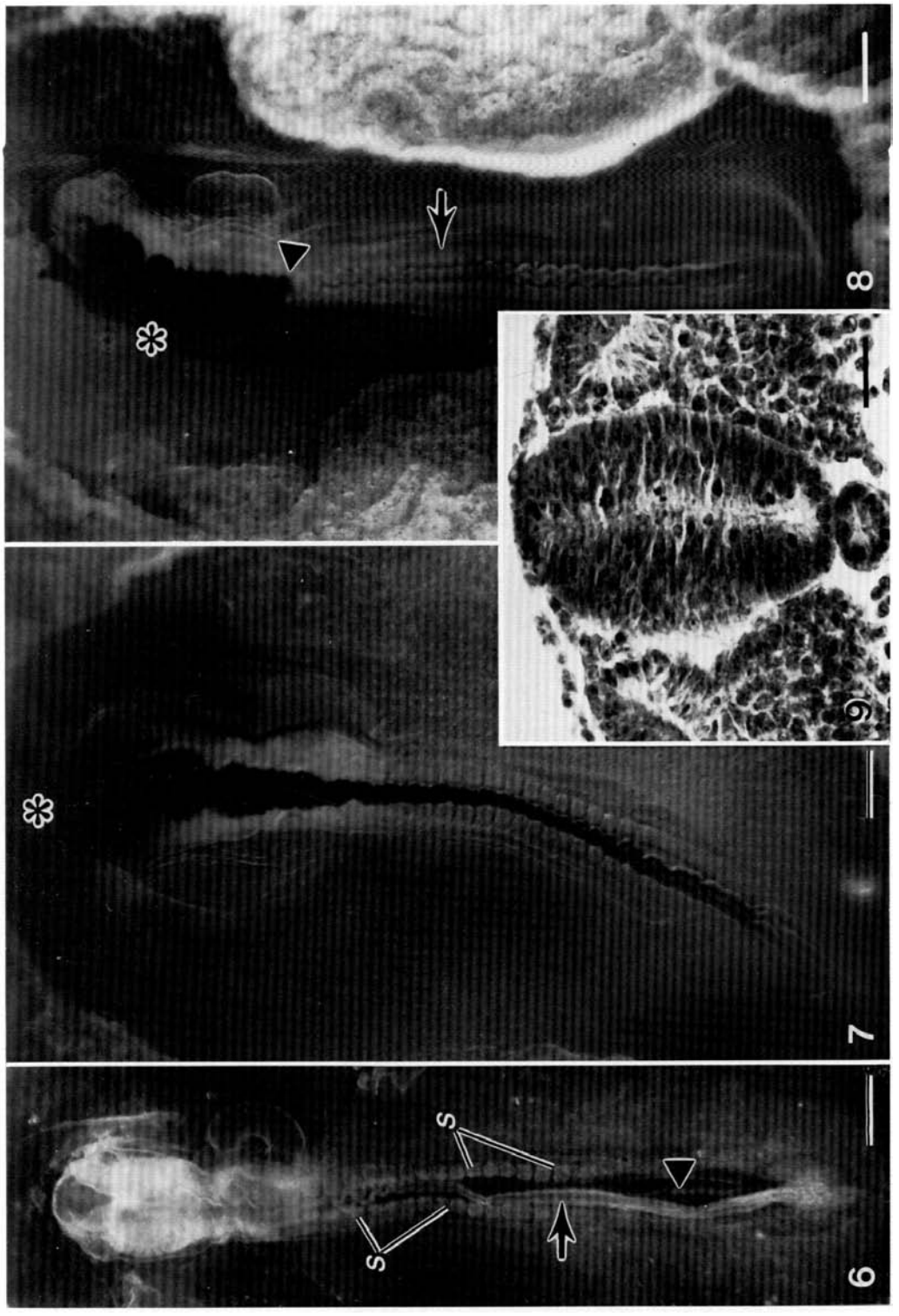
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Chemical treatments

Chemical treatments were used to remove perineural cellular and extracellular materials because they could not be removed completely by microsurgery. Two groups of experiments were performed. In the first group, embryos were treated with EDTA/trypsin, trypsin alone, EDTA alone, EGTA alone, or EDTA plus divalent cations. Data are summarized in Figs 6–13 and Table 1. Mixtures of EDTA/trypsin loosened the spinal cord from adjacent structures so that tissues could be readily teased apart with tungsten needles (Fig. 6). The spinal neurocoel

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Fig. 1. Dorsal view of a living embryo at stage 13−. Stereopair viewed with epi-illumination. The blastoderm was placed in culture at stage 12. 6 h later, its neuraxis was transected (arrow), the roof plate of the cranial transected end of the spinal cord was slit and the brain ventricles were injected with dye through the roof of the midbrain. Dye flowed caudad only to about the level of the third pair of somites (arrowhead). The rostral border of the injected brain is indicated by the asterisk. Bar, 500 μm.

Fig. 2. Dorsal view of a living embryo at stage 17 viewed with transillumination. The blastoderm was placed in culture at stage 11− and cultured for 18 h. Upon injection, dye flowed caudad to the transected level (arrow). Bar, 500 μm.

Fig. 3. Dorsal view of a living embryo at stage 13−. The blastoderm was placed in culture at stage 12+, tissues lateral to the neuraxis were extirpated bilaterally and the blastoderm was cultured for 4 h. It was impossible completely to remove the somites microsurgically without damaging the neural tube so their medial portions were left in place. Upon injection, dye flowed caudad only to about the level of the third pair of somites (arrowhead). Arrow, level of the transverse section shown in Fig. 5. The rostral border of the injected brain is indicated by the asterisk. Bar, 500 μm.

Fig. 4. Dorsal view of a living embryo at stage 12. The blastoderm was placed in culture at stage 11, tissues lateral to the neuraxis were extirpated bilaterally, the spinal cord was transected and the blastoderm was cultured for 4 h. Upon injection, dye flowed caudad only to about the level of the first pair of somites (arrowhead). The rostral border of the injected brain is indicated by the asterisk. Bar, 500 μm.

Fig. 5. Paraffin transverse section through the level indicated by the arrow in Fig. 3. The spinal neurocoel is occluded. Dorsal and ventral pores are present; such pores are a normal feature of occluded spinal cords of control embryos and do *not* indicate that precocious reopening was under way. *n*, notochord; *s*, somite remnants. Bar, 25 μm.

Fig. 6. Dorsal view of a living embryo at stage 13−. The blastoderm was placed in culture at stage 12+ and treated with EDTA/trypsin for 2 h. The spinal cord (arrow) was separated with a tungsten needle from the somites (*s*) and notochord (arrowhead). Bar, 500 μm.

Fig. 7. Dorsal view of a living embryo at stage 12. The blastoderm was placed in culture at stage 12− and treated with EDTA/trypsin for 2 h. Upon injection, dye flowed caudad to the caudal end of the spinal cord. The rostral border of the injected brain is indicated by the asterisk. Bar, 500 μm.

Fig. 8. Dorsal view of a living embryo at stage 13−. The blastoderm was placed in culture at stage 12 and treated with trypsin alone for 3 h. Upon injection, dye flowed caudad only to about the level of the second pair of somites (arrowhead). Arrow, level of the transverse section shown in Fig. 9. The dorsal borders of the injected mid- and hindbrains are indicated by the asterisk. Bar, 500 μm.

Fig. 9. Paraffin transverse section through the level indicated by the arrow in Fig. 8. The spinal neurocoel is occluded. Bar, 25 μm.

reopened precociously, even when mechanical tissue separation was not done (Fig. 7). Trypsin alone had no effect on occlusion (Figs 8, 9), whereas EDTA or EGTA alone caused precocious reopening (Figs 10, 11). Many of the cells of the spinal cord and especially those of the somites and endoderm were rounder in EDTA- or EGTA-treated embryos than in control embryos (Fig. 11). The effects of EDTA on occlusion and cell morphology were eliminated when divalent cations were added to EDTA in slight molar excess (Fig. 12). This suggests that EDTA causes reopening probably by a calcium- or magnesium-mediated event, not by some side effect unrelated to EDTA's chelating ability. Also, EDTA or EGTA had no effect when injected with dye directly into the brain ventricles (Fig. 13). In the one case where precocious reopening resulted, considerable leakage occurred from the injection site; therefore the chelating agent probably acted extraluminally.

In the second group of experiments, major components of the extracellular matrix were degraded without causing gross tissue separation. Data are summarized in Figs 14–17 and Table 1. The glycosaminoglycan component of the perineural extracellular matrix was degraded with *Streptomyces* or testicular hyaluronidase. The spinal neurocoel remained occluded (Figs 14, 15). Collagenase was used to degrade collagen, a ubiquitous major component of the basement membranes. The spinal neurocoel again remained occluded (Fig. 16). Large gaps were present in basement membranes after collagenase treatment and irregular neuroepithelial cell basal protrusions extended through the discontinuities (Fig. 17).

Depolymerization of the cytoskeleton

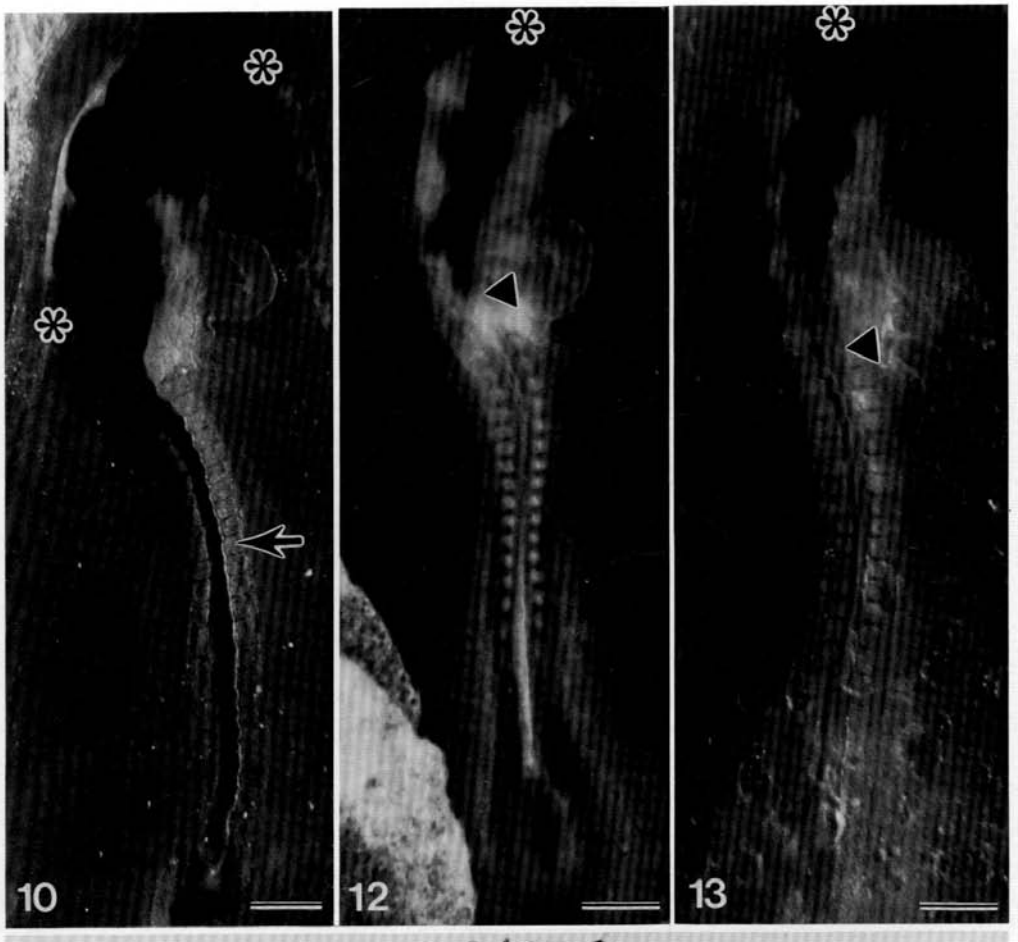
Embryos were treated with cold or cytochalasin D to depolymerize microtubules and microfilaments, respectively (Figs 18–25; Table 1). Neither treatment

Fig. 10. Dorsal view of a living embryo at stage 12+. The blastoderm was placed in culture at stage 12+ and treated with 3% EDTA alone for 10 min. Upon injection, dye flowed caudad to the caudal end of the spinal cord. Arrow, level of the transverse section shown in Fig. 11. The rostral border of the injected brain is indicated by the asterisk. Bar, 500 μm.

Fig. 11. Paraffin transverse section through the level indicated by the arrow in Fig. 10. The spinal neurocoel has reopened. Cells of the spinal cord and especially those of the somites and endoderm have rounded. Bar, 25 μm.

Fig. 12. Dorsal view of a living embryo at stage 13−. The blastoderm was placed in culture at stage 12+ and treated with EDTA plus magnesium for 1 h. Upon injection, dye flowed caudad only to about the level of the first pair of somites (arrowhead). The rostral border of the injected brain is indicated by the asterisk. Bar, 500 μm.

Fig. 13. Dorsal view of a living embryo at stage 12+. The blastoderm was placed in culture at stage 12+ and the brain ventricles were injected with EDTA plus 1% toluidine blue through the roof of the midbrain. The tip of the injection micropipette was left in position for 1 h and the midbrain was reinjected periodically throughout this period. Dye failed to flow caudad beyond the level of the first pair of somites (arrowhead). The rostral border of the injected brain is indicated by the asterisk. Bar, 500 μm.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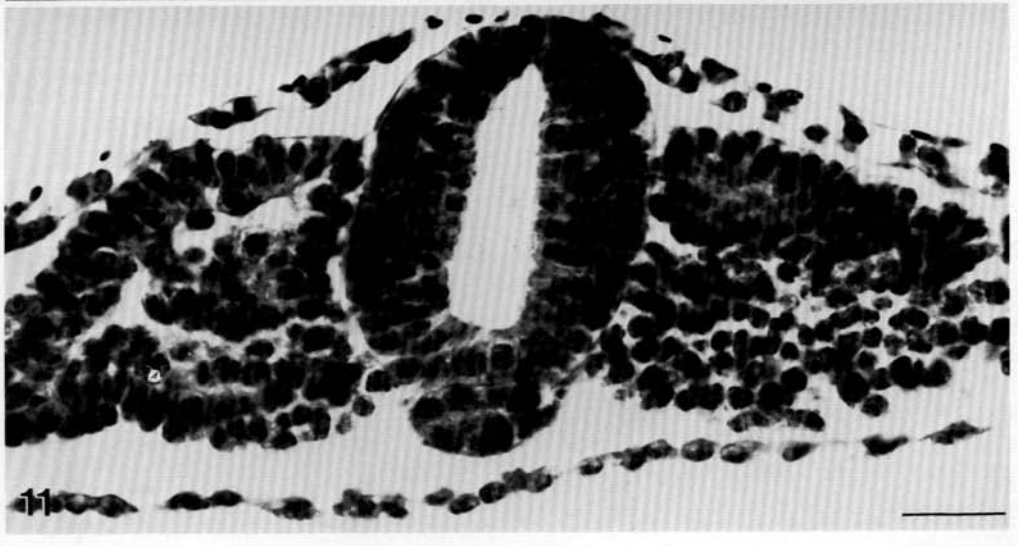
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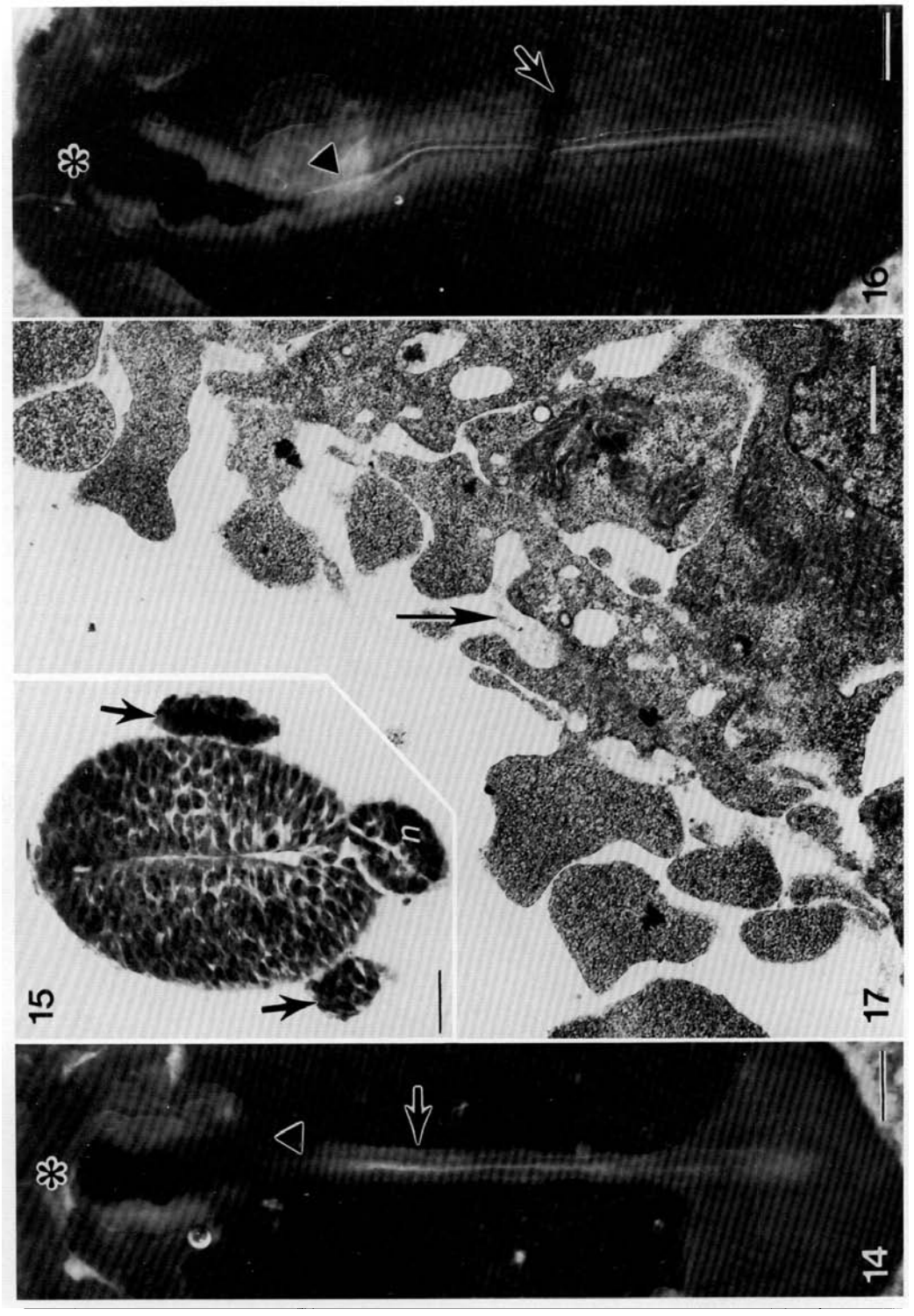
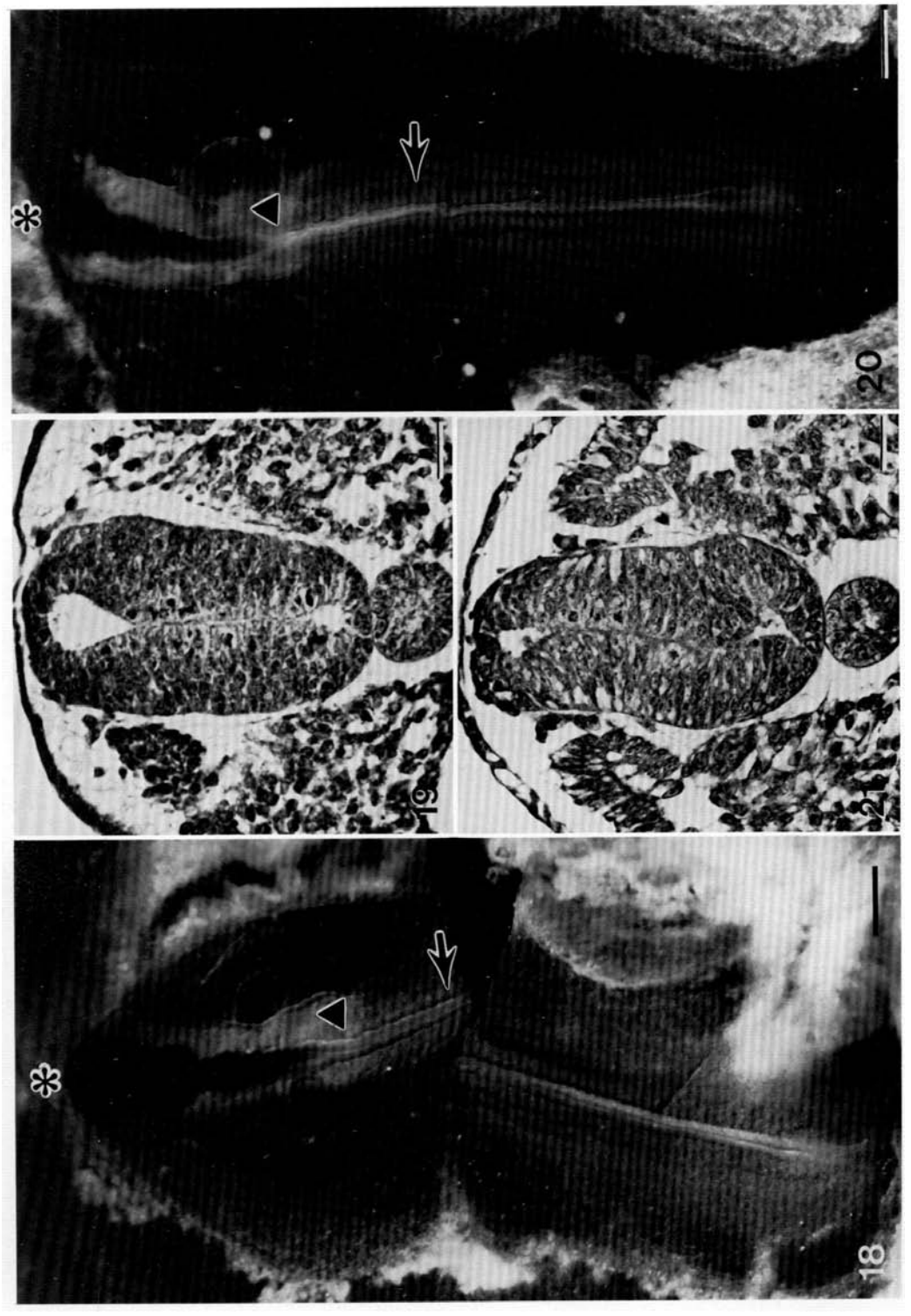
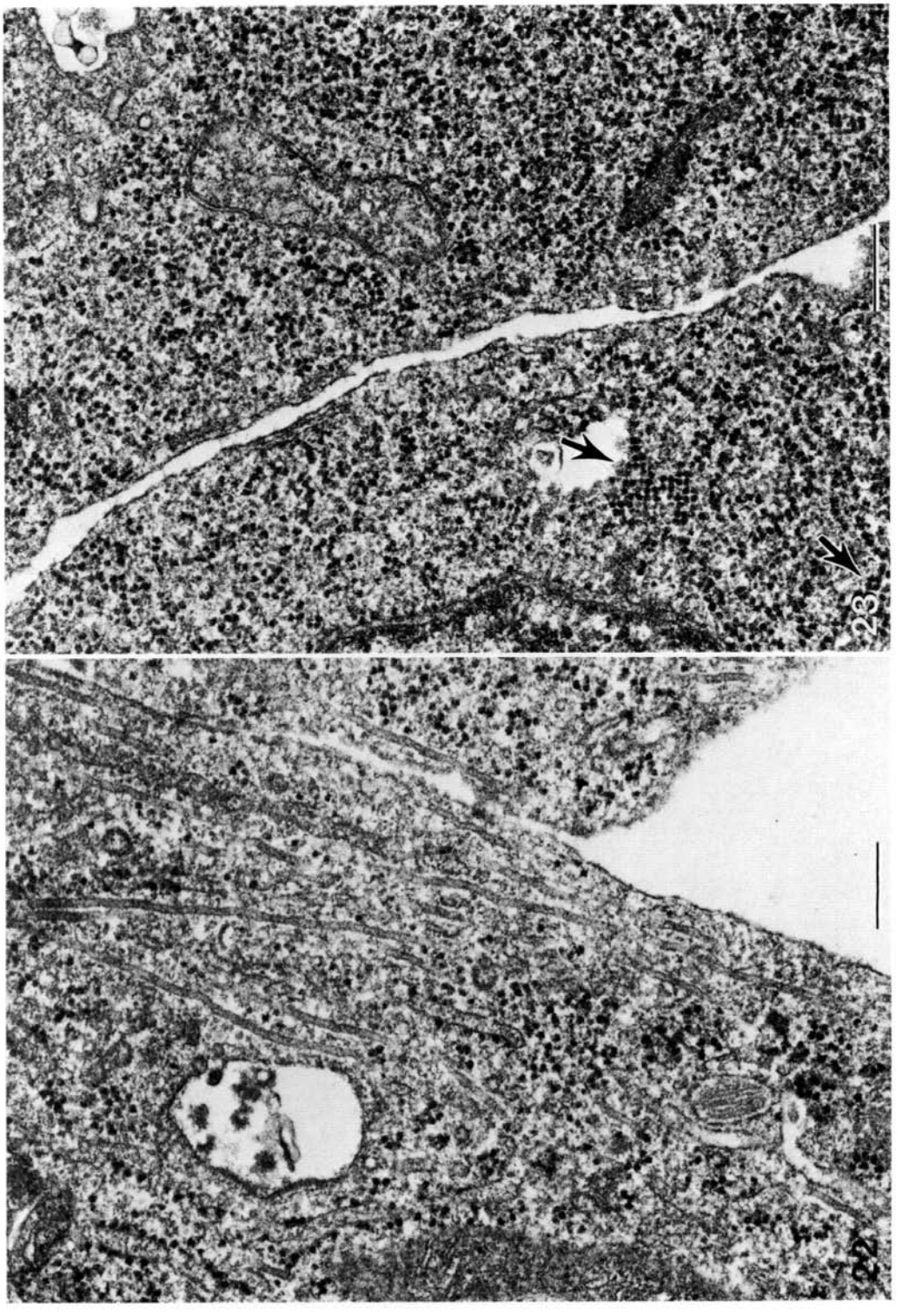


Figs 14–17. For legends see p. 41



Figs 18–21. For legends see p. 41



affected occlusion. Spinal cords of cold-treated embryos had thinner walls and larger dorsal and ventral pores than those of control embryos, suggesting that the heights of the elongated neuroepithelial cells were reduced substantially, but occlusion was still present (Fig. 19). Apices of spinal cords of embryos treated with cytochalasin D showed evidence of cellular disruption and even cell death in many cases, but occlusion was also still present (Fig. 21). Transmission electron microscopy confirmed that cold treatment depolymerized paraxial microtubules (cf. Figs 22, 23). Crystalline arrays of ribosomes were frequently generated by cold treatment (Fig. 23). Similarly, apical bands of microfilaments were absent in neuroepithelial cells from embryos treated with cytochalasin D (cf. Figs 24, 25).

Fig. 14. Dorsal view of a living embryo at stage 11+. The blastoderm was placed in culture at stage 11, tissues lateral to the neuraxis were extirpated bilaterally and the blastoderm was treated with testicular hyaluronidase for 3 h. Upon injection, dye flowed caudad only to about the level of the first pair of somites (arrowhead). The rostral border of the injected brain is indicated by the asterisk. Arrow, level of the transverse section shown in Fig. 15. Bar, 500 μm.

Fig. 15. Paraffin transverse section through the level indicated by the arrow in Fig. 14. The spinal neurocoel is occluded. Arrows, somite remnants; *n*, notochord. Bar, 25 μm.

Fig. 16. Dorsal view of a living embryo at stage 13+. The blastoderm was placed in culture at stage 13− and treated with collagenase for 3 h. Upon injection, dye flowed caudad only to about the level of the first pair of somites (arrowhead). The rostral border of the injected brain is indicated by the asterisk. Arrow, transected neuraxis. Bar, 500 μm.

Fig. 17. Transmission electron micrograph of the basal surface of the occluded spinal cord from a stage 13 embryo treated with collagenase for 2 h. Basal protrusions and basement membrane gaps have formed. Arrow, remnant of basement membrane. Bar, 0·5 μm.

Fig. 18. Dorsal view of a living embryo at stage 12−. The embryo was cooled to 4°C for 24 h *in ovo*. Upon injection, dye flowed caudad only to about the level of the first pair of somites (arrowhead). The rostral border of the injected brain is indicated by the asterisk. Arrow, level of the transverse section shown in Fig. 19. Bar, 500 μm.

Fig. 19. Paraffin transverse section through the level indicated by the arrow in Fig. 18. The spinal neurocoel is occluded. The dorsal pore (and possibly the ventral) is larger than those of control embryos, and the lateral walls of the neural tube are thinner. Bar, 25 μm.

Fig. 20. Dorsal view of a living embryo at stage 13−. The embryo was treated with cytochalasin D *in ovo* for 4 h. Upon injection, dye flowed caudad only to about the level of the first pair of somites (arrowhead). The rostral border of the injected brain is indicated by the asterisk. Arrow, level of the transverse section shown in Fig. 21. Bar, 500 μm.

Fig. 21. Paraffin transverse section through the level indicated by the arrow in Fig. 20. The spinal neurocoel is occluded. Bar, 25 μm.

Fig. 22. Transmission electron micrograph of neuroepithelial cells in a lateral wall of the occluded spinal cord from a stage 12 control embryo. Paraxial microtubules are evident. Bar, 0·25 μm.

Fig. 23. Transmission electron micrograph of neuroepithelial cells in a lateral wall of the occluded spinal cord from a stage 12 experimental embryo. The blastoderm was cooled to 4°C for 24 h. Paraxial microtubules are absent. Arrows, crystalline arrays of ribosomes. Bar, 0·25 μm.

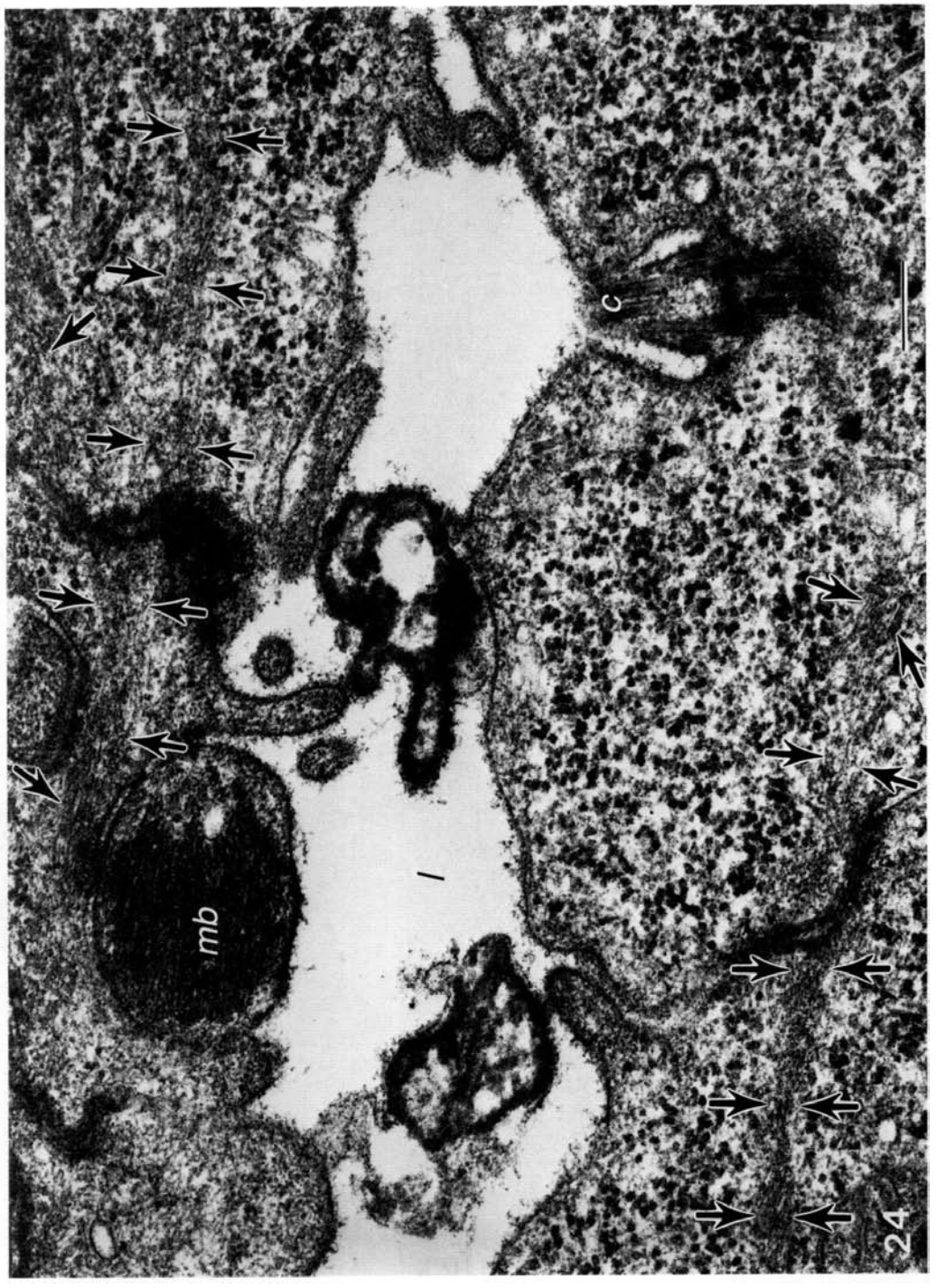


Fig. 24. Transmission electron micrograph of neuroepithelial cells of the occluded spinal cord from a stage 12 control embryo. Apical bands of microfilaments are evident. *c*, cilium; *l*, luminal aspect of occluded spinal cord; *mb*, midbody (i.e. remnant of a telophase bridge); arrows, microfilament bundles. Bar, 0·25 μm.

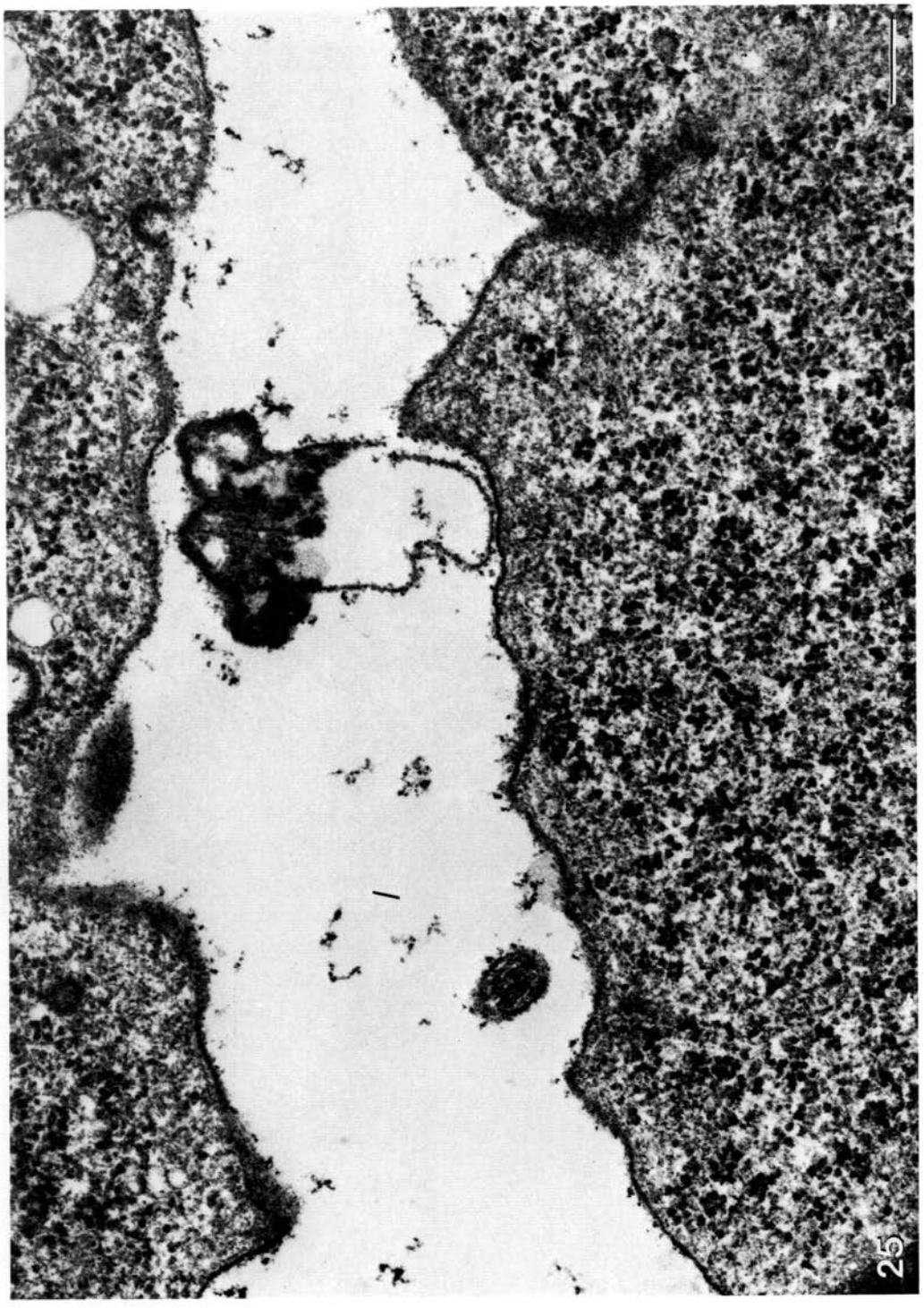


Fig. 25. Transmission electron micrograph of neuroepithelial cells of the occluded spinal cord from a stage 12 experimental embryo. The blastoderm was treated with cytochalasin D for 4 h. Apical bands of microfilaments are absent. *l*, luminal aspect of occluded spinal cord. Bar, 0·25 μm.

DISCUSSION

Our experiments eliminate roles for several postulated factors in *maintaining* occlusion. We suggested previously that ten factors might be involved in this process, five intrinsic and five extrinsic (Schoenwolf & Desmond, 1984*b*). Major roles for three intrinsic factors were eliminated by ultrastructural studies, i.e. interdigitating, crossluminal neuroepithelial cell protrusions; crossluminal intercellular junctions; and heavy cell surface coats. Such factors occur only rarely (Schoenwolf & Desmond, 1984*b*). Below, we will discuss the additional factors eliminated by the present study and we will speculate on what remaining factors might be involved. Our studies have not examined the *initiation* of occlusion. Thus, it is possible that some factors shown not to maintain occlusion actually initiate it. Future studies are needed to deal with this possibility.

Extrinsic factors

The roles of five postulated extrinsic factors (three mediad pushing forces and two stretching forces) were examined in the present study. Mediad pushing forces might be generated by the somites, perineural extracellular matrix, or expanding surface ectoderm. All of these possible forces were eliminated by microsurgical intervention. In addition, embryos with near-naked neuraxes were treated enzymically to degrade major components of the extracellular matrix. Precocious reopening did not occur. These results are somewhat surprising. We expected that at least somites would be involved in occlusion because previous mapping studies showed occlusion occurred only in areas flanked by somites (Desmond & Schoenwolf, 1985). Our results and this expectation could be resolved if somites *initiate* but do not maintain occlusion, or if somites assist in maintaining occlusion not by providing mediad pushing forces (which would be eliminated when lateral portions of the somites and surrounding tissues are extirpated), but by contributing to a girdle-like collar, presumably apposed tightly to the spinal cord. The former possibility has not been tested. The latter is at least suggested by the present study because complete removal of this collar (with EDTA or EGTA) caused precocious reopening. However, it is uncertain whether reopening in this case was due to the removal of the collar, interference with some divalent-cation mediated event or to cell rounding, an event that occurred consistently in the presence of chelating agents (Fig. 11). This rounding is clearly an artifact, not associated with the normal process of reopening (Schoenwolf & Desmond, 1986). New experiments will be needed to examine the role of perineural components in occlusion to clarify this issue.

Our experiments have examined also the roles of possible stretching forces in maintaining occlusion. Two postulated factors were examined: craniocaudal tension generated by elongation of the embryo and expansion of the blastoderm over the yolk; and dorsoventral tension generated by pulling by the elongated notochord, which is attached *via* extracellular matrix to the ventral aspect of the neural tube. Such tensions were eliminated (or at least reduced markedly) in

two ways: removal of the blastoderm from the yolk and its explantation; and transection of the neuraxis. Neither procedure caused precocious reopening, eliminating a major role for possible craniocaudal or dorsoventral tensions in maintaining occlusion.

Intrinsic factors

The roles of two postulated intrinsic forces were examined in the present study: microtubule-mediated cell elongation (especially in the lateral walls of the spinal cord); and microfilament-mediated apical neuroepithelial cell constriction (especially in the floor plate of the spinal cord). Precocious reopening did not occur after depolymerization of microtubules and microfilaments. However, neuroepithelial cell shapes were not altered grossly by these procedures. Thus, factors other than microtubules and microfilaments must by involved in maintaining the shapes of cells in organ rudiments (e.g. packing forces, cohesive forces). This notion is supported by the observation that cell separation and rounding occurred after treatment with chelating agents. It seems unlikely that such events would be due only to the action of these agents on the cytoskeleton because divalent-cation-mediated events are ubiquitous. Therefore, it is possible that non-cytoskeletal factors maintaining the shapes of neuroepithelial cells also play important roles in maintaining the shapes of organ rudiments, such as the occluded spinal cord. Further experiments are needed to clarify this issue.

One final intrinsic force possibly involved in maintaining occlusion deserves further consideration: cell-surface adhesion molecules. Surface coats are not abundant in the occluded neurocoel when embryos are preserved with fixatives containing ruthenium red or cetyl pyridinium chloride (Kaufman, 1983; Schoenwolf & Desmond, 1984*b*). Nevertheless, certain cell surface components, such as cell adhesion molecules (CAM), could play major roles in occlusion and not be detected by these relatively crude methods. It is interesting to note that N-CAM (neural) and L-CAM (liver) are maintained on the surfaces of chick ectodermal cells at gastrula and neurula stages (reviewed by Edelman, Hoffman, Chuong & Cunningham, 1985; Thiery, Boucaut & Yamada, 1985). However, further studies are required to determine whether CAM or other types of apical adhesive coats are present during stages of occlusion, and, if so, what precise role they might play in this process.

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