Embryonic Brain Enlargement Requires Cerebrospinal Fluid Pressure

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The brain of the chick embryo begins to enlarge abruptly on the second day of incubation. Shortly thereafter, major flexures and torsions of the brain occur, and many bulges and furrows appear. The onset of enlargement coincides with closure of the spinal canal which makes the neural tube a closed compartment filled with cerebrospinal fluid. We propose that cerebrospinal fluid pressure is a necessary driving force for normal brain enlargement. We have experimentally tested this hypothesis by intubating brains of chick embryos and comparing brain cavity and tissue volumes in normal and intubated embryos. The increase in cavity volume is greatly reduced, whereas brain tissue continues to grow at a reduced rate and folds into the ventricles.

INTRODUCTION

The brain of the chick embryo rapidly begins both to enlarge and to change shape on the second day of incubation (stage 12 in the staged series of Hamburger and Hamilton, 1951). During the next 26 hr, torsion, flexure, and extensive bulging and furrowing of the brain occurs (Fig. 1). We suspected that rapid enlargement of the brain begins when the neural canal closes in the spinal cord.

The literature was ambiguous as to when the spinal canal closes [Witschi, 1956 (stage 14); Vaage, 1969 (stage 12)]. By looking at serial sections of staged chick embryos, we determined that closure has occurred in most cases by stage 12. At this time, the brain ventricles and anterior spinal canal become a closed compartment filled with cerebrospinal fluid. The closure of the spinal canal (Fig. 2) occurs over a distance of 0.4 to 0.5 mm, beginning about

0.3 mm posterior to the heart and ending about 0.6 to 0.7 mm anterior to the rhomboidal sinus.

Once the spinal canal closes, a positive cerebrospinal fluid pressure (2.5–3.0 mm H_2O) can be measured in the cavity of the mesencephalon of chick embryos. These measurements were made by Jelínek and Pexieder (1968, 1970), and they called attention to the probable role of cerebrospinal fluid pressure in further morphogenesis of the central nervous system.

Coulombre (1956) demonstrated that intraocular pressure is necessary for normal expansion of the eye. Coulombre intubated eyes of 4-day-old chick embryos and found that the overall diameter of the eyes failed to increase. In the absence of intraocular pressure, the sclera, choroid, and pigmented retinal epithelium stopped expanding, but the sensory retina continued to grow and folded into the ocular lumen. In an abstract, Coulombre and Coulombre

FIG. 1. Selected stages of living chick embryos showing extensive enlargement and change in shape of the anterior portion of the neural tube over a 35-hr period. (A) Stage 11 embryo, 40–45 hr old; (B) stage 12 embryo (45–49 hr), about 5 hr older than stage 11. At stage 12, the spinal canal closes. (C) Stage 14 embryo, 50–53 hr old; note the changes in brain size and shape during the 5 hr between stages 12 and 14 as compared to the changes during the 5 hr between stages 11 and 12 before the spinal canal closes. (D) Stage 18 embryo, 65–69 hr old; bulges and furrows have become more obvious, and flexures and torsions of the head have occurred. (E) Stage 20 embryo, 70–72 hr old. All photographs are the same magnification. Bar = 1.0 mm.
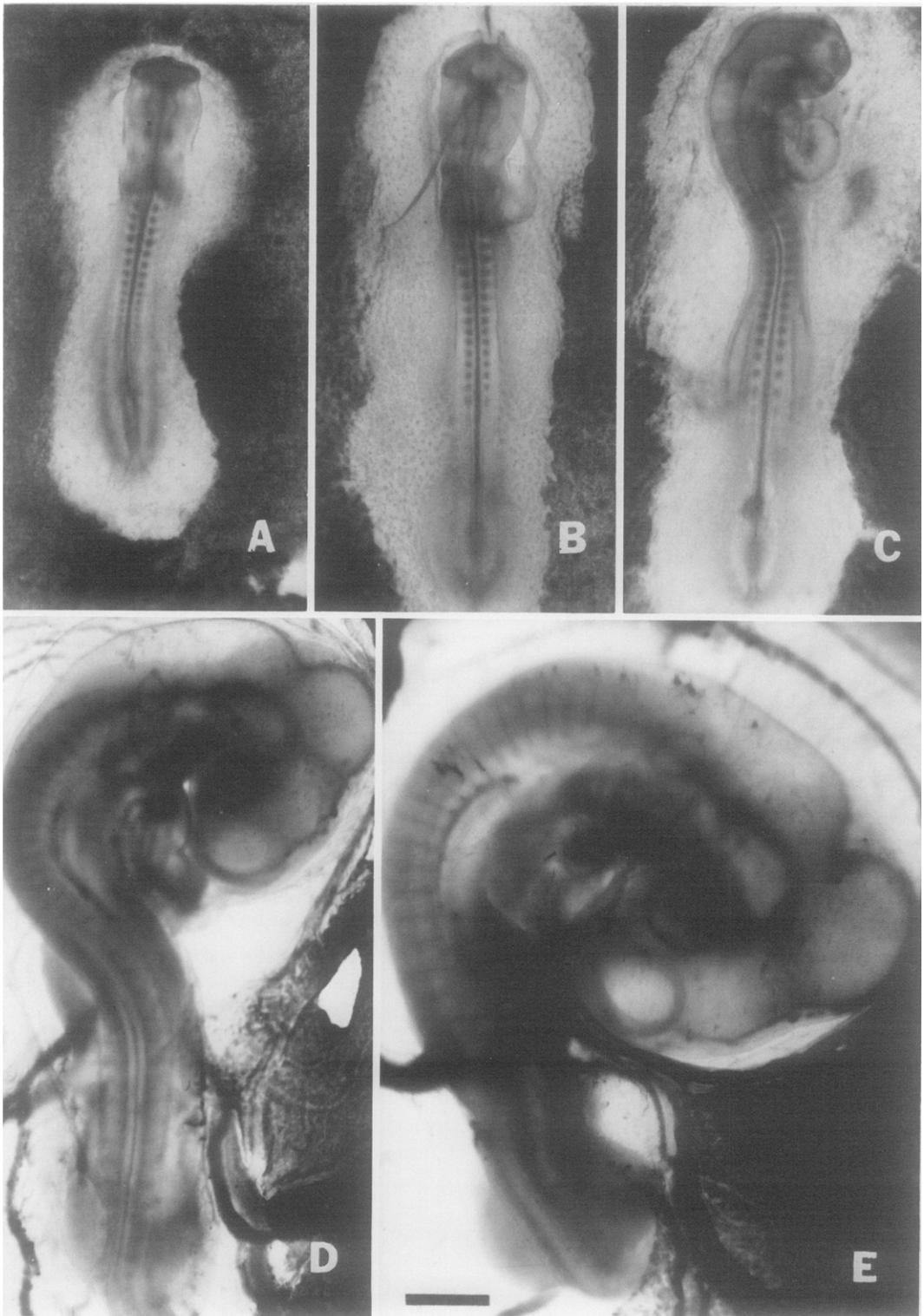
Figure 1
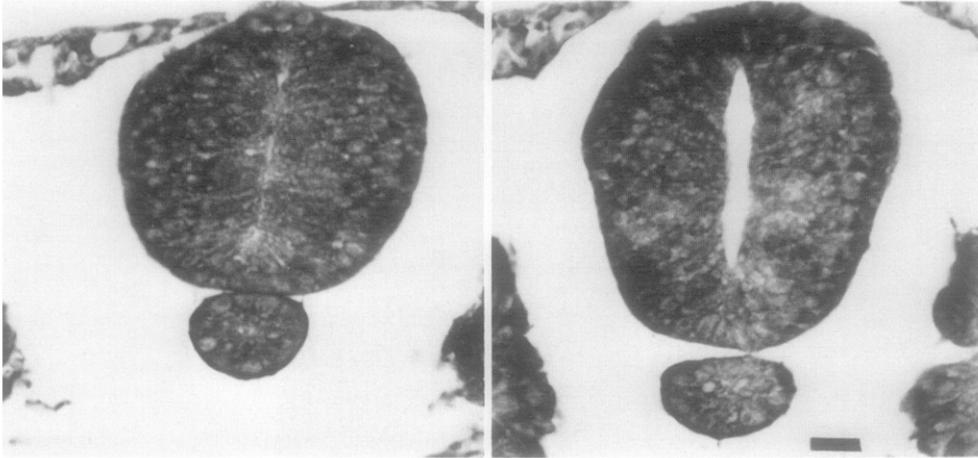
FIG. 2. Cross-sections of the spinal cord from an embryo fixed in glutaraldehyde at stage 12. The spinal canal is closed (left) for a distance of 0.4 mm (71 sections) in the region about midway between the heart and the open rhomboidal sinus. For comparison, an open region of the spinal canal is shown (right). This section was caudad to the most posterior extent of the closed portion. Bar = 0.01 mm.

(1958a) also reported folding of the myelencephalic roof of 3-day-old chick embryos which they had intubated in the myelencephalon.

We have made a series of intubations of the brains of young chick embryos to test whether positive cerebrospinal fluid pressure is a driving force in brain enlargement and shaping.

Embryos at stage 18 (about 3 days old) were the smallest that could be conveniently intubated. We intubated at stage 18 and observed and measured the changes that occurred in the next 24 hr (to stage 24). In early embryos, the most practical way to measure growth is to measure the volume changes which we have done.

Frequently, cell proliferation does not lead to immediate growth in embryos. Measures of cell proliferation are not, therefore, adequate measures of growth, nor do they tell anything about changes in cavity volumes, matrix accumulation, etc.

Nevertheless, mitotic activity is an important component of early brain enlargement, so we will review some of the many investigations of the kinetics of cell proliferation in the early chick brain. We have concentrated on studies in the mesenceph-

alon between the stages we have investigated, stages 18–24.

At these stages, the neural tube of the chick embryo consists of a homogeneous population of ventricular cells arranged as a pseudostratified columnar epithelium (Fujita, 1963, 1964). Ventricular cells (as defined by the Boulder Committee, 1970) are cells located near the lumen of the neural tube that exhibit nuclear migration to and away from the lumen. The nuclei undergo mitosis only when at the lumenal surface and synthesize DNA peripheral to the lumen (Sauer, 1935; Watterson, 1965; Sauer and Chittenden, 1959; Fujita, 1962, 1963, 1964; Källén and Valmin, 1963; Langman et al., 1966).

It is clear from a report of Källén (1961) that there is a burst of cell proliferation in the mesencephalon of chick embryos between stages 19 and 24. An increase in cell number in the optic tectum of the mesencephalon between Days 4 and 5 can also be inferred from the data of Cowan et al. (1968). They report a doubling in the number of mitotic figures per 10,000 μm^2. (Total cell numbers were not reported.)

Generation time of ventricular cells in the embryonic optic tectum increases as

embryos become older. Fujita (1962) reports that the generation time of optic tectal cells for a 6-day-old chick is triple that for comparable cells of a 1-day-old chick embryo, and Wilson (1973) notes that the generation time of optic tectal cells of a 6-day-old chick (15 hr) is double that for a 3-day-old chick (8 hr).

We can conclude from these reports that, between stages 18 and 24, there is a rapid increase in the number of ventricular cells of the mesencephalon and also an increase in the time that it takes these cells to complete a mitotic cycle. Figure 3 illustrates the changes in cell proliferation around the time of our experiments.

MATERIALS AND METHODS

Fertilized eggs from White Leghorn chickens were incubated at 38.5°C for 2 days in the shell and then were transferred to petri dishes for incubation according to the method of Auerbach *et al.* (1974).

Intubation technique. Stage 18 embryos in Auerbach culture were intubated in the cavity of the metencephalon (Fig. 4). To gain access to the head, the vitelline membrane and amnion were removed. A small square (0.25 cm²) of sterile nylon was placed in the subcephalic pocket to support and immobilize the head. An incision was made (with an electrolytically sharpened tungsten needle) at the site of intubation. A sterile glass tube filled with chick Ringers solution was inserted into the brain cavity through the incision. The appearance of fluid flowing through the tube to the outside was the criterion for a successful intubation. Preliminary intubations were made with tubes of various diameters. The smallest internal tube diameter that produced consistent results was 0.30 ± 0.01 mm. This was the diameter chosen for experiments. All tubes were 20 mm long which allowed the free end of the tube to be covered with fluid on top of the tolk. The open end of the tube must rest on the nylon to keep the tube in place as the embryo moves about. Most intubated em-
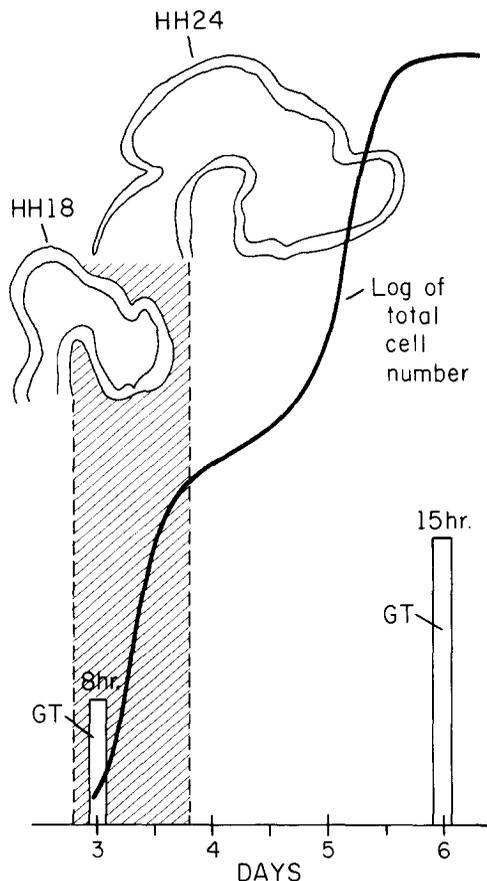
FIG. 3. During the 24-hr interval of brain intubations (cross-hatching) between 2.75 days (stage 18) and 3.75 days (stage 24) of incubation, there is a marked increase in the total number of cells in the mesencephalon (the curve is from Källén, 1961, who did not label his ordinate). The generation time (GT) of ventricular cells in the mesencephalon is indicated by the bars (from Wilson, 1973). The outline drawings of the brains at stages 18 and 24 are to scale and illustrate the dramatic increase in both tissue and cavity in this time period.

bryos were incubated for 24 hr. Some were incubated for shorter or longer periods (4 to 48 hr) to establish that 24 hr of incubation was the best time to examine experimental results. Sham controls were intubated at stage 18 with a solid rod the same size as the experimental tubes and were fixed, sectioned, and measured 24 hr later at stage 24. All controls were prepared at the same time as experimental embryos, and they were incubated together.
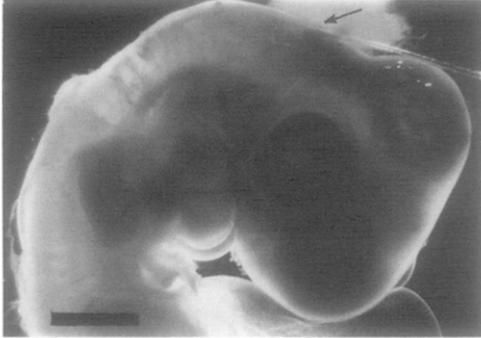
FIG. 4. A stage 24 embryo that was intubated in the metencephalon 24 hr before the picture was taken. The arrow indicates the site of tube entrance into the brain cavity. Bar = 1.0 mm.

Volume measurements. Live embryos were excised from the yolk and immediately fixed in Bouin's fixative. Viability was checked before fixing to insure that we did not look at postmortem changes, and the glass tubes were removed from experimental embryos before fixation. Serial sagittal sections were cut at 5.76 μm on a calibrated microtome. To obtain volumes of the brain tissue and cavities, images of each section of prosencephalon, mesencephalon, and presumptive cerebellum were projected onto uniformly dense paper, and tracings were made. These traced images were cut out and weighed. The area of these paper images was determined by comparing the weight of the cutout images of sections to the weight of paper squares of known area. The actual area of tissue and cavities was calculated by dividing the area of paper cutouts by the magnification squared. Volume was then obtained by multiplying the area by the thickness of the sections.

This method of determining volume is very time consuming. For example, once serial sections are made, the tracing, cutting, and weighing of the sections of a stage 26 embryo takes approximately 200 hr and involves about 850 sections. Therefore, our number of measured cases is small. Volumes of stage 26 embryos measured planimetrically take one-fourth of the time and agree within 1% of volumes

measured by the weighing method. Therefore, a planimeter was used to measure volumes of some of our cases. As controls, we have measured nine brains at stage 18, five at stage 24 (24 hr later), and five at stage 26 (48 hr later). Four brains of experimental embryos intubated at stage 18 and incubated for 24 hr have been measured. One sham-control brain was measured.

The volume measurements are not accurate absolute values because of shrinkage during fixation, but we assume that shrinkage affects different stages and different embryos to the same extent, so that the relative differences in volume between stages and between normal and experimental embryos should be reliable. (See, for example, Summerbell, 1976).

We have made some volume determinations using every fifth or every fourth section for comparison with our data using every section. These comparisons show that, for stage 18 embryos, every fifth section may be used with less than 1% error from the value obtained using all sections to determine the volume. However, the error increased to 10% when using every fifth or fourth section of stage 26 embryos. We recommend using every section for older more complexly shaped embryos.

RESULTS

After 24 hr of intubation, regardless of the site of intubation, the entire central nervous system, including the eye, is severely affected by the lack of cerebrospinal fluid pressure. Without this pressure, the nervous tissue of the neural tube folds into the lumen of the neural tube, and the sensory retina of the eye folds into the ocular cavity (Fig. 5).

In general, our results show that the longer the embryo is intubated, the more often folding of nervous tissue is seen, and the greater is its extent. Data based on a random sample of 66 successful intubations showed that nervous tissue folding was found in none of the cases intubated only 4–10 hr (six examples). In embryos
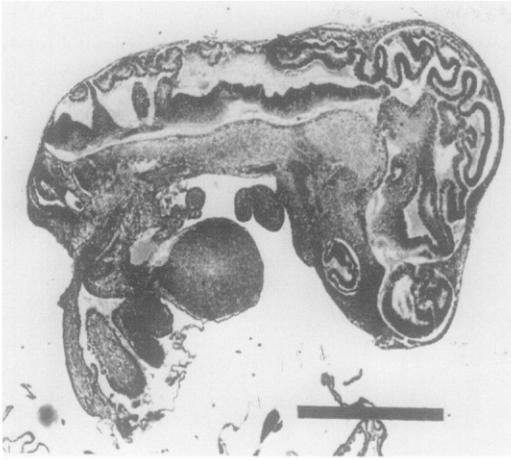
FIG. 5. Extensive folding of the nervous tissue into the neural cavities of the spinal cord and brain in an embryo intubated at stage 18 and fixed and sectioned 24 hr later (at stage 24). This parasagittal section includes the eye to illustrate that folding of sensory retinal tissue also occurs when the brain is intubated. Bar = 1.0 mm.

intubated 10–24 hr, nervous tissue folding was found throughout the neural tube in 75% of the cases (9 of 12 examples). In seven embryos intubated 24–48 hours, 100% showed nervous tissue folding in the mesencephalon, the hind brain, and the spinal cord. Of this group, 89% also showed folding in the diencephalon, 78% also showed folding in the eyes and telencephalon. (Those cases that failed to fold in the anterior brain parts may have closed off the ventricular lumen with folded nervous tissue anterior to the site of intubation, making the anterior areas closed fluid systems capable of supporting continued anterior brain expansion.)

Exact timing experiments show that at least 8 hr of intubation are necessary to produce any nervous tissue folding. This probably reflects the time needed for a complete cell cycle. We have chosen 24 hr of intubation time to maximize positive results. According to the data of Wilson (1973), two or more cell cycles could occur in this 24-hr period. In control cases (six examples), 100% of the embryos intubated 24 hr with solid glass tubes had the same

appearance as the nonintubated embryos (Fig. 6).

The parts of the brain that we have measured are those anterior to the rostral border of the first rhombomere. In normal control embryos, the total volume of these parts increases 29-fold over a 48-hr period between stages 18 and 26 (Fig. 7). This increase is due to a 50-fold increase in cavity volume and an 18-fold increase in tissue volume. The impressive increase in cavity volume is two and a half times more than the increase in tissue volume. The rate of increase of volume of both cavity and tissue is slightly less between stages 24 and 26 than between stages 18 and 24.

Experimental embryos were intubated for 24 hr between stages 18 and 24. Changes in volumes of cavities and tissue of brains of experimental and control embryos are shown in Fig. 8. The volume of the sham control lay within the range of the normal controls. A normal stage 18 embryo increases its cavity 10-fold in 24 hr, compared to a 1.6-fold increase in cavity size for intubated embryos. While the near doubling of the tissue volume of intubated embryos is significantly greater than that of the stage 18 controls at the 95% confidence level using the Mann–Whitney U-test for nonparametric data, it is apparent that this increase in tissue volume is less than the increase in tissue volume found in normal and sham controls in the 24-hr period.

DISCUSSION

Our intubation experiments show that cerebrospinal fluid pressure is necessary for normal brain enlargement and shaping. When positive cerebrospinal fluid pressure is lacking, the ventricles expand to only a fifth of normal size. Brain tissue, however, continues to grow at a reduced rate and folds into the ventricles. Since the brain ventricles and spinal canal are a single compartment, one would expect to find the results of intubation throughout the whole nervous system, regardless of
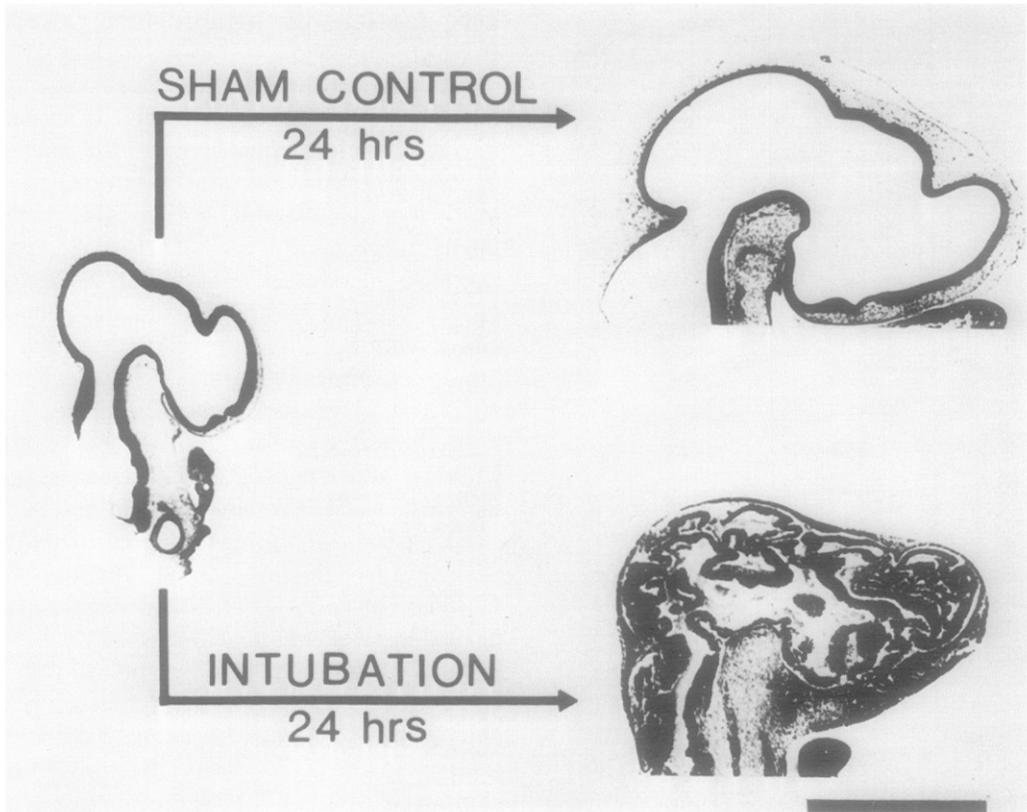
FIG. 6. Midsagittal sections of heads of embryos, illustrating brain shape and size at the time of intubation (left) and 24 hr later in embryos intubated with a solid glass rod (top, sham control) or a hollow glass tube (bottom). Embryos were at stage 18 (65-69 hr) when intubated (left) and at stage 24, 24 hr later (right). Release of cerebrospinal fluid pressure through the hollow tube results in brain tissue folding, improper shaping, and a threefold less total volume increase than sham controls (Fig. 8). Bar = 1.0 mm.

the site of intubation. All parts of the central nervous system were affected by intubation in the metencephalon. The eye was also affected which suggests that positive intraocular pressure in these 3- to 5-day-old eyes depends on positive cerebrospinal fluid pressure. Probably, the brain ventricle effectively communicates through the optic stalk with the optic lumen.

The fact that a hydraulic system is a driving force in morphogenesis of the brain is not a unique occurrence in embryogenesis. Coulombre's (1956) example in the older chick eye has already been discussed. Chapman (1975) reviews the versatility of hydraulic systems in transmitting force in many different instances of invertebrate morphogenesis and locomotion. Wilhelm

His, writing in 1888 (Coleman, 1967), was impressed by the fact that the embryo might utilize mechanical forces to shape itself. He reminded his colleagues that "Embryology and morphology cannot proceed independently of all reference to the general laws of matter–to the laws of physics and mechanics." Boyden's experiments in 1924 suggested that normal development of the allantois is dependent upon the mechanical distention provided by excretions of the mesonephric kidney.

A positive cerebrospinal fluid pressure is necessary for a normal increase in volume of the brain ventricles. The brain is essentially blown up somewhat like a balloon. The wall of the "balloon" consists of brain tissue, mesenchymal cells, matrix, and the
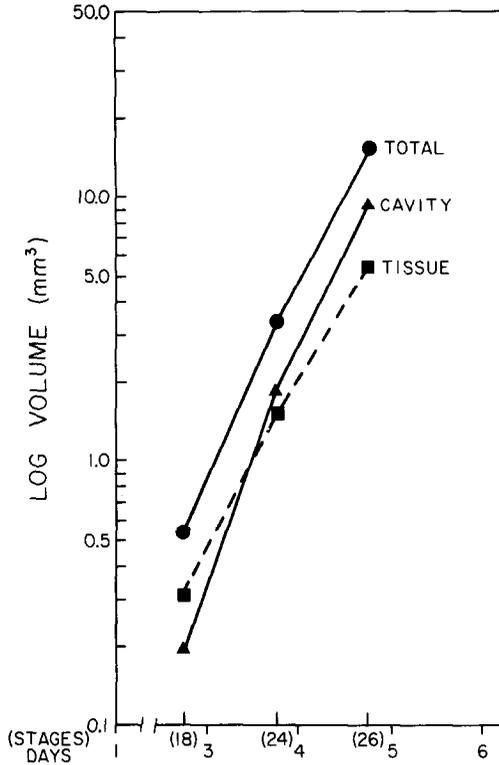
FIG. 7. Average total volumes of the brain (anterior to the rostral border of the first rhombomere) at three stages are compared to the average cavity volumes and brain tissue volumes. See text (Materials and Methods) for numbers of cases in each group. The time intervals between stages 18 and 24 and between stages 24 and 26 are 24 hr each.

epidermis (Fig. 9). The mesenchyme will later form meninges, skull, muscles, and dermis. Just as an expanding balloon can be shaped by local application of resistance to expansion, subtle differences in the resistance to expansion of the brain wall, mesenchyme, and epidermis may shape the brain.

Bergquist and Källén (1953) suggested that brain shaping is the result of intrinsic patterning of mitosis, migration, aggregation, and differentiation of brain cells. The contribution of these processes may, in some cases, be in conjunction with the expansive force of cerebrospinal fluid pressure and the differential resistance of the tissues.

We agree with Weiss (1955) who stated that gross mechanical factors do not create the differentials manifested in cave-ins, outpocketing, fissures, or folds, but merely translate them into more conspicuous configurations.

Coulombre and Coulombre (1958b) describe the differentiation of scleral cartilage in a ring around the corneal limbus.
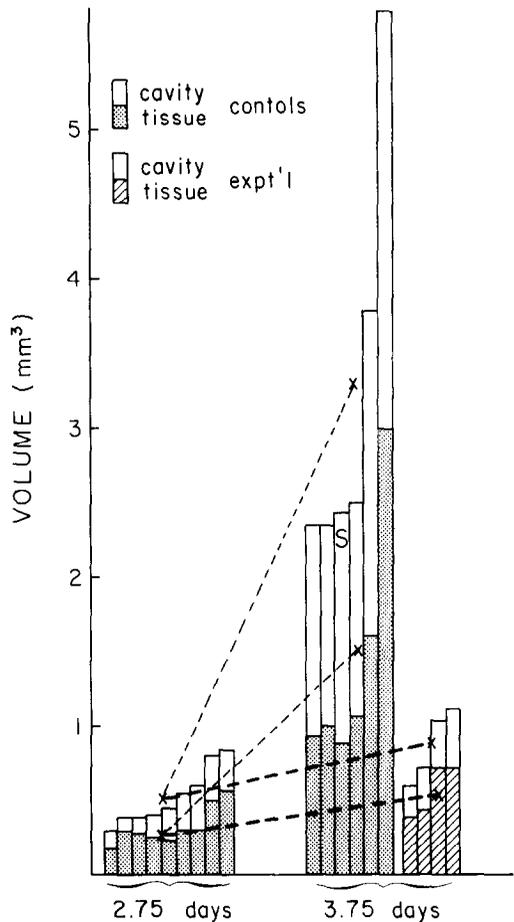
FIG. 8. The total volume of the brain is composed of cavity volume and tissue volume. Each bar indicates the total brain volume and the proportions that are cavity and tissue. The volumes measured include all parts of the brain anterior to the first rhombomere. Measurements are shown for nine normal controls at 2.75 days (stage 18) and 3.75 days (stage 24) for five normal controls, a sham control (S) and four experimental embryos that had been intubated since stage 18. Lines connect the mean volumes (X) of controls (light dashes) and experimental cases (heavy dashes).
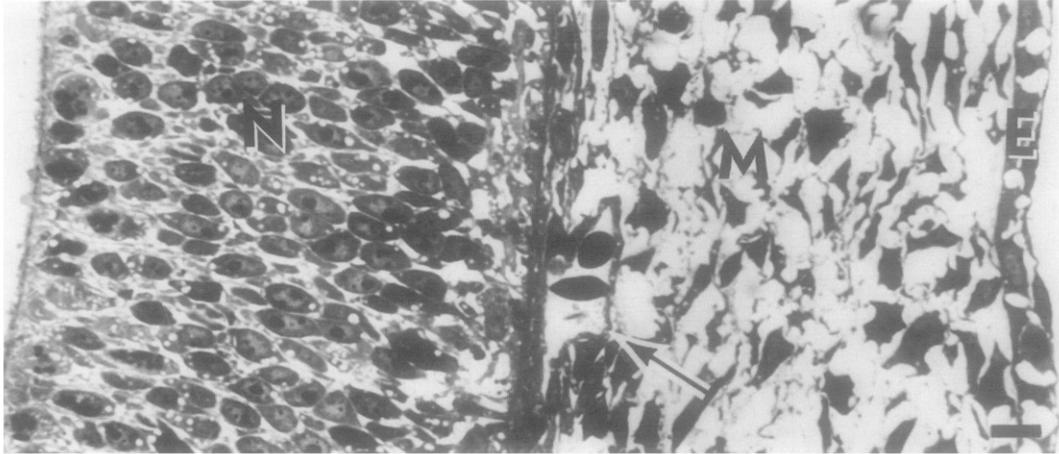
FIG. 9. Plastic section (1.0 μm thick) through the dorsal wall of the diencephalon of a stage 24 embryo. The brain cavity is visible (left). The mesenchyme (M) between the neural tissue (N) and epidermis (E) has not yet segregated into discrete meninges, skull, muscles, dermis, etc., but the area is vascularized (arrow). Bar = 0.01 mm.

From observations and experiments, they conclude that this scleral ring (by resisting expansion of the eyeball) is instrumental in the differential shaping of the cornea and the rest of the eyeball.

The folding of the nervous tissue into the lumen of the brain that we see in our intubated embryos appears to be very similar or identical to the brain abnormalities that Patten (1952) described in aborted human fetuses and which he called "neural overgrowth". Other investigators have experimentally produced folding of nervous tissue within the ventricles in chick embryos which appears similar to our results and to the brain anomaly Patten (1952) described. However, they seem to regard the folding of brain tissue as an abnormal hypertrophy of the nervous tissue without regard for the fact that the ventricle may be reduced in size (Sjodin, 1957; Bergquist, 1959; Burda, 1968).

Bergquist (1960) measured planimetrically the volume of cerebral hemispheres of 4- to 8-day-old chick embryos and found no differences in hemisphere tissue volume between normal embryos and embryos in which he had experimentally produced "neural overgrowth". He found the number of mitoses in "overgrown" hemispheres to be greater than normal and concluded that the folding of the nervous tissue in brains with "overgrowth" was similar to the development of neuromeric bulges described earlier (Bergquist and Källén, 1953). Wilson (1974) has measured cell cycles in the overgrown optic tectum. Compared to normal, the cell cycle is longer, and the duration of mitosis is twice as long in overgrown brain tissue in 3- to 6-day-old chick embryos. Wilson points out that counting mitoses does not measure proliferative activity without knowing changes in generation time. She concludes that, since the cell cycle slows down, the term "overgrowth" is a misnomer.

Jelínek (1961) also has produced "neural overgrowth" experimentally by lowering or removing cerebrospinal fluid pressure. He concluded that the abnormality was not an overgrowth but rather a folding of the tissue into a cavity space that had remained smaller than normal because of experimental manipulation. This conclusion was based on volume estimates done by eye.

It is apparent from our experiments and from volume measurements that folding of

nervous tissue into the lumen is not a case of overgrowth. There is actually an under-production of neural tissue and a considerably smaller cavity. Upon consideration of the variety of manipulations that produce this syndrome, it seems likely that brain folding may result from one primary defect, the lack of adequate cerebrospinal fluid pressure.

In conclusion, we want to emphasize the fact that our findings suggest that normal brain enlargement involves a mechanical force, cerebrospinal fluid pressure, as well as an increase in cell number and size. In the absence of cerebrospinal fluid pressure, brain tissue growth is deficient and grossly disorganized. Since the embryo utilizes cerebrospinal fluid pressure to enlarge the brain, it may also utilize differential resistance along the neural tube to shape the early brain. Localized resistance to brain expansion could be due to regional characteristics of brain tissue, mesenchyme, matrix, or epidermis, and these tissues could be interacting.

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