The carbonic anhydrase inhibitor methazolamide was used to test the hypothesis that carbonic anhydrase activity plays a role in the early morphogenesis of the chick eye. Methazolamide was administered topically to eyes of 4-7-day-old chick embryos in shell-free culture. Either continuous application of drug solution with a miniosmotic pump or a single daily application of drug resulted in significant reduction in embryonic eye growth relative to sham-treated controls. *Invest Ophthalmol Vis Sci* 30:783-785, 1989

Carbonic anhydrase-II (CA-II) is expressed to very high concentrations in the early embryonic chick eye. Quantitative and immunohistochemical analyses have shown that the level of CA-II in the embryonic optic neural ectoderm reaches a peak between days 4 and 5 of development. This corresponds to the time in development when the choroid fissure closes and the eye becomes a closed, chambered organ. At this time, CA-II constitutes 3% of the total tissue protein of the optic neural ectoderm and is also present in the lens. Hence, the early embryonic eye can be thought of as a contiguous sphere of ectoderm-derived tissues which is further distinguished by its uniquely high concentration of CA-II.

Following closure of the choroid fissure, the embryonic eye begins rapid spherical expansion and growth. This growth is dependent on intraocular pressure. Since CA-II activity influences intraocular pressure in adult mammals, we decided to examine the possibility that the enzyme also influences intraocular pressure and hence eye morphogenesis in the embryo.

**Materials and Methods.** These investigations adhered to the ARVO Resolution on the Use of Animals in Research. Fertile White Leghorn chick eggs were purchased from the University of Florida Division of Poultry Science. The eggs were incubated in a forced-draft humidified egg incubator (Sears, Jacksonville, FL) at 38.8°C. On day 3 of incubation, the shells were removed and the contents were placed in culture according to Dunn. The embryo cultures were then placed in a humidified CO₂ incubator and the CO₂ concentration was regulated to 1%. Treatment of embryos began after 1 day in embryo culture, that is, on the day during which the choroid fissure closes.

Methazolamide was provided by Dr. Thomas Maren (University of Florida). Methazolamide (5 mM) was prepared in Tyrode's solution. Two drug treatment paradigms were used. First, solutions of methazolamide or vehicle (Tyrode's) were loaded into Alzet (Alza Corp., Palo Alto, CA) miniosmotic pumps according to the manufacturer's specifications. The pumps were attached to a capillary tube which was also filled with the drug or vehicle solution. The end of the capillary tube was then positioned over the exposed eye of an embryo (Fig. 1). The pump itself was submerged in phosphate-buffered saline in a test tube which was attached to the side of the culture vessel (Fig. 1). The specific pumps that were used delivered 1 μl/hr when submerged in physiological saline. Eye growth was monitored by daily measurement of eye diameter with a dissecting microscope fitted with an eyepiece micrometer. The volume of the eye was calculated assuming a spherical eye shape.

In the second drug application paradigm, 40 μl of methazolamide or Tyrode's solution was injected into the space between the head ectoderm and the extraembryonic membranes. Embryos lie on their side, which results in the formation of a depression in the extraembryonic membranes beneath the embryo. The eye is by far the largest protuberance of the head and thus forms the deepest pocket in the extraembryonic membranes. Thus, fluids injected into the depression in the membranes naturally flow down and pool around the down-side eye. In this drug paradigm, 40 μl was administered once each day. After 3 days, or the equivalent of 7 days of total development, embryos were harvested. Eyes were dissected free of surrounding tissues and the diameters measured and volumes were calculated.

**Results.** In preliminary studies we injected various doses of radiolabeled CA-II-inhibiting drugs into chick eggs. In these cases we used radiolabeled acetazolamide, a compound whose structure and activity is very similar to that of methazolamide. Evaluation of the distribution of radioactivity in the embryo indicated that most drug became sequestered in the yolk. Little drug accumulation in the eye could be achieved by directly injecting the drug into the egg. Inhibition of the physiologically important activity of CA-II requires sufficient inhibitor to block available enzyme by 99%. Thus we sought a more direct method of delivering CA-II inhibitors to the embryonic eye.
Fig. 1. This figure depicts the topical administration of methazolamide-containing or control Tyrode's solutions to chick embryos in culture. Alzet miniosmotic pumps immersed in saline delivered 1 μl/hr via the capillary pipette to the surface of the embryo's exposed eye.

Figure 2 shows the effects of topical administration of 5 mM methazolamide on the growth of embryonic chick eyes. In this experiment, methazolamide was applied with Alza miniosmotic pumps. The experiment was started (time 0) on day 4 of development (ie, 1 day after the embryos were removed from the shell). Eye diameters were measured daily. Drug (Treated) and sham (Control) solutions were prepared and embryos were treated "blind" to assure that no investigator bias would occur in the measurements. The calculated volumes of the eyes are plotted versus the days of incubation following the initiation of treatment. Error bars show the standard deviations for each point. Each point was derived from 9–15 embryos. At the outset each group consisted of 15 embryos. At termination of the experiment, the control group had lost six embryos to fungal contamination and the treated group three.

The results of this experiment indicate that the topically administered methazolamide caused a decrease in the rate of spherical expansion of the growing embryonic eye. A student t-test showed the two groups to be significantly different ($P < 0.01$) on both days 2 and 3 of treatment. Gross observation and histological analyses of the harvested eyes failed to detect any other differences between the two groups of eyes.

The results obtained with the second drug administration paradigm were qualitatively similar to those presented in Figure 2. In this experiment 40 μl of 5 mM methazolamide in Tyrode's or Tyrode's alone was injected into the space surrounding the bottom eye of a 4-day-old embryo. The embryos were then allowed 3 more days in culture with daily administration of drug or Tyrode's. At the termination of the experiment the bottom eyes of each embryo were dissected free of surrounding tissue and then measured. Again, drug and control solutions were administered blind to prevent investigator bias. Each trial for this treatment paradigm consisted of four to eight embryos. The experiment was repeated five times. The results showed a very consistent 25% reduction in the size of eyes treated in this manner. The contralateral eye from each of these embryos was also evaluated and no consistent effects of drug treatment were noted. This suggests that the effects of the drug were limited to the bottom eye, which actually contacted the drug solution as the injected solution pooled around the protrusion of the lower eye. Again, gross observation and histological analyses showed no other marked effects on eye development from methazolamide exposure.

Discussion. The results presented here support the hypothesis that CA-II activity can influence the morphogenesis of the vertebrate eye. Methazolamide is a specific inhibitor of CA activity with solubility characteristics superior to those of acetazolamide (Dia-ox$^\text{®}$). We chose to use this sulfonamide inhibitor of CA because of its documented capacity to penetrate the eye when administered topically. Methazol-amide has CA inhibitory characteristics similar to those of acetazolamide and can bring about complete...
inhibition of CA-II at micromolar concentrations.\textsuperscript{4,6} We used millimolar concentrations of drug in our experiments due to the routes of administration. It is likely that most of the drug that we administered in either protocol diffused away from the intended site of action fairly quickly, thus necessitating high concentrations of drug during treatment. The specificity of methazolamide for carbonic anhydrase is well documented,\textsuperscript{4} and thus the effects we have measured are likely due to inhibition of carbonic anhydrase. Immunohistochemical studies with both polyclonal and monoclonal antibodies indicate that CA-II is the specific carbonic anhydrase isoenzyme which is found in the embryonic eye.\textsuperscript{1,7} Thus it is likely that the effects on eye growth that we have described are due to inhibition of CA-II activity. Other interpretations are possible, based on more indirect action of our drug treatments, but certainly the simplest interpretation is that CA-II activity is involved in eye growth.

The mechanism by which CA-II activity could generate morphogenetic intraocular pressure is presumably similar to the way CA-II influences the secretion of aqueous humor in the mature eye. Briefly, the $\text{HCO}_3^-$ generated by the action of CA-II, and other ions (Cl\textsuperscript{-}, Na\textsuperscript{+}, H\textsuperscript{+}) are transported by the epithelial cells such that a net osmotic imbalance results in the closed chamber of the eye. The osmotic imbalance results in the passive movement of water into the optic compartment, generating intraocular pressure.\textsuperscript{3} Coulumbre's work clearly demonstrated that intraocular pressure is critical for normal eye morphogenesis.\textsuperscript{2} In Coulumbre's studies, embryonic chick eyes were penetrated with micro-catheters which relieved the pressure differential between the inside and outside of the eyes. In those studies eye growth was stopped nearly completely.\textsuperscript{2} Our efforts provided only a partial inhibition of eye growth. Hence, it is possible that CA-II activity contributes only to a portion of the morphogenetic intraocular pressure. Additionally, our drug administration paradigms probably resulted in incomplete inhibition of CA-II for the duration of the experiments.

Coulumbre hypothesized that the osmotic pressure in the embryonic eye is generated through the secretion of the glycosaminoglycans and proteoglycans that are components of the vitreous humor. It is likely that this is true at some point in development. Our study seems to indicate that at the initiation of eye expansion, CA-II and mechanisms similar to those responsible for the secretion of aqueous humor in the adult eye also play a role in morphogenesis of the embryonic eye.

Key words: carbonic anhydrase-II, morphogenesis, eye development, methazolamide, chicken

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