Direct observation of secretory pumping in vitro of the rabbit eye ciliary processes

Influence of ion milieu and carbonic anhydrase inhibition

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The function of the ciliary processes in the rabbit eye has been studied by a new technique with a slit lamp and photographic registration in in vitro experiments on living processes. The appearance of the processes and their shrinkage during the course of an experiment have been taken as a measure of secretory activity. The secretory activity was shown to be potassium dependent, and could be blocked by carbonic anhydrase inhibition. It is thought that this new technique of obtaining information on secretion in the ciliary processes in the eye might be of use in studies on other secretory problems, too.

In a previous article, the membrane potentials from the ciliary processes in the rabbit eye were described. It was incidentally found that during the course of an experiment the ciliary processes gradually became thinner. Though unimportant in these experiments, this observation was nevertheless considered to be of interest. A further investigation seemed to be justified on the assumption that the secretory activities of the ciliary processes caused the shrinkage.

A ciliary process consists of a loose stroma bordered by two epithelial layers. The latter take part in the secretion of aqueous or some of its constituents. These constituents are derived from the stroma and moved across the ciliary epithelial layers. Under normal conditions in the intact eye this would not influence the appearance of the processes. In an excised living ciliary process, however, where the continuous supply of constituents from the capillaries is cut off, secretion or pumping is dependent upon a limited and irreplaceable amount of material in the stroma. If we regard a ciliary process as a bag, and there were no additional supply of constituents to the stroma, the secretory activity would stop when the supply of constituents in the stroma was exhausted. The epithelial layers have then pumped the constituents (plus water) from the inside to the outside and we could expect the ciliary process bag to be empty or shrunken. On the other hand, if there were no shrinkage, this could be due to the fact that the pump mechanism had stopped. An objective observation

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of the appearance of the processes and their time variations during various conditions would indirectly give information of secretion and of the factors that influence secretion. A preliminary report of these findings of the author has been given by Barany.2

Material
Male albino rabbits weighing about 2 kilograms were used throughout the experiments. They were fed a diet of hay, oats, and water ad libitum.

Methods
In order to study secretion or pumping in in vitro experiments with excised living ciliary processes, the following points were considered important. The epithelial layers should border compartments (that is, in this case, stroma and bath) with identical or, at least, known composition, and it should be possible to calculate shrinkage objectively. Furthermore, as secretion was measured by shrinkage of the processes, it was important that secretion was prevented as much as possible up to the point of commencement of recording.

Operation procedure. During this phase of the procedure, the stromal content of the ciliary processes was replaced with a buffer solution by perfusion through a carotid artery in the living animal. Since it was desirable to obtain the largest possible processes in the succeeding in vitro experiments, measures were taken to fill the processes maximally and to prevent shrinkage. Only one eye from each animal could be used.

The animal was anesthetized by an intravenous injection of 5-allyl-5-isopropylbarbituric acid (Numal)*, 0.6 c.c. per kilogram of body weight. The carotid veins and arteries were dissected free on both sides of the neck. Heparin, 5,000 I. U. (Vitrum, Sweden), was given to prevent blood clotting. A cannula connected to a reservoir containing the perfusion fluid was inserted into one artery. Perfusion was carried out at a pressure of approximately 300 cm. H2O. The perfusion fluid was kept ice cold, and continuously bubbled with a N2/CO2 mixture (N2 = 95 per cent, CO2 = 5 per cent). In order to replace the blood volume by perfusion fluid, the artery was cut open below the cannula. The corresponding vein was also opened. Perfusion was carried on for 3 minutes, and the fluid appearing from the opened vessels was then blood-free. The veins and arteries on both sides were then ligated, except the part of the artery connected to the perfusion apparatus. The connection to the perfusion fluid under 300 cm. H2O pressure was maintained about 17 minutes. In order to facilitate the development of edema in the processes, the eye on the perfusion side was opened well behind the limbus. The eye as well as the perfusion fluid were kept ice cold throughout. The eye was then enucleated.

In vitro preparation and recording. The iris and ciliary region were dissected free under ice cold buffer solution and attached to a plexiglass cylinder (Fig. 1). The cylinder was then placed in a

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*Rocher Laboratories.
bath, which consisted of a plexiglass box filled with 50 ml. buffer solution and equipped with arrangements for temperature regulation and gas bubbling. These preparations took about 5 minutes.

Shrinkage of the ciliary processes was studied indirectly by measuring a transverse optical section of the processes intermittently during the course of an experiment. A slit lamp focused transversely on the ciliary processes gave an optical section of 50 μ thickness of the processes. According to the anatomy of the ciliary zone, the “middle” section was used where the processes are erect and vascular.3 Pictures of this section were taken intermittently during the course of an experiment lasting 60 to 90 minutes.

After arrangement of the specimen and photographic adjustment, the first pictures were taken in the ice cold solution. This was recorded as zero time. (Time from operation: blood-buffer change, 3 minutes; stasis, 17 minutes; and emaciation and preparation, 5 minutes. Zero time thus meant about 25 minutes after death.) The ice cold solution was then replaced by a solution at 30° C. without disturbing the arrangements (Fig. 1). Temperature was kept at desired values within ± 1.0° C. with the aid of a thermostat. The gas mixture used was O2/CO2 (95 per cent O2, 5 per cent CO2). Samples for pH measurements were taken intermittently from the bath during an experiment, and checked with a glass electrode. Standard experiments were performed at 30° C. and a pH of 7.4. In other experiments the temperature was varied between 20° to 41° C. and the pH between 7.0 and 7.7.

Calculations. The area of the transverse section of a ciliary process was measured by planimetry on enlarged photographs. About six processes were obtained on each photograph, 1 to 4 of which were suitable for planimetry. The size of the processes could vary between different animals but to a lesser extent in the same individual. Percentage values instead of absolute values were thus used. The zero time value was taken as 100 per cent. The percentage values of the different processes were averaged for each time. The single eye (or animal) was treated as the statistical unit.

Solutions and drugs. Krebs4 solution nr 1 was ordinarily used as buffer. The chemicals were of analytical grade, except for the organic salts.

Fig. 2. Drawings made from photographs obtained during the course of a standard experiment (1 to 90 minutes).

Fig. 3. An optical section through the ciliary processes (same experiment as that in Fig. 2). A, At start of secretion (1 minute); B, After 90 minutes.
which were available only as "purified." In some experiments, potassium or calcium was replaced by sodium in the Krebs solution. The perfusion fluid and bathing solution were always identical.

Another solution used, but containing no ions, was Macrodex (Pharmacia, Sweden) (containing 6 per cent dextranum hydrolysatum Mr. 75,000, and made blood isotonic with glucose).

Results

Standard experiments. In standard experiments the ciliary processes gradually shrank during the course of an experiment (60 to 90 minutes). The transverse section area of the processes was reduced by about 25 per cent during the first 10 to 20 minutes, reaching a maximum reduction of about 40 per cent after 40 to 60 minutes (Figs. 2 to 4). The mean one hour value for the remaining section area in standard experiments was 54.9 (standard error of the mean ± 5.4, n = 10). After one hour there was only an insignificant further reduction, so 60 minutes was thus considered as an end point. Standard experiments were performed at pH 7.4 ± 0.05 and at a temperature of 30° ± 1°C.

Variations in experimental conditions.

Temperature. Two other temperature regions besides the standard temperature of 30°C were also tested. In the range 20° to 24°C (12 eyes) as well as in the range 35° to 41°C (4 eyes), there was less shrinkage or pumping. The mean one hour value for the remaining area was 70 and 75 per cent, respectively.

pH. Curves from experiments performed at pH 7.0 and pH 7.7 did not deviate significantly from the standard curve at pH 7.4 (Fig. 4).

Substitution of ions. No change could be observed when calcium was replaced by sodium in the buffer solution (Fig. 5).

However, if potassium was replaced by sodium the slope of the curve decreased, that is, there was less shrinkage of the ciliary processes, and after one hour the mean value was still more than 80 per cent. These results demonstrate that the potassium ion is a fundamental component in the normal secretion of the ciliary processes.

Carbonic anhydrase inhibition experiments. In the preliminary experiments it was not possible to influence the shrinkage of the ciliary processes by carbonic anhydrase inhibition with acetazolamide (Diamox). Failure was met whether the drug was put into the bathing solution and/or in the perfusion fluid and/or injected intravenously one-half hour before perfusion. Average and well above average doses were tried. These experiments were performed at a pH of 7.4. If the experiments were repeated in an acid or alkaline
medium, which in itself did not influence the shrinkage process, the results showed that there was no effect at pH 7.7 compared to pH 7.4; but at pH 7.0 a blocking effect on secretion by acetazolamide could be established (Fig. 6).

If a pH of 7.0 was used, this blocking effect could be reproduced without previous intravenous injection, the drug only being put into the bath solution 5 minutes after the start of secretion or pumping. This effect could be demonstrated down to \(10^{-3}\)M. There was no or little difference in the slope of the curves with doses between \(10^{-3}\) and \(10^{-6}\)M. The carbonic anhydrase inhibition was registered as a marked decrease in the slope of the shrinkage curve, reaching a final mean one hour value of about 70 per cent of the initial section area (Fig. 7).

**Discussion**

In analogy to other transport systems, the ciliary epithelial layers have been regarded as a pump system transporting ions from the inside stroma to the outside posterior chamber.

If, as in the present in vitro preparations, there is no further supply of constituents to the stroma, then "the bag" of a ciliary process will gradually be pumped empty. The shrinkage of a process might thus be regarded as a picture of the transporting secretory process. The use of identical physiologic solutions on both sides of the epithelial layers rules out any changes due to osmotic effects. In this type of experiment it is not possible to study work against concentration gradients since the ciliary process is open at its base.

That shrinkage was due to a pumping effect was further established by the fact that no shrinkage occurred when no ions were available.

The cross-section pictures of a process with a thin slit-lamp beam gave a satisfactory registration of shrinkage (pumping). It is possible that this new technique of obtaining information on secretion in the ciliary processes in the eye might be of use in studies on other secretory problems, too. Less shrinkage than normal would mean a blocking of secretion, and a more pronounced shrinkage an increased secretion. Only the former alternative—a blocking effect—could be established in the present experiments. If an increased secretion could be achieved, it is possible that it would be demonstrated more as an accelerated shrinkage than a further absolute shrinkage, since the 50 to 60 per cent value after one hour might be a lower limit value.

In previous experiments where the mem-

**Other perfusion fluid.** In order to study the effect on the shrinkage of the processes when no penetrating ions or molecules were available, some experiments were performed with a glucose-dextran solution. There was no shrinkage during these experiments (2 eyes, 5 processes).
brane potentials of the ciliary epithelial layers were studied, the potentials were found to be potassium dependent. No membrane potentials of the ciliary epithelial layers were found in potassium-free milieu. In accordance with these findings it was shown in the present experiments that the slope of the shrinkage curve was significantly less in potassium-free milieu than in standard experiments. Thus, in a completely different type of experiment, the functions of the ciliary epithelial layers have been found to be potassium dependent.

The results from experiments with acetazolamide (Diamox) are interesting from several points of view. In the aforementioned experiments with membrane potentials, these were, in preliminary experiments, found to be decreased in the presence of a carbonic anhydrase inhibitor. The results could, however, not be reproduced and were thus not included in the final study. In the present experiments it was possible to block partially the secretion process by Diamox, registered as a prevention of normal shrinkage of the processes, but only when the pH was lowered to 7.0. It might be possible that the failure to reproduce the decrease of
the membrane potentials in the presence of Diamox was caused by a pH effect.

The theory that the carbonic anhydrase enzyme system takes part indirectly in secretion by providing buffering capacity to the secretory cells has been outlined previously. If the secretory cells, as in our experiments, are presented an acid milieu, the cells will have greater difficulties in ridding themselves of their acidic excess that will result from their production of an alkaline secretion. A greater strain will be laid upon the buffering capacity, and an intact carbonic anhydrase system might be essential. If it is blocked by an inhibitor such as Diamox, the resulting diminished secretion will be discovered more easily.

In a different type of experiment, Wistrand and Maren have shown that metabolic acidosis will cause a drop of intraocular pressure in rabbits, and carbonic anhydrase inhibition reduced the intraocular pressure even further. In the present experiments the capacity of the cells was sufficient to maintain normal secretion in an acid milieu or in the presence of Diamox, but when they had to work in an acid milieu and were deprived of carbonic anhydrase, the load apparently became too much and secretion dropped.

REFERENCES