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Adenosine Stimulation of Fluid Transport Across Rabbit Corneal Endothelium

J. Fischbarg*, J.J. Lim, and J. Bourguet

Departments of Physiology and Ophthalmology, College of Physicians and Surgeons, Columbia University, New York, and Département de Biologie, Centre d'Energie Nucléaire, Saclay, France

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Summary. The rate of fluid transport across rabbit corneal endothelium has been measured with an automatic volumetric method. The present resolution of the procedure is 1–3 nanoliters, and intervals of measurement can be made as small as seconds. In the presence of glucose, oxidized glutathione (GSSG), and adenosine, the maximal rates were 6.2 ± 1.0 μl/hr cm^2, and 8.2 ± 0.8 μl/hr cm^2 if a large portion of the stroma was dissected away. In the presence of glucose and GSSG only, the rates were lower, namely 3.7 ± 0.5 μl/hr cm^2. The rates consistently increased or decreased when adenosine was added or deleted, respectively, during given experiments. The stimulation of fluid transport by adenosine was in the order of 40–50%. The results raise the possibility that this transport mechanism might be subject to metabolic control.

The purpose of the work presently reported was to develop a procedure that would allow accurate and experimentally convenient measurements of the rate at which fluid is actively transported across corneal endothelium. Other methods which have been satisfactorily used for the early studies of fluid movements in this preparation have, nevertheless, shortcomings which preclude their use for some types of experiments. The specular reflexion microscope method (Maurice, 1968; Dikstein & Maurice, 1972) has lower resolution (± 1 μm for a 2 cm^2 area, or 200 nl); in addition, since stromal thickness is measured only along the optical axis, possible uneven variations in stromal volume in other regions could go undetected; lastly, and perhaps most importantly, the actual rate of fluid transport corresponds to the rate of corneal thinning measured with such method only down to some 450 μm corneal thickness, since below that value the rising stromal imbibition pressure destroys that correspondence. Measurements of meniscus displacements (Maurice,

* *Present address:* Physiological Laboratory, Cambridge CB2 3EG, England.

1972) avoid these difficulties, but are cumbersome to perform if great accuracy and time resolution is desired. Other procedures employed to measure fluid movements in other preparations such as serial weighing (in frog skin, Huf, 1935; urinary bladder, Bentley, 1958; or gallbladder, Diamond, 1962), marker dilution (in ileum, Curran & Solomon, 1957), or serial photography of a split-drop (in kidney tubule, Gertz, 1963) all have limitations similar to some of those exposed. Since the rate of fluid transport presently considered is comparatively small (some 200 nl per min across a 1.75 cm^2 area), it is possible that some of the characteristics of this transport mechanism which might have gone undetected could be uncovered with higher-resolution procedures. In order to move towards that goal, we have adapted for this purpose a method originally developed for the measurement of osmotic flow across urinary bladder (Bourguet & Jard, 1964). This earlier instrument has been modified so that its present accuracy is 1–3 nl. The main technical aspects of the method and some of the measurements that have become possible constitute the subjects of the present communication. Some of the present results have appeared in Abstract form (Fischbarg, Lim & Bourguet, 1975).

Materials and Methods

Earlier System (Bourguet & Jard, 1964)

Briefly stated, that system consists of a microinjection unit driven by a motor with low inertia. When fluid moves across the preparation out of a closed volume the fluid level in a detector unit falls, electrical contact is interrupted and the motor turns until the fluid injected reestablishes contact, thereby maintaining a constant volume. The volume injected is given by the voltage read from a potentiometer that turns with the motor. At predetermined intervals, an auxiliary motor mechanically resets the potentiometer to zero. The height of the consecutive bars obtained by plotting voltage vs. time with a chart recorder gives a visual indication of the average rate of fluid movement during each one of the intervals.

Present Design

Most of the basic ideas of the earlier system were incorporated into the present design. A schematic diagram of the present system is shown in Fig. 1. A stepping motor was substituted in place of the regular motor. An electronic counter now displays the number of steps that the motor moves, and which corresponds to defined volume increments. As before, this volume is recorded graphically as a function of time. The number of motor steps is also available from the counters for a printer and for further numerical processing of the data. A timer resets the counter to zero at preset intervals. Aside from the electronic modifications, the most critical need to be met was the refining of the volume detector to one capable of operating at the nanoliter level.

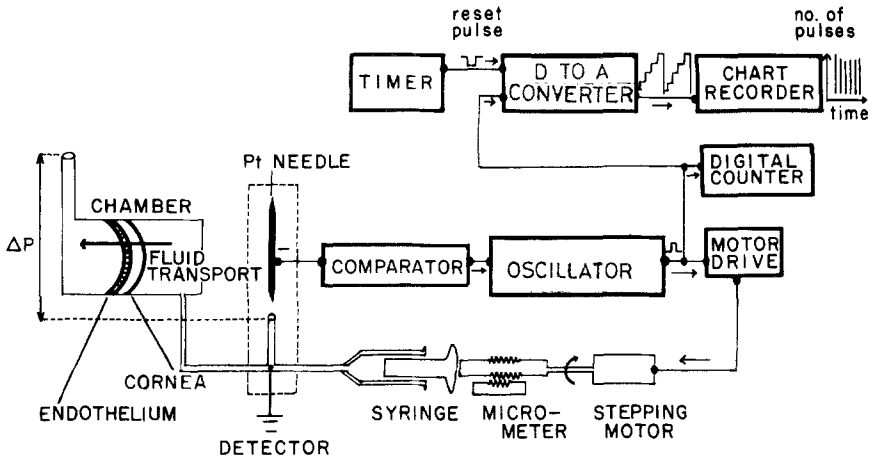

Fig. 1. Schematic diagram of the experimental chamber, microinjection device and electronic setup employed

Development of the Detector Unit

The sensing element originally adopted, following the existing one (Bourguet & Jard, 1964) consisted of a sharp needle that could make electrical contact with saline emerging from the tip of a piece of glass tubing. The present aim was to reduce the volume displacement needed to interrupt electrical contact ("dead volume") to preferably not more than 5 nl. In our early attempts, a Pt needle was electrolytically sharpened (Frank & Becker, 1964) to a tip of 5–7 μm, and was positioned directly facing the opening of a 0.8 mm o.d. glass capillary tubing. Here and in what follows, the saline solution customarily used (163 mM NaCl, or "isotonic" saline) was made to displace with an injection device consisting of an "Agla" (Burroughs Wellcome, Durham, N.C.) syringe connected to a micrometer. The stepping motor used (The Superior Electronic Co., Bristol, Conn.) drove the micrometer through a 50:1 speed reducer (Designatronics Co., New Hyde Park, N.Y.). Dead volumes with this method were in the order of 20–30 nl. In order to reduce them further, efforts were concentrated upon the reduction of the needle tip diameter. Success was ultimately reached by using commercially available Pt needles of 1 μm tip diameter (No. 31-10-03, Frederick Haer & Co., Ann Arbor). With these, the dead volume can be as low as 1–3 nl, provided that they are frequently cleaned with solvents and HNO_3. A micromanipulator and a dissection microscope were used to position the needle tip facing the center of the glass tubing opening (Fig. 2). The glass tubing and the rest of the detector were immobilized by screws against the same plate to which the manipulator is fastened; the base plate rides on blocks of porous rubber, which insulates it from most vibrations. With further improvements in the design of the electronic comparator connected to the detector, conventional KCl-filled glass microelectrodes could also be used instead of the Pt needles; this allows 1 nl resolution under most conditions. It was also judged useful to immerse the capillary and needle tip in mineral oil, which avoids evaporation and decreases the surface tension difference at the interface, allowing the needle to pierce the meniscus more readily.

Mechanical Drive

The subject of the mechanical syringe drive deserves mention. It was found that, at the level of accuracy needed, the slightest misalignment between the turning shafts

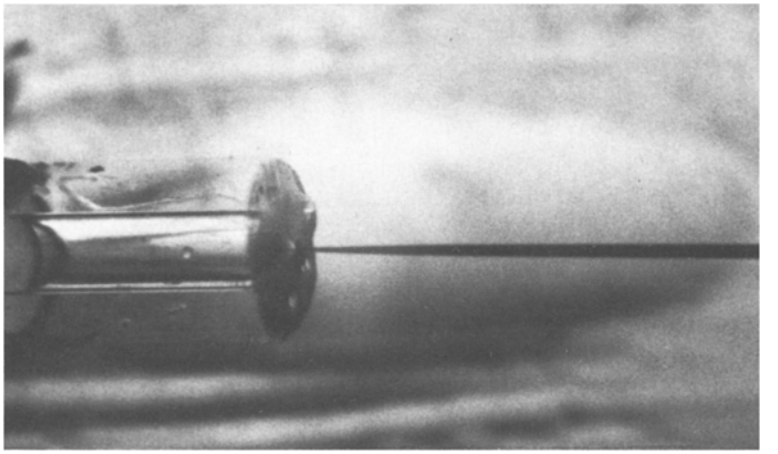

Fig. 2. Photograph of the detector of volume changes. Electrical contact is established between Pt needle at right and saline at the tip of the capillary at the left. The outside diameter of the capillary is 800 μm and the inside diameter 300 μm. The assembly is immersed in mineral oil

of the motor, slip clutch, micrometer and syringe axis could result in somewhat uneven injection and, quite especially, uneven aspiration. A universal joint (T166, P.I.C., Ridgefield, Ct.) was interposed between motor and clutch, and the other components were fastened to a specially-machined concentric mount. Best results were achieved when the syringe plunger was rigidly coupled to the micrometer shaft through another universal joint. It was helpful to lubricate the plunger with silicone grease.

Electronic Design

A schematic diagram of the present setup is shown in Fig. 1. The detector operates a comparator; this one enables the oscillator that drives the stepping-motor. The motor steps are transformed into voltage increments by a 12-bit digital-to-analog converter, which allows a maximum of 4096 steps or 4096 nl per interval. An electronic timer resets the D to A converter and counter to zero at preset intervals that can be varied at will, and disables the oscillator during the resetting interval. A more detailed description of some of these aspects will appear in another publication (Fischbarg, Wankowicz, Skezeres & Bourguet, *in preparation*).

Temperature Control

Lucite jacketed chambers were designed and built for this purpose; since additional openings in the chamber often resulted in troublesome leaks, success was ultimately achieved with a chamber endowed with only one opening into which a rubber stopper pierced by a piece of stainless steel tubing could be forced so as to close the chamber while allowing connection to the detector. The temperature of the bath whose water is circulated through the jacket was adjusted so that the chamber contents would remain at 36.80 °C. Constancy of this value is important for the present purposes, since, due to the chamber size, temperature oscillations of 0.003 °C would produce a displacement of some 3 nl due to volume expansion of water. The temperature control was therefore made to include

a proportional controller (Fisher & Scientific Co.) with a standard bath heater (FE, Haake Instruments). A sensor was chosen for its very low thermal inertia (Yellow Springs #520 thermistor). With this procedure, oscillations were ± 0.014 °C, with a period of about 1 min, and long-term drift only some 0.008 °C/hr.

Tissue Movements

Piston-like displacements of the cornea were seen to result in large errors, and the preparation therefore had to be rigidly supported. A satisfactory method was to shape a stainless steel wire cloth (100 mesh, Small Parts, Inc., Miami, Florida) as a semisphere (radius: 7.8 mm) using a metal mold and a hydraulic press. This net was secured inside the chamber; the hydrostatic pressure subsequently imposed was adequate for immobilizing the cornea against this support.

Tubing Connections

Stainless steel tubing (gauge 21, Small Parts, Inc.) was used for all the connections between the chamber and the detector system, except for the bare minimum of gauge 22 teflon tubing (I.C.O./Rally Corp., Palo Alto, California) used where flexible bends were needed. As for the configuration of the connections, since each motor step delivers to the fluid a sharp impulse, the fluid could move in either of 2 directions (*cf.* Fig. 1): (1) up the capillary into the detector, or (2) into the experimental chamber, producing a piston-like displacement of the cornea. The second possibility is clearly undesirable, and attempts were made to minimize it. A satisfactory solution was achieved by designing the connection to the detector unit so that the segment of tubing leading to the chamber opens very close to the detector's end. The hydraulic resistance between that point and the chamber is therefore some 15 times larger than that to the free end of the detector's capillary.

Dissection and Mounting

Some of the techniques employed for dissection of rabbit eyes have been detailed before (Dikstein & Maurice, 1972; Fischbarg & Lim, 1974). For the present work, the globe, held by its conjunctiva, was cut and dissected so that only the endothelium and the supporting stroma and sclera were left. For some experiments, in addition, prior to opening the globe, a large portion of the stroma was cut away by making a deep circular incision with a corneal trephine and separating the outer portion by blunt dissection following the lamellar planes. The eye was subsequently placed on an extension of the chamber, where the stroma came to rest against the supporting wire net. Suction (ca. -20 cm H_2O) kept it in that position. The conjunctiva was everted, pulled down the outside of the extension and tied to a rim. The two half chambers were then clamped together. After this, the inside was filled with the experimental solution, and all its outlets were closed; subsequently, the outer chamber was filled and manipulated so that no bubbles were left in it. The closure of all outlets on the inside prevented large corneal movements during this procedure. The only outlet in the outside chamber was closed by means of a stopper through which a piece of stainless steel tubing allowed the displaced fluid to flow out of the chamber. The pressure head employed for these experiments (10 cm H_2O) was established, the proper outlets were open and the measurements begun. After an initial settling period, the preparations usually began to pump fluid some 5–10 min after mounting.

The solutions employed were as follows: "regular solution" (RS) contained (in mM) NaCl 110, $NaHCO_3$ 39, $KHCO_3$ 3.8, KH_2PO_4 1.0, $MgSO_4$ 0.86, $CaCl_2$ 1.7, glucose 6.9, adenosine 5.0, oxidized glutathione (GSSG) 0.1. "Glucose medium" (BSG) was identical to the one above except for the fact that no adenosine was present, having been replaced by sucrose on an osmolal basis. Bubble formation inside the warm chambers was prevented by allowing the solutions to be used to come to gaseous equilibrium by warming them to some 40 °C for about half an hour immediately prior to the experiments.

Results and Discussion

Oscillations in the Rate of Fluid Movement

In some of the experiments in which high time-resolution (e.g., 30 sec intervals) was used, oscillatory or uneven behavior of the rate measured was apparent in varying degrees (*cf.* Figs. 7 and 8). In order to learn whether they were of cellular origin or due to limitations inherent in this and similar methods, control experiments were performed in artificial systems. The accuracy of the present volumetric method was evaluated by using a second analogous instrument which withdrew fluid from a closed system at constant rates and determining how closely the rate of injection of the present instrument followed those rates. As Fig. 3 shows, the agreement was total within the experimental error. Subsequently, the rate of leak induced by a hydrostatic pressure head across the 70 cm length of connecting hypodermic tubing (gauge 21, stainless steel) was measured. The hydraulic resistance computed for this tubing on the basis of Poiseuille's equation is some 2×10^5 g/cm^4 sec; as Fig. 4 shows, in this case the flow induced is recorded without oscillations, aside from the ones coming from the experimental limit of resolution. Since the hydraulic resistance of the endothelial layer is, however, much higher (7.2×10^{10} g/cm^4 sec, Fischbarg, Warshavsky & Lim, 1977) than that of the tubing, further experiments were conducted using higher hydraulic resistances. Fig. 5 shows the behavior of the hydraulic leak induced across a 15.6 cm length of gauge 30 hypodermic tubing, which has a hydraulic resistance of 1.3×10^8 g/cm^4 sec. Once more the present method measures the rates without showing major oscillations; the long period shown is comparable in duration to some experiments with the endothelial preparation. Fig. 6 shows, however, that if the hydraulic resistance is increased further, oscillatory behavior is apparent at rates below 2–3 μl/hr, which are at the low end of our experimental range. The hydraulic restriction of 1.5×10^{10} g/cm^4 sec was obtained here by compressing a length of teflon tubing with a micrometric caliper.

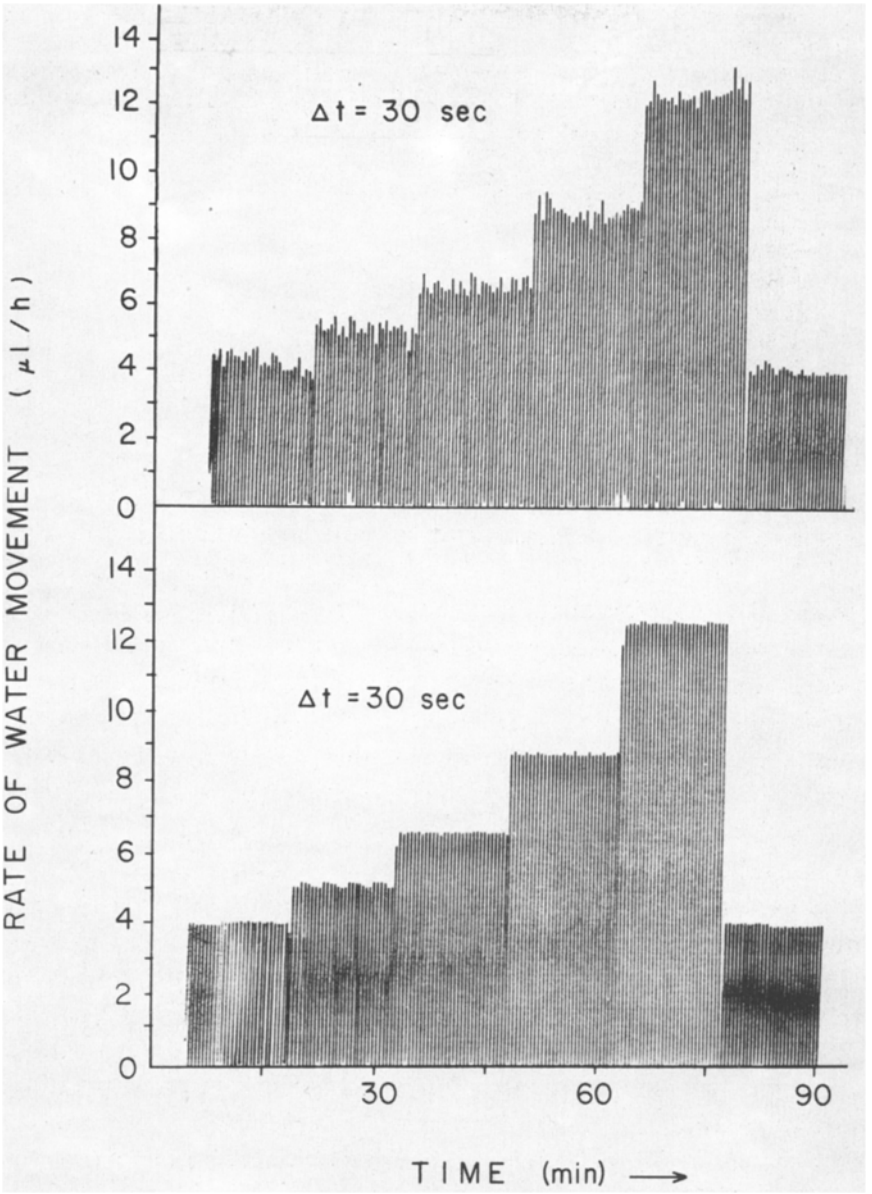

Fig. 3. Rate of fluid injection into the tubing and detector system, as compared to the rate of withdrawal simultaneously induced by a second microsyringe drive. The recording at top represents the rate of injection, obtained under command of the sensor, and "slaved" to the rate of withdrawal represented at the bottom. Time intervals: 30 sec. T=22 °C. Several rates of withdrawal and corresponding rates of reinjection were tested near the usual range of rates due to endothelial transport. The apparent delay is only due to the mechanical arrangement of the two pens in the chart recorder; changes in the "slave" rate actually took place nearly 2 sec after a change in the "driving" rate

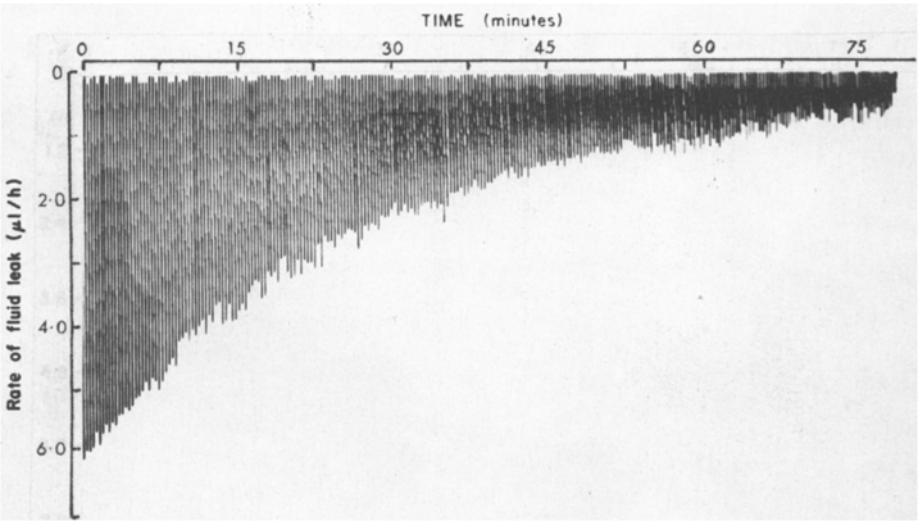

Fig. 4. Rate of leak of saline out of a 70-cm 21-gauge length of stainless steel connecting tubing. A pressure head was established by adding a small volume increment to the experimental chamber to which the tubing was connected. $\Delta P = 1$ mm H_2O; time intervals: 20 sec; T = 22 °C

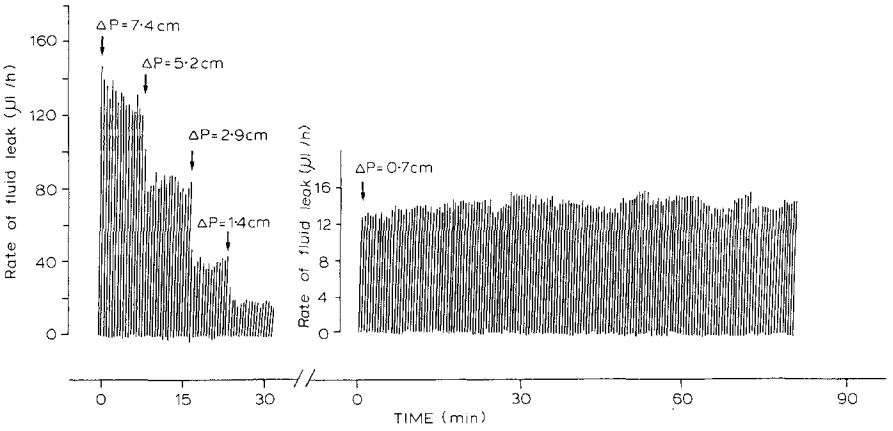

Fig. 5. Rate of leak of saline through a length of 30-gauge hypodermic tubing. The hydrostatic pressure head was adjusted as shown. Time intervals: 30 sec; T = 20 °C

In such a simplified system, a straightforward explanation for the oscillations is that the water-air (or water-oil) interface in the detector would introduce some distortion due to its surface tension; the interaction of the meniscus with the detector's capillary end is certainly complex and could change in its modality (from bulk water movements across

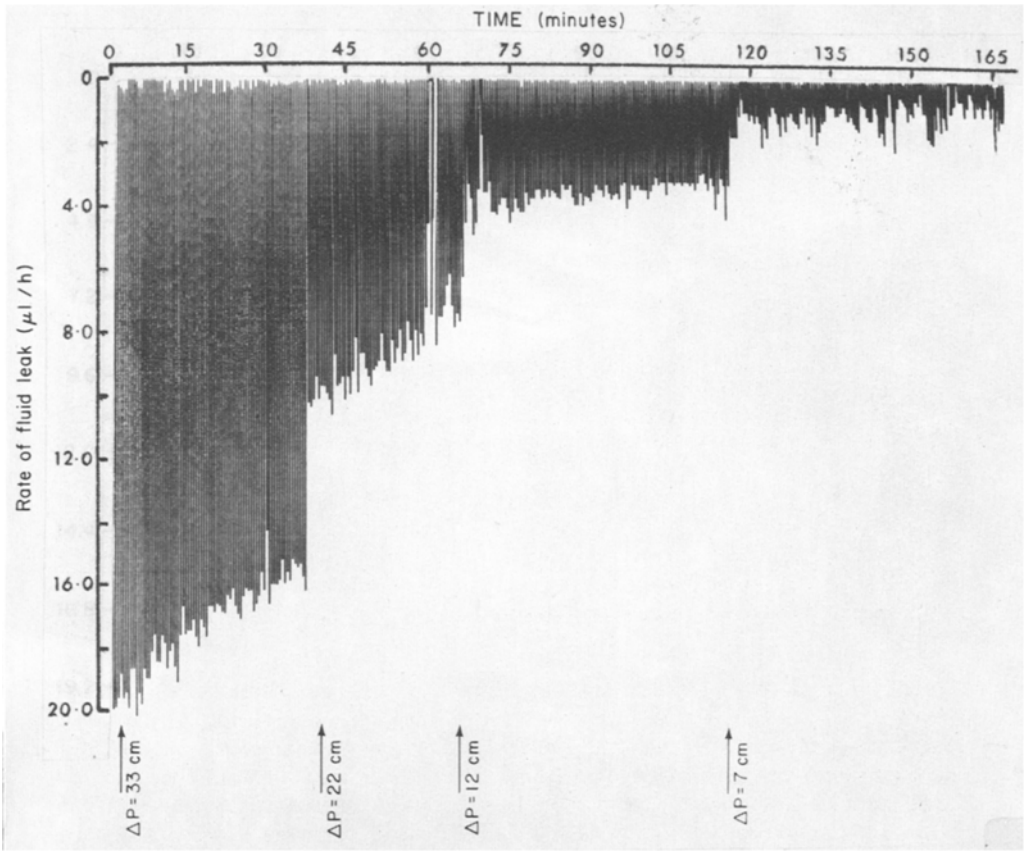

Fig. 6. Rate of leak of saline through a hydraulic restriction caused by compression of 16-gauge teflon tubing with a micrometric caliper. The different rates were obtained by adjusting the hydrostatic pressure head as shown. Time intervals: 30 sec; T=26 °C

the entire capillary area to a rubber-membrane like behavior) depending on the water-glass wall interaction. This explanation is borne by the fact that, in other experiments with an artificial system, the volume fluctuations that result in oscillatory behavior at low rates were seen to be of the same order of magnitude as the volume of the spherical segment (or convex meniscus) of water present at the capillary end.

The presence of oscillations therefore appears linked with transitions in the detector's behavior; however, much as these technical aspects might limit some types of measurements, for the present purpose they pose no problem due to the fact that they average out along sufficiently long time intervals. The desirable length of those intervals depends in turn on the magnitude of the rates being measured. As an illustration,

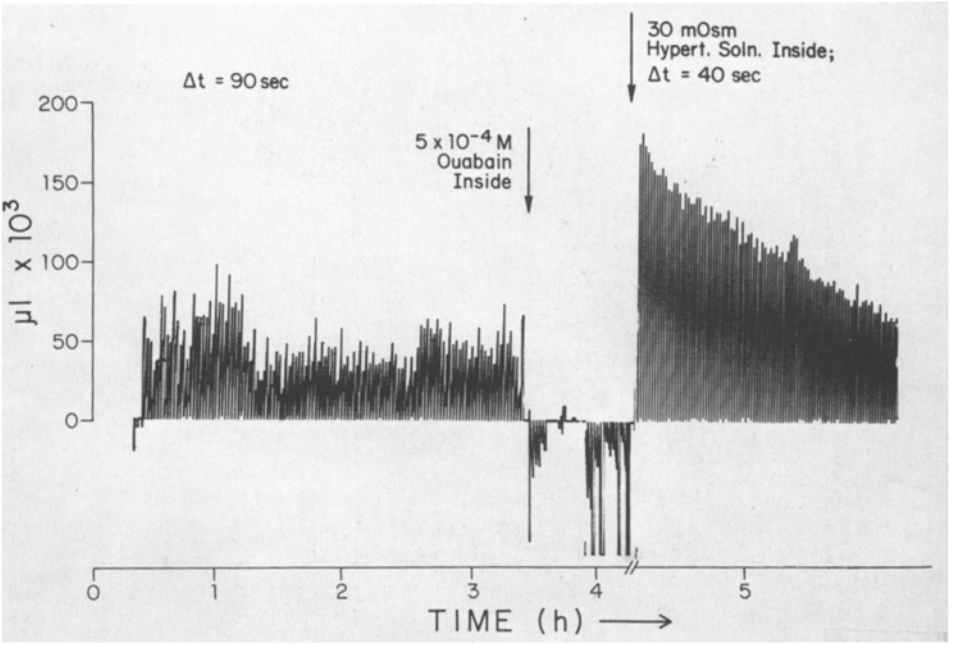

Fig. 7. Approximately 2/3 of the stroma were dissected off this preparation. After mounting, it was bathed by RS on both sides and the rate of transport was recorded. At the arrow, the solution on the inside (aqueous) side was replaced by one containing ouabain 5×10^{-4} M. The fluid pump was inhibited and the fluid movement reversed its direction, since ΔP was 10 cm H_2O. After that, the solution on the inside was replaced with RS made 30 mOsm hypertonic through the addition of sucrose, and the osmotic flow was recorded. In order to keep the tracings within the recorder's scale, the time intervals were changed as indicated. Here and in the following figures, T = 36.80 °C

Fig. 7 shows oscillations in the rate of fluid transport using 90-sec intervals; on the other hand, when an osmotic flow is imposed which entails a larger rate of water movement, the oscillations are minimal even with the use of high time-resolution (time intervals of 20 sec). For the case of the experiments shown in Figs. 9–13, in order to optimize the observation of the relative rates, a relatively low time resolution (time intervals of 4 min) was chosen that all but eliminated the oscillations.

Role of the Stroma

Of necessity, most of the experiments presently reported were performed with a "normal" preparation constituted by the endothelium attached to Descemet's membrane and the stroma. The presence of the stroma introduces a factor which merits discussion. In principle, after denuding it and placing it in contact with saline, the stroma swells

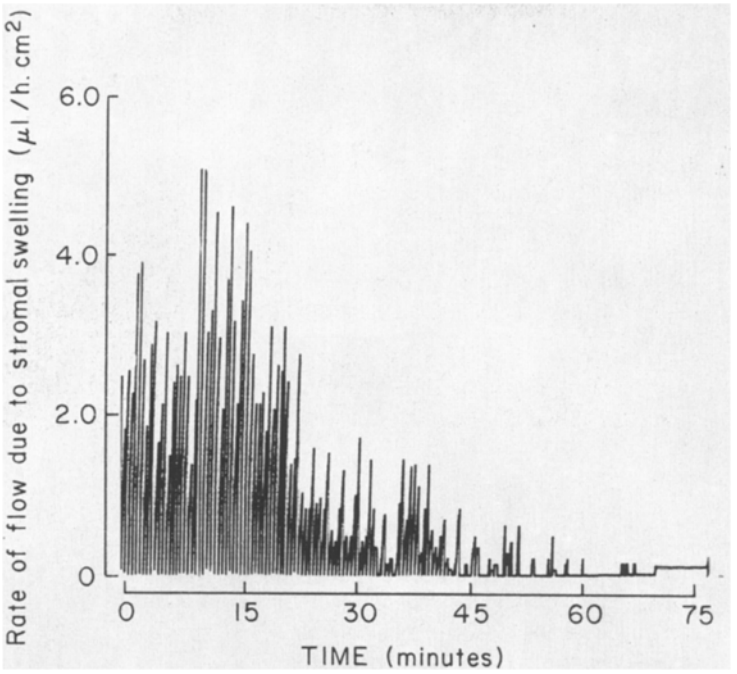

Fig. 8. Both epithelium and endothelium were scraped off. After mounting, the preparation was bathed by silicone oil on the inside and regular solution on the outside. The rate at which stromal swelling withdrew fluid from the outside chamber is plotted against time. Time intervals were 30 sec

for some 90–120 min from its initial 350–400 μm to some 700 μm, where it stabilizes. This displacement, since the stroma is supported by a net, would induce a movement of fluid into it of some 18 μl/hr cm^2, which would add up to the fluid transport being measured. When the stroma was present, therefore, the rates obtained during the first two hours after the preparation was mounted were not used for the purpose of numerical analysis. After this initial period, however, the stroma was not a significant factor; this fact was suggested by the previous evidence on this matter and was confirmed here. It is known that the value of the rate of fluid transport obtained from the rate of corneal thinning (MacRobbie-Ussing-Maurice technique) agrees very well with that obtained in the present experiments and with the rate measured from linear displacements of menisci (Maurice, 1972); we confirmed here (*see below*) that substantial dissection of the stroma did not reduce the rate of transport (Maurice, 1972). Our further observations on this matter are that: (a) preparations can pump continuously for up to 9 hr under the present conditions, while the stromal thickness stabilizes after 2 hr when viewed

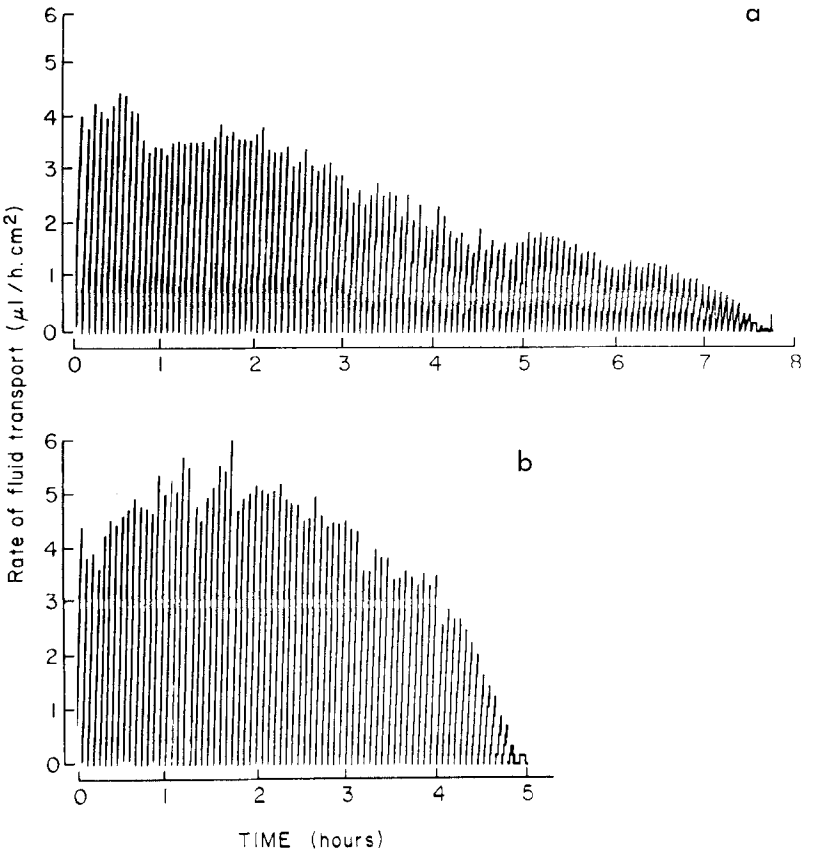

Fig. 9. Paired experiment with the two eyes of a rabbit. (*a*) The preparation was mounted as usual and bathed with regular solution on both sides. (*b*): prior to mounting, some 2/3 of stroma was dissected away; after mounting, the preparation was also bathed in regular solution. The records show the magnitude and time course of the rate of fluid pumping in the two cases. Time intervals: 4 min

under the specular microscope; (b) ouabain abolishes fluid transport and leaves instead a slow hydraulic leak (Fig. 7), both of which are expected regardless of the presence of stroma. To the extent that these observations, while suggestive, bring only indirect evidence, two other types of control experiments were performed; (c) both the epithelium and endothelium were scraped off and, after mounting the remaining stroma in the usual way, the inside was bathed with silicone oil while the outside was placed in contact with saline as usual. Under these conditions, fluid movement out of the outside chamber can only be due to stromal swelling. Fig. 8 shows as an example one such experiment in which stromal swelling indeed took place only during the initial 70 min; both the rate of swelling

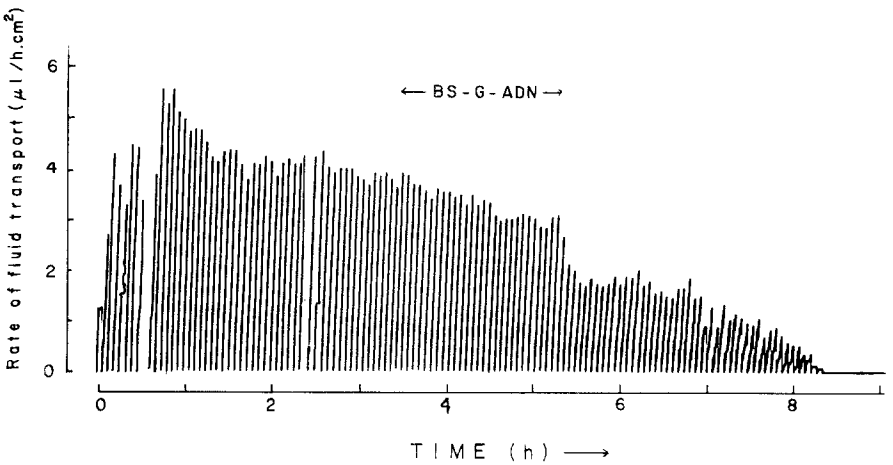

Fig. 10. Preparation bathed with RS on both sides. Time intervals: 4 min

and its duration in this experiment were among the highest seen. (d) Lastly, paired simultaneous experiments were run in which one preparation had most of its stroma dissected, as shown by histologic observation, while the one coming from the fellow eye served as control. Fig. 9 shows an example of the rates obtained.

The preparations with the stroma dissected actually displayed a rate higher than that in control preparations, which is probably due to the fact that the stroma, when present, adds to the hydraulic barrier across which fluid must pass. These experiments were a confirmation of several others in which preparations with most of its stroma dissected away had been seen to pump fluid at normal rates. While undoubtedly the presence of the stroma cannot be ignored, the present experiments add to the existing evidence which suggests that after its initial swelling its role is reduced to that of an additional barrier.

Effect of Adenosine

Fig. 10 shows a representative plot of the rate of fluid transport as a function of time in the presence of adenosine, that is, with RS. In this type of experiments, the high initial rates, attributed to the stromal swelling artifact, fell somewhat during the first hour of incubation and stabilized at a value somewhere between 4–8 $\mu l/hr \times cm^2$. When the preparations approached the fifth hour of incubation, the rates began to fall again until pumping stopped and passive reverse flow ensued, driven by the existing hydrostatic pressure. The behavior of preparations perfused initially with BSG was different in that the rates near which the

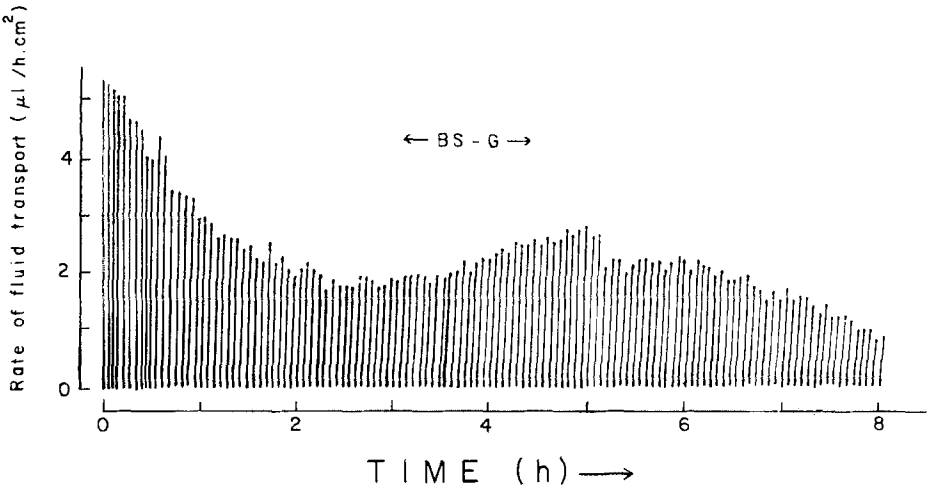

Fig. 11. Preparation bathed with BSG on both sides. Time intervals: 4 min

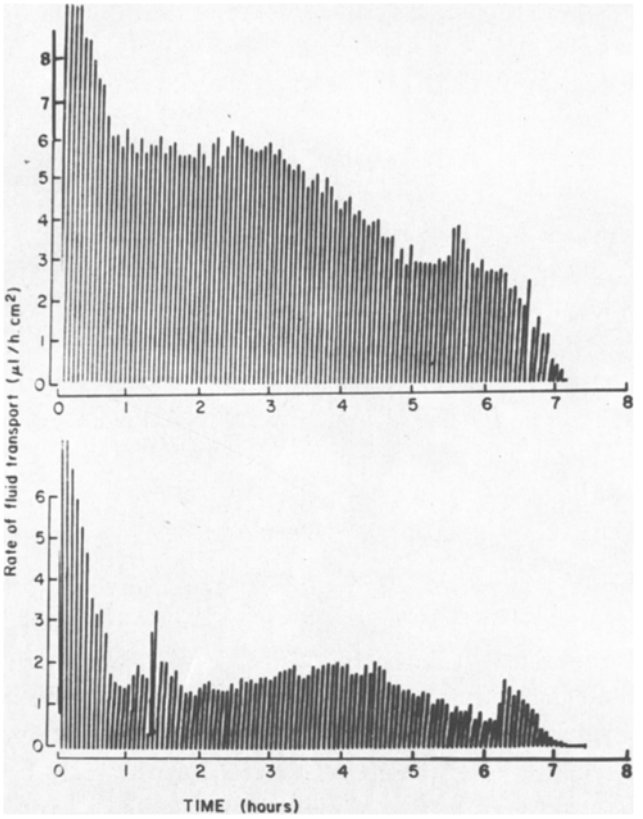

Fig. 12. Paired experiment; top: RS on both sides; bottom: BSG on both sides. Time intervals: 4 min

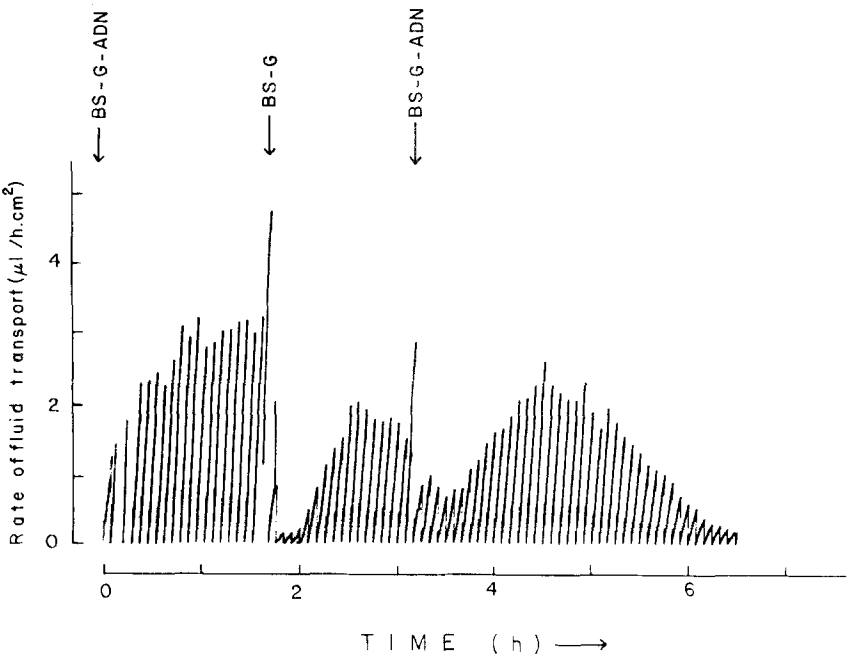

Fig. 13. Preparation bathed initially with RS on the inside and BSG on the outside. At the arrows, the inside solution was replaced as shown. Time intervals: 4 min

preparations stabilized were lower (3–5 $\mu l/hr \times cm^2$). An example is shown in Fig. 11. In the average, the maximal steady-state rates observed were $6.2 \pm 1.0\ \mu l/hr \times cm^2$ (SEM, $n = 12$) with RS, and $3.7 \pm 0.5\ \mu l/hr \times cm^2$ ($n = 8$) with BSG. The reference to rates being presently made implies that a period long enough for a reasonable estimate of an average rate has been considered. Although that period varies with each experiment, it usually ranged between 2 and 5 min. Average rates were either found by eye from the number of motor steps recorded graphically, or were calculated from the number of motor steps accumulated in the counter printout for given periods. Both methods gave comparable results.

The higher rates recorded in the presence of adenosine were attributed to stimulation by this nutrient. The comparative effects of glucose and adenosine were tested by 31 solution replacements performed in the course of 14 experiments. Examples of other types of experiments which demonstrate stimulation are shown in Figs. 12 and 13. Fig. 12 is representative of four paired experiments in which one preparation was bathed by RS while the other one (from the fellow eye) was simultaneously made to pump in the presence of BSG. As can be seen, the preparation bathed with RS pumps at a higher rate for several hours, until its

rate decays to the level of that bathed with BSG. In the presence of BSG, the rate is relatively stable and reaches a nearly constant level early in the experiment. It can also be seen that both preparations pump for approximately the same length of time after *in vitro* mounting, which might mean that some critical nutrients absent from this simplified medium would be exhausted at that same time, regardless of the previous rate of pumping. Also to be noted is the increase in rate that takes place near the end of the experiment, shortly before the preparation is to exhaust its capability to pump. This phenomenon was a common observation, and indicates that some cellular compensating mechanisms might be at play at that point, trying perhaps to preserve cellular function under adverse conditions. In yet another type of experiment, the preparations were initially bathed with RS on the inside and BSG on the outside; after a steady rate was obtained, the RS on the inside was replaced by BSG and the rate recorded, and finally RS was once more substituted on the inside. An example is shown in Fig. 13. The fact that the rate in the presence of BSG is smaller than the one before and after is consistent with the behavior detailed above. It should be noted that the 5 mM adenosine concentration employed here and in the past (Dikstein & Maurice, 1972) is probably higher than strictly necessary, and small amounts that might have diffused across the endothelium and remained in the stroma could account for a somewhat delayed effect of its replacement observed at times; thorough washing was usually necessary in order to observe the change in rates.

While preservation of Na transport in red blood cells by adenosine has been known to occur for some time (Post & Jolly, 1957), and while in the present preparations the inclusion of adenosine in the perfusing medium was previously reported to be beneficial for fluid transport (Dikstein & Maurice, 1972), a direct quantitative demonstration such as the present one was not apparently available. Regarding the mechanism by which adenosine exerts its effect, the main possibilities that can be considered are: (1) it could serve as substrate for ATP synthesis; (2) it could be deaminated and cleaved, yielding ribose phosphate to be used for ultimate ADP→ATP conversion via the pentose phosphate cycle; (3) it might contribute to the synthesis of presumed regulators of cellular activity such as cyclic AMP. In the case of the red blood cell, the evidence points to the sparing of ATP afforded by the replenishment of substrate for adenosine deaminase as a dominant factor, since those cells cannot perform *de novo* synthesis of adenosine (*cf.* Bishop & Surgenor, 1964). Whether the same is true for the endothelium is not known, although

some support for that possibility comes from the fact that adenosine maintains comparatively high ATP levels in there and its absence leads to decreased ATP levels (Anderson, Fischbarg & Spector, 1973). The possibilities listed under *1* and *2* above might therefore result in higher ATP levels and subsequently higher rates of fluid pumping than in the presence of glucose, just as experimentally observed. Whether this "reserve capacity" of the transport system would have a role when physiological conditions and stimuli would prevail remains to be established.

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