

COMMUNICATIONS

ELECTRICAL POTENTIAL ACROSS THE ISOLATED CILIARY BODY OBSERVED *IN VITRO**

BY

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THE existence of a potential difference (P.D.) across the ciliary body has been demonstrated in intact animals by Lehmann and Meesmann (1924), Berggren (1960), and Cole (1961), whilst recently Holland, Mallerich, Bellestri, and Tischler (1960) have studied the potential across the isolated ciliary body of the cat. It has been shown that, *in vivo*, there is a relationship between the blood-aqueous P.D. and the rate of aqueous flow (Cole, 1961), and it is evident that further information about the electrical electrochemical behaviour of the barrier would throw light upon the transport processes concerned in the formation of the aqueous humour. One of the limitations encountered when using intact animals is that it is impossible to impose very wide changes upon the composition of the fluids bathing the two sides of the barrier. Were it practicable to measure the alterations of potential across the barrier caused by modifying the fluids on either side it seemed likely that some of the electrochemical properties of the barrier could be inferred, and for this reason a technique has been developed for measuring the potential difference developed across isolated pieces of ciliary body placed between two chambers containing saline media the composition of which could be varied.

Materials

The pieces of ciliary body were excised from ox eyes obtained from the abattoir and which normally reached the laboratory within 30 minutes of the death of the animal. Immediately after their removal the eyes were placed in a buffered and oxygenated "NaCl" medium (see below, Table I), cooled to 1°C. and kept in a vacuum flask for transport to the laboratory. There they were opened by an equatorial incision and the lens and vitreous removed from the anterior segment. A radial cut was made through the ciliary body and cornea, and the ciliary body, together with some vitreous and loose tissue lying between it and the sclera, was pulled away from the remainder of the anterior segment. This tissue was then placed in oxygenated "NaCl" or "SO₄" medium at 37°C. until required.

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The composition of the saline media used in the course of this series of experiments is shown in Table I. In the "SO₄" and "EtSO₄" solutions, the chloride ion was replaced with sulphate or ethyl sulphate (as ethyl sodium sulphate, C₂H₅NaSO₄) respectively, whilst in the "Choline" and "Mannitol" media the sodium was replaced by choline (as choline chloride, (CH₂(OH).CH₂.N(CH₃)₃Cl) or Mannitol. The "Mannitol 470 mM/L" medium was designed to be approximately isotonic with the "Na₂SO₄ 300 mEq Na/L" medium, and solutions containing varying concentrations of sodium ion were obtained by combining the latter with the former. The "High-K" media were obtained by replacing some of the sodium in the "NaCl" medium with potassium. All media contained 7.0 mM/L glucose and 10 mM/L glutamate. Turner, Eggleston, and Krebs (1950) have shown that glutamate is required for the maintenance of normal intracellular potassium concentrations in isolated retina and its presence appeared to assist in maintaining steady potentials in the isolated ciliary body.

TABLE I
CONSTITUTION OF VARIOUS SALINE MEDIA (mEq (or mM) per litre)

Medium	Na	Cl	K	Mg	SO ₄	Glucose	Glutamate	Choline	EtSO ₄	Mannitol	†Tris/Nitric Acid Buffer and pH 7.45
"NaCl"	150	150	3.0	0.9	0.9	7.0	10.0	—	—	—	15.4
"SO ₄ "	150	0	3.0	0.9	76.0	7.0	10.0	—	—	—	15.4
"Choline"	0	150	3.0	0.9	0.9	7.0	10.0	150	—	—	15.4
"EtSO ₄ "	150	0	3.0	0.9	0.9	7.0	10.0	—	150	—	15.4
"Mannitol"	0	0	3.0	0.9	0.9	7.0	10.0	—	—	300	15.4
*"Na ₂ SO ₄ " (300 mEq Na/L)	300	0	3.0	0.9	151	7.0	10.0	—	—	—	15.4
"Mannitol" (470 mM/L)		0	3.0	0.9	0.9	7.0	10.0	—	—	450	15.4
"High K"	147 to 113	150	6.0 to 40.0	0.9	0.9	7.0	10.0	—	—	—	15.4

* This solution was combined with varying proportions of the "Mannitol (470 mM/L)" medium to give a series of solutions containing from 10 to 300 mEq Na⁺ per litre.

† "Tris" stands for tris (hydroxy methyl) amino methane: CH₂OH.C(NH₂). (CH₂OH)₂.

Methods

The two "Perspex" vessels between which the isolated tissue was placed are shown in Figs 1 and 2 (opposite). The outer semicircular compartments (B and B') contained water at 37°C. circulating from an external thermostatically controlled water-bath, whilst the inner compartments, D and D', contained the bathing media, oxygenated through H and H' (Fig. 1). In use the two halves were clamped by the bolts E and E' (Fig. 2), with the piece of tissue (E, Fig. 1) situated on the platform G (Figs 1 and 2). Each inner compartment was connected to a calomel/KCl electrode *via* an agar/NH₄NO₃ bridge in polythene tubing (C and C', Fig. 1). The openings A and A' were used either for the removal of some of the bathing fluid or for applying an electric current from an external source in order to estimate the membrane resistance.

A segment, measuring about 1 × 1 cm., was cut from the ring of isolated ciliary body and iris and placed upon a square of gauze with its inwards facing (epithelial) side uppermost. The gauze and tissue was then trimmed to fit easily upon the platform, G, of the one half of the perspex chamber. The other half was then slipped over the two bolts (E and E', Fig. 2) and the two sections were bolted securely together so that the two semicircular inner compartments were separated

by a piece of ciliary body and supporting tissue some 0.40 cm. diameter having its outer (stromal) side in D' and its inner (epithelial) side in D (Fig. 1).

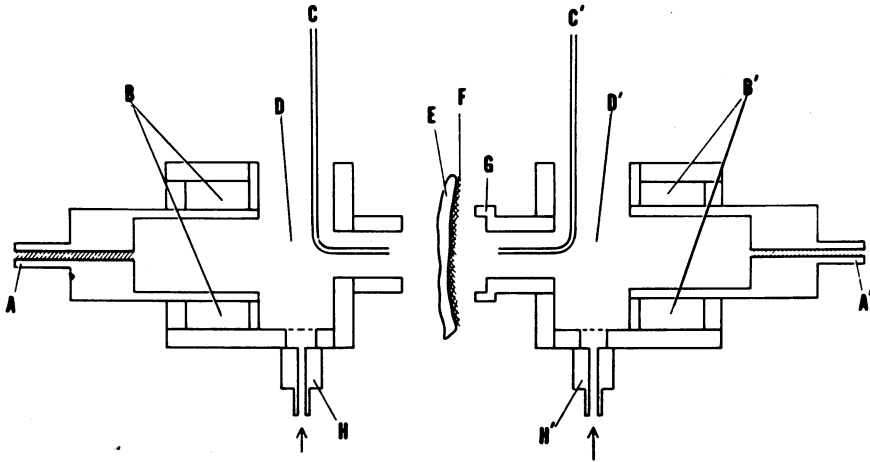


FIG. 1.—Apparatus used for measuring P.D. across isolated blood-aqueous barrier. Vertical section $\times 1.5$ approx.

- A, A' = side arms for removal of fluid;
- B, B' = water jacket (maintained at 37°C. from external water-bath);
- C, C' = agar/NH₄NO₃ electrodes in polythene tubing for measurement of P.D.;
- D, D' = compartments containing bathing media on inner and outer sides respectively of isolated tissue;
- E = piece of isolated ox ciliary body, inner (epithelial) surface facing towards D and outer (stromal) surface towards D';
- F = gauze support on outer (stromal) side of isolated tissue;
- G = platform;
- H, H' = inlets for oxygen to bathing fluids.

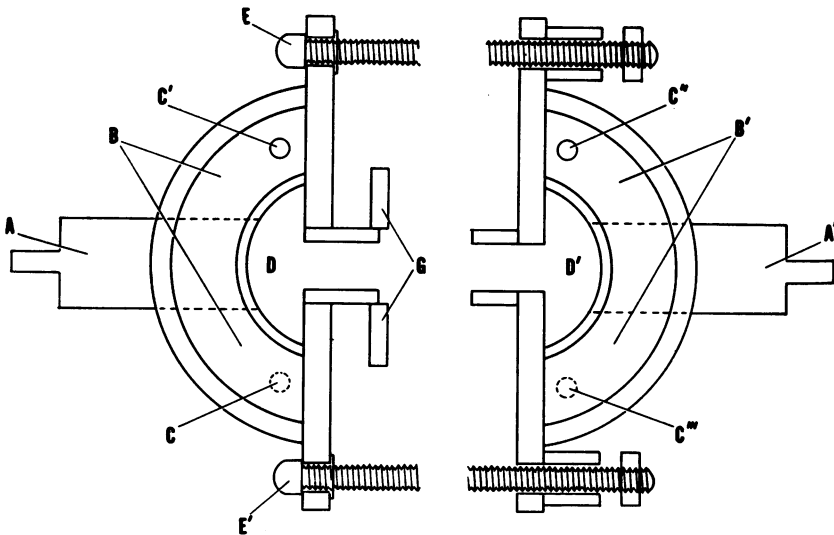


FIG. 2.—Apparatus used for measuring P.D. across isolated blood-aqueous barrier. Plan $\times 1.5$ approx.

- A, A' = side arms for removal of fluid;
- B, B' = water jacket (maintained at 37°C. from external water-bath);
- C, C', C'' = entrances and exits for water to water jacket;
- D, D' = compartments containing bathing media on outer and inner sides respectively of isolated tissue;
- E, E' = bolts used for clamping two parts of apparatus together when tissue is in position;
- G = platform

A typical cross-section of an isolated ciliary body is shown in Fig. 3. The platform and any protruding edges of tissue were dried with cotton wool and liberally covered with silicone grease (Silicone Stopcock Grease, Edwards High Vacuum Ltd., Crawley, Sussex). The outer water-jacket was then connected to the reservoir and circulating pump and the two oxygen inlets to a cylinder of gas. The two inner compartments were filled with the appropriate saline medium and the whole device mounted on a clamp. The L-shaped agar/ NH_4NO_3 bridges were mounted on either side of the tissue with their tips fairly close to it (Fig. 1, C and C'). The electrodes were connected to a high impedance millivoltmeter with an input impedance of 10^{13} ohms (Vibron Electrometer 33B, Electronic Instruments Ltd., Richmond, Surrey) the output of which was fed to a pen writer through a simple transistor amplifier.

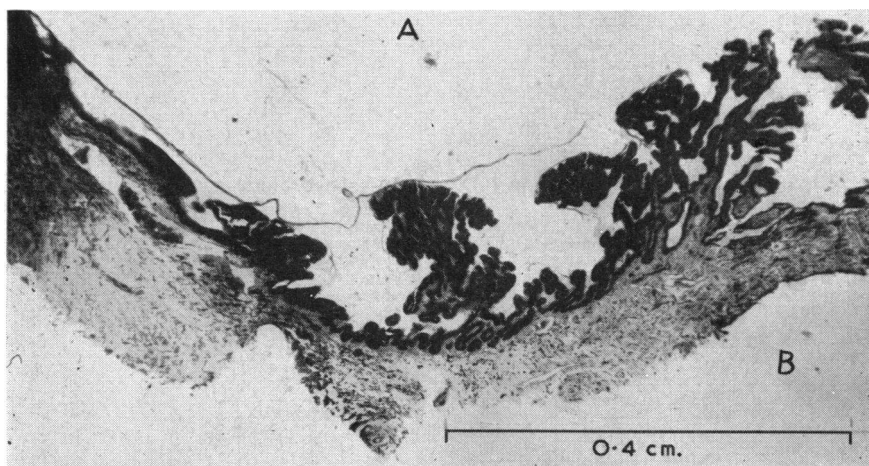


FIG. 3.—Cross-section of isolated ox ciliary body (Mallory's stain). Horizontal line, 0.4 cm., represents diameter of aperture between inner and outer saline compartments (Fig. 1).

A = inner (epithelial) side of tissue
B = outer (stromal) side of tissue.

In estimating the membrane resistance, a current from an external source was applied *via* calomel electrodes and agar/Ringer bridges across A and A' (Fig. 1). The P.D. between C and C' was measured as before for a series of steady currents at intervals of $20 \mu\text{A}$ over a range of -80 to $+80 \mu\text{A}$, for which the current-voltage relationship was linear. The area of barrier available for the passage of current at G was 0.125 cm^2 and the results were expressed in ohms/cm^2 .

In some cases, pieces of isolated ciliary body were fixed in Zenker's solution, embedded in paraffin and sections 10μ thick were cut and stained with Mallory's stain in order to show the position of the epithelium and stromal connective tissue in relation to the saline compartments.

Results

This work has been chiefly concerned with alterations of the composition of the fluid medium on the outer (stromal) side of the barrier, the results being shown in Table II (opposite).

TABLE II

CHANGES IN BARRIER P.D. CAUSED BY REMOVAL OF CHLORIDE OR SODIUM IONS FROM FLUID ON OUTER (STROMAL) SIDE OF ISOLATED CILIARY BODY

Outside Medium	P.D. "NaCl" Medium Outside	Δ P.D.
"NaCl"	3.80 ± 0.26	—
"SO ₄ "	—	$+3.00 \pm 0.36$
"EtSO ₄ "	—	$+3.19 \pm 0.39$
{ "Mannitol" "Choline" }	—	-2.97 ± 0.50

Values are given as the mean \pm its standard error and at least ten observations were obtained in each case.

With the "NaCl" medium as the inside solution, it is seen that substitution of the "SO₄" or the "EtSO₄" for the "NaCl" medium outside significantly increased the P.D., whereas replacement of sodium ion with choline or Mannitol outside significantly decreased the P.D. Typical results in individual experiments are shown in Figs 4 and 5, and in Fig. 6 (overleaf).

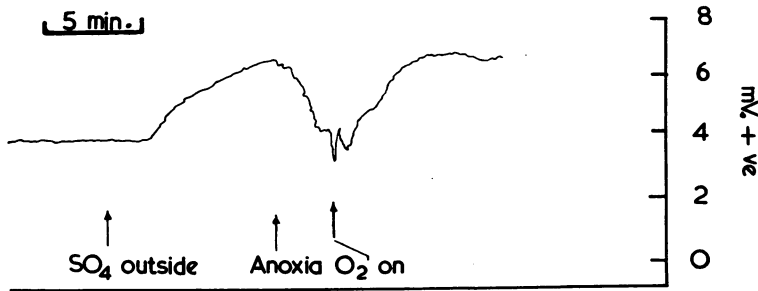


FIG. 4.—Recording of ciliary body P.D. during substitution of SO₄ for Cl⁻ on outer (stromal) side. A rise in P.D. was reversibly reduced by anoxia.

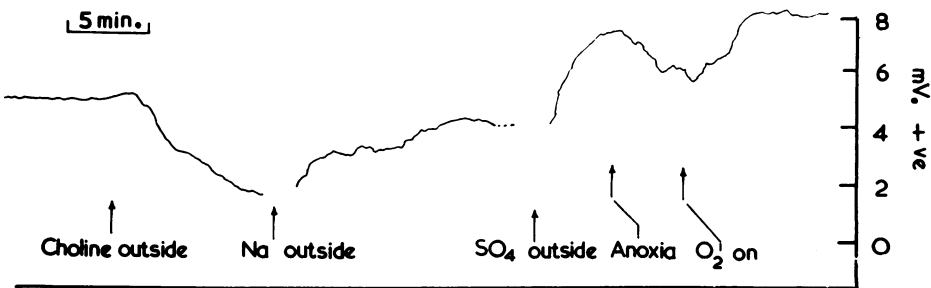


FIG. 5.—Recording of ciliary body P.D. during substitution of choline for Na⁺ on outer (stromal) side. A fall in P.D. was restored by replacement of Na⁺ and increased by substitution of SO₄ for Cl⁻. Anoxia caused a reversible fall in P.D.

Fig. 7 (overleaf) shows results from a number of experiments where the inner (epithelial) side was in contact with the "Na₂SO₄ 300 mEq Na./L" medium

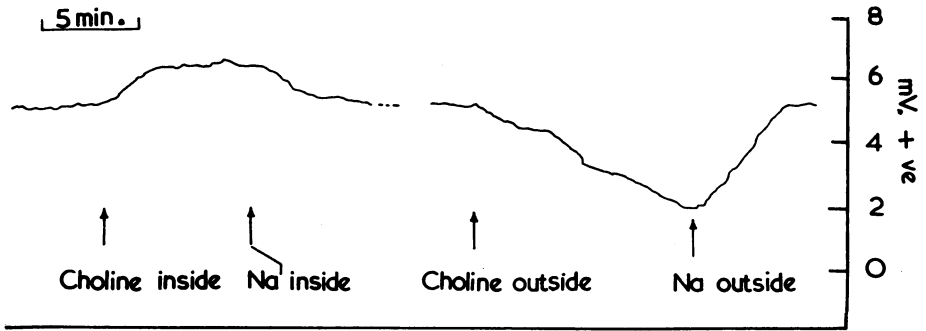


FIG. 6.—Recording of ciliary body P.D. during alteration of sodium ion concentration at inner (epithelial) surface. Reduction of $[Na_i]$ increased P.D., whereas, at a later stage in the experiment, reduction of $[Na_o]$ (by substituting choline for Na^+) decreased P.D.

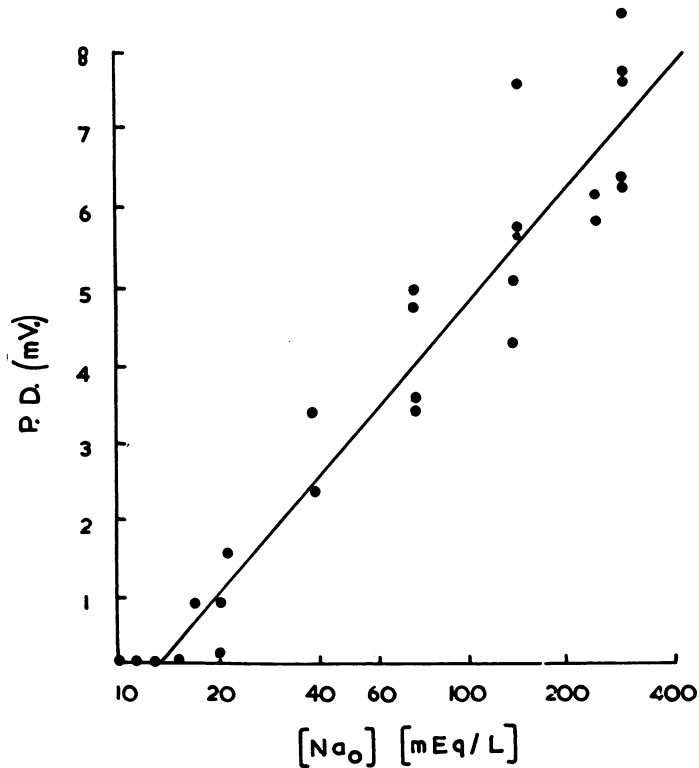


FIG. 7.—Relationship between ciliary body P.D. (ordinate) and outside sodium concentration $[Na_o]$ (abscissa).

The scale of the abscissa is logarithmic and the regression line represents the equation:
 $E = 4.5 \log [Na_o] - 4.28$.

Points represent values from individual experiments.

throughout, but the sodium concentration on the stromal side was varied from 10 to 300 mEq/L by adding sodium to the "Mannitol 470 mM/L" medium.

Reduction of the inside sodium ion concentration caused small increases in P.D. (Fig. 6 and in Fig. 8) but increasing the inside concentration of potassium ("NaCl" medium outside) resulted in a fall (Fig. 9).

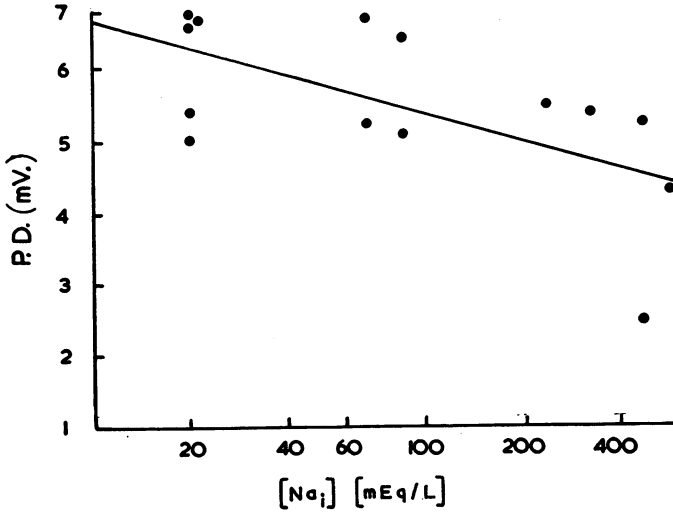


FIG. 8.—Relationship between ciliary body P.D. (ordinate) and inside sodium concentration [Na_i] (abscissa). The scale of the abscissa is logarithmic and the regression line represents the equation: $E = 7.9 - 1.25 \log [Na_i]$. Points represent values from individual experiments.

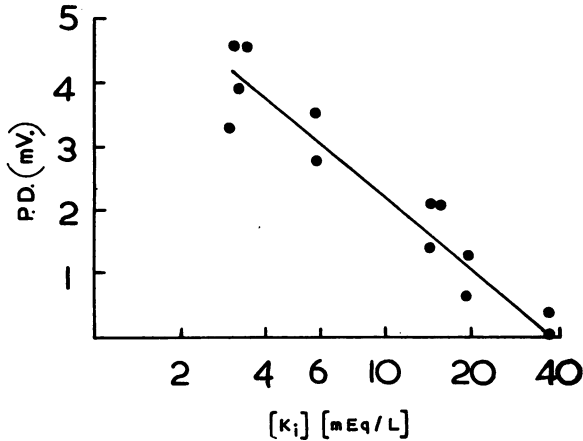


FIG. 9.—Relationship between ciliary body P.D. (ordinate) and inside potassium concentration [K_i] (abscissa). The scale of the abscissa is logarithmic and the regression line represents the equation: $E = 6.0 - 3.8 \log [K_i]$. Points represent values from individual experiments.

Anoxia produced by stopping the aeration on both sides of the barrier reduced the P.D. (Figs 4 and 5) as did 5×10^{-4} 2,4:dinitrophenol and 1×10^{-3} cyanide added to the inside medium (Figs 10 and 11, Table III). In all these cases the inside solution was the "NaCl" medium.

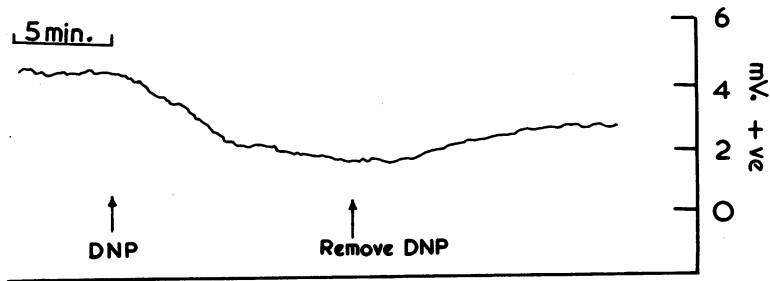


FIG. 10.—Recording of ciliary body P.D. in the presence of 5×10^{-4} M dinitrophenol in the inside medium.

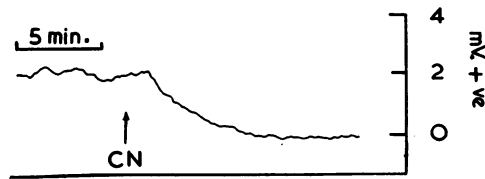


FIG. 11.—Recording of ciliary body P.D. in the presence of 1×10^{-3} M cyanide in the inside medium.

TABLE III
ACTION OF METABOLIC INHIBITORS UPON BARRIER P.D. OF ISOLATED CILIARY BODY

Inhibitor	P.D. in Control Period	Δ P.D.
—	3.89 ± 0.31	—
DNP (inside) 5×10^{-4} M	3.57 ± 0.25	-1.67 ± 0.39
CN' (inside) 1×10^{-3} M	4.07 ± 0.41	-2.40 ± 0.37
Anoxia (both sides)	3.95 ± 0.31	-2.62 ± 0.51

Values are given as the mean \pm its standard error and at least eight observations were obtained in each case.

The electrical resistance of the barrier was 61.3 ± 5.1 ohms per cm^2 exposed tissue, but owing to the extensive folding of the ciliary epithelium the resistance per cm^2 epithelial surface would be considerably greater than this.

Discussion

The maintenance of a P.D. with similar solutions on each side of the isolated barrier and its dependence upon adequate oxygenation provides evidence that the tissue remains active *in vitro* under the present experimental conditions. Although its value might vary considerably from one eye to another it appeared to remain relatively stable for 30 to 60 minutes in individual cases. In all cases the inner (epithelial) side was positive to the outer (stromal) side in accord with the findings in intact eyes *in situ*. The P.D. described by Holland and others (1960) was measured between the anterior surface of the iris and the scleral surface of the ciliary body (termed by these authors the "stromal surface") and the posterior surface of the ciliary body (termed the "neuro-epithelial surface"), and its polarity was the reverse of the blood-aqueous P.D. reported by Lehmann and Meesmann (1924), Berggren (1960), and Cole (1961). It was also the reverse of the P.D. observed in the present experiments, and in comparing these findings it should be remembered that in this report the term "stromal side" refers only to that part of the ciliary body which has been detached from the inner surface of the sclera and does not include the anterior iridic surface. This distinction may account for the discrepancy between the results of Holland and others (1960) and both the present findings and the blood-aqueous P.D. in intact eyes. The mean P.D. ("NaCl" medium both sides) of $+3.80 \pm 0.26$ mV is rather less than that which is found in intact rabbit eyes ($+6.20 \pm 0.52$ mV; Cole, 1961) using an entirely different technique.

The chief advantage of *in vitro* experimentation is that it affords freedom to alter the solute composition on either side of the membrane independently and so enables the action of certain individual ions upon the P.D. to be studied independently. An analogy between the formation of the aqueous humour and the transport of sodium and water through frog skin has been suggested by Cole (1959, 1960); and Ussing and his collaborators (Ussing and Zerahn, 1951; Koefoed-Johnsen and Ussing, 1958; Ussing, 1960a, b), working on the active transport of Na^+ through frog skin, have shown that such transport is normally associated with passive movement of chloride ion and that the observed membrane P.D. is the resultant of the active carriage of positive sodium ions and the passive migration of negative chloride ions, the latter penetrating the membrane fairly readily. If the Cl^- ion is replaced by the SO_4^{2-} ion, which penetrates very much more slowly, the P.D. rises because the electrical "leak" due to anion penetration has been greatly decreased. Furthermore, since the existence of the P.D. depends upon transport of Na^+ , the removal of this ion from the outward-facing side of the membrane will lower the observed P.D.

If this analogy between frog skin and the blood-aqueous barrier is valid, it follows that there would be active transport of Na^+ from blood to aqueous characterized by a barrier P.D. with the inner (epithelial) side positive to

the stroma. This P.D. would be increased by the substitution of a large, slowly penetrating anion (such as SO_4'' or EtSO_4') for Cl' and would be reduced in the absence of Na^+ on the stromal side. All these effects have been observed with the isolated ciliary body preparation used in these experiments (Table II; Figs 4 and 5); a rise of P.D. occurred when chloride on the stromal side of the barrier was replaced with sulphate or ethyl sulphate, and there was a fall when Mannitol or choline was substituted for sodium. It was evident that the rise which occurred with the " SO_4 " medium was dependent upon aerobic metabolism, since it was subject to inhibition by anoxia (Figs 4 and 5), and these results strongly suggest that there is a mechanism capable of transporting sodium ion actively from the stromal side of the ciliary body into the aqueous humour.

It appeared that there was a fairly consistent relationship between the concentration of sodium in the outer compartment ($[\text{Na}_o]$) and the barrier P.D. when anion movement was decreased by the substitution of SO_4'' for Cl' on both sides of the barrier. Although the thickness of the tissue made it difficult to be certain of the actual ion concentrations obtaining at the cell surfaces, it is clear that the P.D. is a function of outside sodium concentration (Fig. 7), indicating that the outer (stromal) surface of the barrier is permeable to sodium ions (*cf.* Koefoed-Johnsen and Ussing, 1958; Ussing, 1960a). However, the barrier P.D. was not entirely independent of the sodium concentration of the inner (epithelial) side, although this effect was less marked and in the opposite direction (Figs 6 and 8). Increasing the concentration of potassium (using the "High K" media) on the inner side reduced the barrier P.D. sharply (Fig. 9) but potassium concentration was without significant effect when applied to the outer (stromal) surface.

These findings indicate that the inner (epithelial) surface of this region of the isolated blood-aqueous barrier as well as the outer (stromal) surface is permeable to sodium ions by passive diffusion, whilst the potassium permeability of the stromal surface is negligible compared with that of the epithelial surface.

Not only was the overall barrier P.D. fairly small when compared with the P.D. developed across frog skin, but the changes which resulted from alteration of $[\text{Na}_o]$, although highly significant ($b=4.5 \pm 0.29$; $P < 0.01$), were much smaller than those originally described by Koefoed-Johnsen and Ussing (1958). It seems possible that the anion permeability of the isolated barrier, even to large ions such as SO_4'' or EtSO_4' , was greater than that of frog skin—an hypothesis which is consistent with the view that the normal barrier is sufficiently permeable for ultrafiltration to play a significant part in the normal formation of the aqueous humour (Cole, 1960). This anion permeability, together with a relatively high permeability to sodium of the inner surface, forms an important distinction from the mechanism proposed for frog skin (Ussing, 1959; 1960a), and it is suggested that the scheme proposed by Koefoed-Johnsen and Ussing (1958) be modified as indicated in

Fig. 12. It should also be borne in mind that the position is further complicated by the presence of two layers of cells, the pigmented and epithelial layers, in the blood-aqueous barrier, both of which are known to contribute to the electrical profile across the barrier (Berggren, 1960).

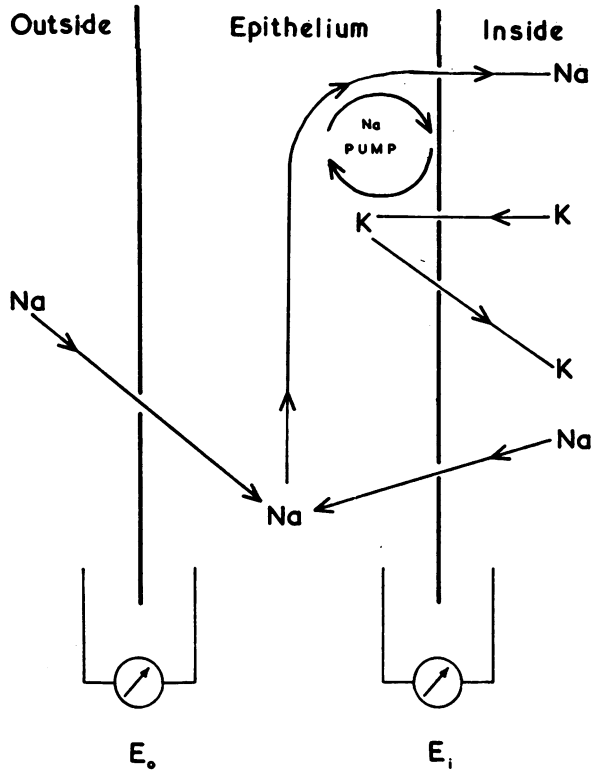


FIG. 12.—Diagrammatic representation of cation permeabilities of blood-aqueous barrier at its outer (stromal) and inner (epithelial) surfaces. For explanation see text.

In the system which is now proposed, the total blood-aqueous P.D. (E) results from the P.D.s at outer (stromal) and inner (epithelial) surfaces; E_o and E_i . The outer P.D. (E_o) would be a function of

$$\frac{RT}{F} \ln \left\{ \frac{P_n[\text{Na}_o] + \Sigma P_a[\text{A}_c]}{P_n[\text{Na}_c] + \Sigma P_a[\text{A}_o]} \right\},$$

where P_a and P_n represent anion and sodium permeabilities; $[\text{Na}_o]$ and $[\text{A}_o]$ represent outside concentrations of sodium ion and of anions, and $[\text{Na}_c]$ and $[\text{A}_c]$ represent the corresponding intracellular concentrations (*cf.* Goldman, 1943; Hodgkin and Katz, 1949; Conway, 1957; Patlak, 1960).

The outer P.D. (E_i) would be a function of

$$\frac{RT}{F} \ln \left\{ \frac{P'_k [K_c] + P'_n [Na_c] + \Sigma P'_a [A_i]}{P'_k [K_i] + P'_n [Na_i] + \Sigma P'_a [A_c]} \right\},$$

where P'_k , P'_n , and P'_a represent potassium, sodium, and anion permeabilities at the inner surface of the barrier; $[K_c]$ represents intracellular potassium concentration, and $[K_i]$, $[Na_i]$, and $[A_i]$ represent the inside (aqueous) concentrations of potassium, sodium and anions (see Fig. 12).

The total barrier P.D. in this system ($E_o + E_i$) will be dependent upon $[Na_o]$, $[Na_i]$, and $[K_i]$, and the effect of altering the inside sodium concentration will be small compared with changes in the outside concentration of sodium so long as $P_n > P'_n$. The result of substituting sulphate or ethyl sulphate for chloride will be to reduce the anion permeabilities, P_a and P'_a , and so to raise the total P.D. Increasing $[K_i]$, by using the "High K" media on the inner (epithelial) side, will tend to decrease the P.D. Although this scheme is obviously incomplete, it seems to go some way towards explaining the observations in the present series of experiments.

Inhibitors.—Both the normal P.D., with "NaCl" medium on both sides, and the elevated P.D. in experiments where SO_4^{2-} or $EtSO_4^-$ was substituted for Cl^- on the outer (stromal) side, were reversibly reduced by anoxia (Figs 4 and 5). The P.D. was also decreased when $10^{-3}M$ cyanide or $5 \times 10^{-4}M$ dinitrophenol was added to the solution on the inner (epithelial) side (Figs 11 and 12), and in these respects the isolated ciliary body potential may be said to behave similarly to frog skin potential (Huf, Doss, and Wills, 1957).

Summary

(1) The electrical potential difference (P.D.) across the isolated ox ciliary body suspended between two saline-filled chambers has been measured; it amounts to 3.80 ± 0.26 mV, the epithelial side being positive to the stroma.

(2) Replacement of chloride in the solution on the stromal side of the isolated tissue by larger anions such as sulphate or ethyl sulphate increased the P.D. The P.D. fell when the sodium of the solution on the stromal side was replaced by choline or Mannitol.

(3) There was a logarithmic relationship between the sodium concentration on the stromal side of the ciliary body and the P.D., and also between the potassium concentration on the epithelial side and the P.D.

(4) The potential was decreased by anoxia or in the presence of cyanide or 2,4-dinitrophenol.

(5) The results indicate the occurrence of active sodium transport from the stromal to the epithelial side of the isolated ciliary body and it is suggested that this process may play an important part in the normal mechanism of aqueous humour formation.

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