Potential, Current, and Ionic Fluxes across the Isolated Retinal Pigment Epithelium and Choroid

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ABSTRACT A flux chamber was utilized for in vitro studies of a membrane formed by the retinal pigment epithelium and choroid of the eye of the toad (*Bufo arenarum* and *Bufo marinus*). A transmembrane potential of 20 to 30 mv was found, the pigment epithelium surface positive with respect to the choroidal surface. Unidirectional fluxes of chloride, sodium, potassium, and calcium were determined in the absence of an electrochemical potential difference. A net transfer of chloride from pigment epithelium to choroid accounted for a major fraction of the mean short-circuit current. A small net flux of sodium from choroid to pigment epithelium was detected in *Bufo marinus*. In both species of toads, however, about one-third of the mean short-circuit current remained unaccounted for. Manometric determinations of bicarbonate suggested an uptake of this ion at the epithelial surface of the membrane but did not provide evidence of a relationship between this process and the short-circuit current.

It is well known that a dc potential of a few millivolts can be recorded across the vertebrate retina, the vitreous humor being positive with respect to the sclera (8). The site of origin of this electric potential has not been unequivocally established. For a long time it was generally accepted that the electrogenic layer is located at the photoreceptor cell level (11, 12). A different position, however, was taken by Noell (18) who postulated, on the basis of pharmacological observations, that the pigment epithelium is the major source of the resting potential of the retina. In addition, he suggested that this potential is a consequence of an active ion transport process taking place in the pigment epithelium cells. More recently, Noell, Crapper, and

Paganelli (20) in measuring the fluxes of several ions between the blood and the ocular cavity in the rabbit found evidence for an active transport of potassium across the retina and toward the vitreous humor.

The initial purpose of this study was to test the hypothesis that the pigment epithelium of the retina generates a dc potential. Electrical recordings were obtained in vitro across a membrane composed of the pigment epithelium and choroid of the toad eye. The existence of a potential difference between the two surfaces of this membrane was clearly established. Then, an attempt was made to investigate some of the functional features underlying the electrical manifestations. This objective was pursued by analyzing the ionic movements through the pigment layers of the eye with the technique developed by Ussing and Zerahn (24) for studies of ion transport in frog skin and other biological membranes (3, 9, 16, 22).

METHODS

The eyes of the toads *Bufo arenarum* Hensel and *Bufo marinus* (Linnaeus) were used throughout this study. The excised eye was bisected at the equator and the posterior hemisphere cut meridionally to exclude the optic papilla. The vascular connections between choroid and sclera were severed with fine forceps, thus releasing the choroid and retina as a unit. Finally, the neural retina was carefully detached and discarded. The resulting membrane, formed by the retinal pigment epithelium and choroid, was mounted between the halves of a lucite chamber (Fig. 1). The surface of the membrane exposed to the bathing solution was only 0.1 cm^2.

The saline solution utilized (1 ml in each compartment of the flux chamber) contained 115.5 mM sodium chloride, 2.0 mM potassium chloride, 2.4 mM sodium bicarbonate, and 1.0 mM calcium chloride. The pH of this solution was 7.9 when equilibrated with air. In experiments performed to observe the effect of a chloride-free medium, the substituting salts were sodium sulfate, methylsulfate or glucuronate, potassium sulfate, methylsulfate or gluconate, and calcium gluconate. When sulfate was the anion used the solution was made isotonic by adding sucrose. The experiments were carried out at room temperature (23° ± 1°C).

Electrical Measurements Transmembrane potentials were measured with a Keithley 150R microvoltammeter or 600A electrometer through agar-saline bridges and calomel electrodes. The sum of electrode and junction potentials was always less than 0.5 mv. The tips of the agar bridges were positioned at 1 mm from the surfaces of the membrane. The resistance of the saline solution between the tips of the agar bridges was about 200 Ω and the voltage drop across this resistance (0.6 to 0.8 mv) was taken into account when short-circuiting the membrane.

Short-circuit currents were measured with a Keithley 600A electrometer or a Keithley 200B voltmeter with the input shunted by a 10 kΩ resistor. The external current was applied to the membrane by means of a variable electromotive force through silver–silver chloride electrodes and agar-saline bridges. The tips of the current bridges were radially centered with respect to the membrane.

Radioisotope Fluxes Only unidirectional fluxes were measured. The total duration of each experiment was about 3 hr. The electrical potential became stable a few minutes after the saline solution was added to the chamber and then the membrane was short-circuited. The radioisotope was added to one of the compartments and after 40 min the collection of samples was started. Samples of 100 μl (replaced with

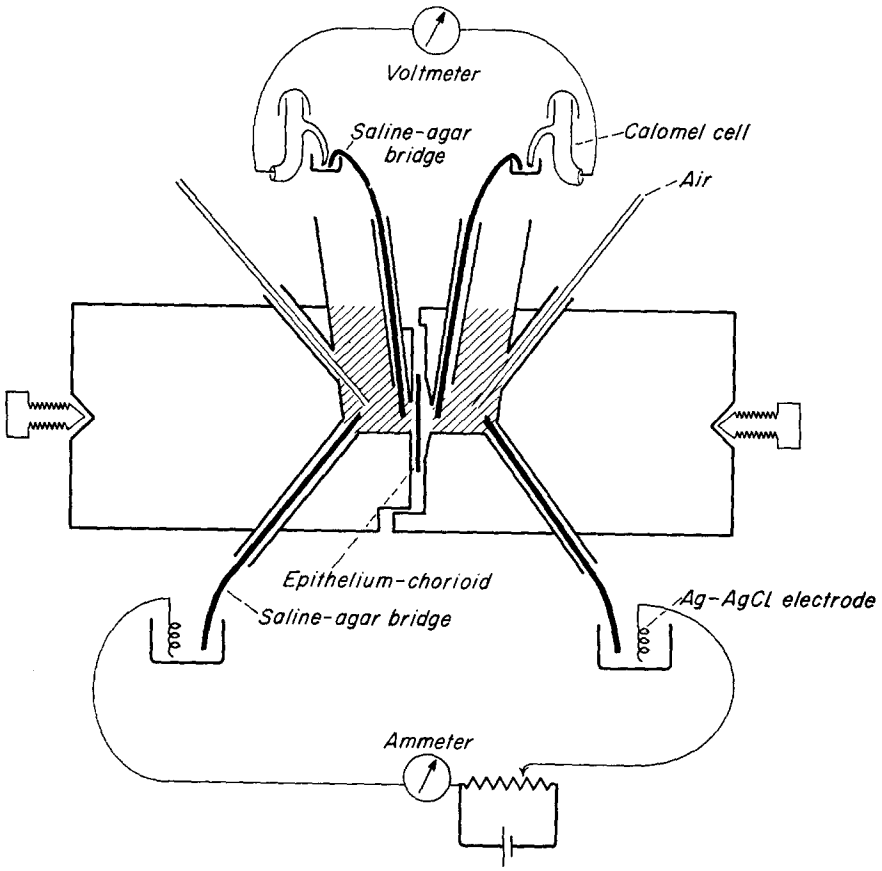

FIGURE 1. Flux chamber assembly. The area of the membrane exposed to the bathing solution was 0.1 cm^2. The surface of one of the half-chambers had a conical recess that provided a clearance of 0.1 mm at the circumference of the central opening. Stirring and aeration were achieved by bubbling water-saturated air.

fresh solution) were obtained every 30 min during 2 hr (4 periods of flux) from the compartment opposite to that in which the radioisotope had been added. Samples of 10 μl were withdrawn from the initially labeled compartment to determine specific activity. The ionic fluxes were then calculated from the specific activity and the amount of isotope traversing the membrane in unit time. Ionic fluxes and short-circuit currents were expressed as μeq cm^{-2} hr^{-1}.

Fluxes of Na^{22}, K^{42}, Cl^{36}, and Ca^{45} were measured in the South American toad,

Bufo arenarum. The activities of Na^{22} and K^{42} were determined in a well scintillation counter. Cl^{36} and Ca^{45} were assayed in a liquid scintillation counter.

Simultaneous fluxes of Cl^{36} and Na^{24} were measured in *Bufo marinus*. In this case the samples were counted, in a low background gas flow detector fitted with a micromil window, immediately after the experiment and again after complete decay of Na^{24}. All counts were made to $\pm 2\%$ standard deviation.

Determination of Bicarbonate Content For this purpose, the experiments were extended to 6 hr and the volume of the bathing solution in each compartment reduced to 0.7 ml, in order to allow larger changes in bicarbonate content and thus facilitate the gasometric determination. The membrane (*Bufo arenarum*) was short-circuited throughout the experiment. Samples of 0.5 ml were finally withdrawn from each compartment of the flux chamber, and the bicarbonate content was measured in a Warburg-type manometer (B. Braun, model V85 with 7 ml flasks) as the acid release of CO_2. When the original saline solution (HCO_3^- 2.4 mM) was assayed under these conditions a manometric reading of 30 mm was obtained. The total bicarbonate content in each compartment of the chamber was calculated from the content in the samples. The fluid lost by evaporation in each compartment (30 to 40 μl) was estimated after obtaining the samples by withdrawing all the remaining bathing solution with Lang-Levy pipettes.

RESULTS

Structure A histological section of a membrane used in the present experiments is shown in Fig. 2. The total thickness of this membrane, formed by the pigment epithelium of the retina and the choroid, is about 150 μ. All the choroidal layers are present, namely: the choriocapillaris (Fig. 2, *c*) which lies immediately beneath the pigment epithelium and is separated from it by Bruch's membrane; the stratum vasculosum (*v*) which is occupied by large vessels; and the suprachoroidea (*s*) which is formed by several strata of melanocytes and fibroblasts embedded in collagen. The elements present in the suprachoroidea are also found within the intervascular septa in the stratum vasculosum and choriocapillaris. Therefore, the connective tissue strands extend uninterruptedly between the choroidal surface of the membrane and the basal part of the pigment epithelium cells.

The pigment epithelium of the retina (Fig. 2, *p*) is formed by a single layer of cuboidal cells which are connected to one another by terminal bars (2, 21). The fine structure of pigment epithelium cells in the frog eye has been described in detail by Porter and Yamada (21); this description is entirely applicable to both species of toads used herein. It should be noted that as a result of the junctional complexes obliterating the intercellular spaces (2), the pigment epithelium of the retina is a continuous cellular layer. On these morphological grounds the pigment epithelium appears to be the only major diffusion barrier within the membrane used in the present work. This assump-

tion is supported by electrical data showing that the pigment epithelium is the site of the so-called R membrane of the eye (1).

Potential Measurements The electrical potential difference recorded across the pigment layers of the toad eye (both species) was usually about 20 to 30 mv, the epithelial surface positive with respect to the choroidal surface. The highest potential registered was 45 mv. The magnitude of this potential was substantially decreased by even the slightest mishandling of the membrane during dissection. In these experiments, only preparations with an initial voltage of at least 20 mv were used.

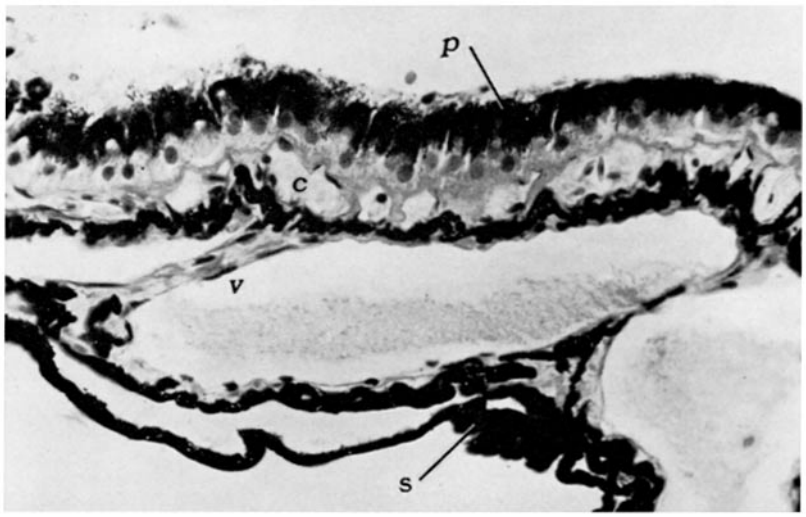

FIGURE 2. Histological section of the pigment epithelium–choroid membrane (*Bufo arenarum*). *p*, pigment epithelium; *c*, choriocapillaris; *v*, stratum vasculosum; *s*, suprachoroidea. PAS-hematoxylin staining. × 300.

During the first minutes the transmembrane potential decreased by several millivolts, but then it remained fairly stable. In preparations which were not short-circuited, potential measurements were not continued beyond 3 hr. In the case of short-circuited preparations, the voltage recorded when opening the circuit was usually the same as the initial voltage even after experiments lasting 6 hr. Changes in illumination had no effect on the potential.

Short-Circuit Current The mean short-circuit current in membranes of *Bufo arenarum* was approximately 40 μamp cm^{-2} (1.5 μeq cm^{-2} hr^{-1}). Direction of the current was from choroidal to epithelial surface. In *Bufo marinus* the current values were somewhat lower (Table IV). The current readings declined throughout the duration of the experiments at a rate of about 4.0 μamp cm^{-2} per hr.

A conspicuous current transient lasting about 1 min occurred when the current circuit was closed. A voltage transient with a similar time course was observed upon opening the circuit. These transients were essentially identical to those thoroughly described by Cooperstein and Hogben (3) in the frog large intestine.

Resistance Values During the flux determinations, the electrical resistance of the membrane was estimated every 30 min from the short-circuit current and the instantaneous voltage change obtained when the current circuit was opened briefly. From the preceding reference to the current and voltage transients, it follows that the membrane cannot be treated as a simple dc resistor. Linderholm (17) has also pointed out that the short-circuit current is not an adequate reference for estimating the electrical resistance in frog skin. Therefore, the resistance values are not intended to reflect accurate measurements. They were obtained only in order to have an additional way to compare different sets of experiments. For this purpose the resistance measurements were considered adequate since the error can at least be said to be equivalent for all the membranes studied.

Since the short-circuit current decreases steadily while the potential remains relatively stable, the resistance values obtained as described above increase with time. The mean electrical resistance is about 450 Ωcm^2 in membranes from *Bufo arenarum* and 600 Ωcm^2 in the case of *Bufo marinus*. In the tables these figures are expressed as conductance values to facilitate comparison with the ionic fluxes.

Ionic Fluxes Unidirectional movements from epithelial surface to choroid will be referred to as *outfluxes*, indicating direction with respect to the eye as a whole. Conversely, unidirectional ionic movements from choroid to epithelial surface will be termed *influxes*. Since the direction of the current is from choroid to pigment epithelium, it must be carried by a net cation influx and/or anion outflux.

The mean chloride fluxes in *Bufo arenarum* are presented in Table I. There is a net outflux of 0.92 $\mu eq\ cm^{-2}\ hr^{-1}$ which accounts for a major fraction of the mean short-circuit current. However, a substantial part of the current does not appear to be carried by chloride. The chloride outfluxes in individual experiments are shown in Table II, together with the parallel short-circuit currents. Only in a few instances were the chloride outfluxes larger than the current.

In an attempt to define the nature of the nonchloride fraction of the short-circuit current, the fluxes of other ions present in the bathing solution were also measured. The unidirectional fluxes of sodium in *Bufo arenarum* are shown to be of the same magnitude in Table III.

Measurements of the unidirectional chloride and sodium fluxes were also carried out in *Bufo marinus*. In this case, simultaneous fluxes of both ions were determined. The values obtained are given in Table IV. There is a net chloride outflux which accounts for about the same fraction of the mean short-circuit current that was found in *Bufo arenarum*. In *Bufo marinus*, however, a small net influx of sodium is observed also. Nevertheless, in *Bufo marinus*, as in *Bufo arenarum*, a fraction of about one-third of the mean short-circuit current remains unaccounted for.

TABLE I

CHLORIDE FLUXES AND SHORT-CIRCUIT CURRENTS ACROSS THE ISOLATED PIGMENT EPITHELIUM AND CHOROID (*Bufo arenarum*)

	No. of experiments	Mean Cl^- flux	Mean short-circuit current	Mean conductance
		$\mu eq\ cm^{-2}\ hr^{-1}$	$\mu eq\ cm^{-2}\ hr^{-1}$	$mmhos\ cm^{-2}$
Outflux (epithelium to choroid)	12	1.47±0.12*	1.44±0.06	2.19±0.08
Influx (choroid to epithelium)	11	0.55±0.10	1.50±0.07	2.08±0.07
Net		0.92‡		

* Standard error of the mean.
‡ $p < 0.001$.

TABLE II

CHLORIDE OUTFLUXES AND SIMULTANEOUS SHORT-CIRCUIT CURRENTS (*Bufo arenarum*)

Experiment No.	Cl^- outflux	Short-circuit currents
	$\mu eq\ cm^{-2}\ hr^{-1}$	$\mu eq\ cm^{-2}\ hr^{-1}$
1	1.57	1.58
2	1.36	1.64
3	2.00	1.43
4	2.39	1.54
5	1.04	1.07
6	1.06	1.42
7	1.45	1.56
8	1.74	1.52
9	1.30	1.45
10	1.07	1.06
11	1.12	1.28
12	1.59	1.80

Fluxes of potassium and calcium were measured in *Bufo arenarum* (Table V). The observed influxes were too small to justify any further consideration of these ions as possible carriers of any important fraction of the current. In the case of potassium, however, the outflux was measured in two experiments and found equal to the influx. The potassium influx was also measured in

one experiment in *Bufo marinus* with the same result that was obtained in *Bufo arenarum.*

Determination of Bicarbonate Changes Bicarbonate is the only ion included in the bathing solution that has not been mentioned in the preceding account. Radioisotope methods were not used to analyze bicarbonate movements because of difficulties in avoiding $C^{14}O_2$ exchange with the atmosphere. An attempt was made to measure bicarbonate manometrically, in

TABLE III

SODIUM FLUXES AND SHORT-CIRCUIT CURRENTS ACROSS THE ISOLATED PIGMENT EPITHELIUM AND CHOROID (*Bufo arenarum*)

	No. of experiments	Mean Na^+ flux	Mean short-circuit current	Mean conductance
		$\mu eq\ cm^{-2}\ hr^{-1}$	$\mu eq\ cm^{-2}\ hr^{-1}$	$mmhos\ cm^{-2}$
Influx	10	0.74±0.13	1.48±0.09	2.40±0.21
Outflux	10	0.79±0.12	1.41±0.03	2.82±0.27
Net		0.05*		

* $p > 0.7$.

TABLE IV

SIMULTANEOUS CHLORIDE AND SODIUM FLUXES AND SHORT-CIRCUIT CURRENTS ACROSS THE ISOLATED PIGMENT EPITHELIUM AND CHOROID (*Bufo marinus*)

	No. of experiments	Mean Cl^- flux	Mean Na^+ flux	Mean short-circuit currents	Mean conductance
		$\mu eq\ cm^{-2}\ hr^{-1}$	$\mu eq\ cm^{-2}\ hr^{-1}$	$\mu eq\ cm^{-2}\ hr^{-1}$	$mmhos\ cm^{-2}$
Outflux	8	1.37±0.06	0.35±0.03	1.13±0.04	1.60±0.06
Influx	8	0.69±0.06	0.48±0.03	1.11±0.06	1.60±0.06
Net		0.68* (outflux)	0.13‡ (influx)		

* $p < 0.001$.
‡ $p < 0.02$.

the expectation that the total content in the bathing solution would decrease in the epithelial compartment of the flux chamber and increase in the choroidal compartment by an amount equivalent to the unidentified fraction of the short-circuit current. As shown in Table VI, the expected decrease was found in the epithelial compartment. However, the solution in the choroidal compartment failed to show changes.

Since this finding could be explained by an asymmetric release of acid from the tissue, pH measurements were taken during three experiments in which the pigment layers were bathed in bicarbonate-free unbuffered saline solution. After 2 hr under short-circuit conditions, the solution in neither the

epithelial compartment nor in the choroidal compartment showed appreciable change in pH.

Other Observations Replacement of chloride by the anions mentioned under Methods brought about a 50 to 60% decrease in short-circuit current; the previous value of the current was regained on return to the chloride medium.

TABLE V

POTASSIUM AND CALCIUM FLUXES ACROSS THE ISOLATED PIGMENT EPITHELIUM AND CHOROID (*Bufo arenarum*)

	No. of experiments	Mean flux	Mean short-circuit current	Mean conductance
		$\mu eq\ cm^{-2}\ hr^{-1}$	$\mu eq\ cm^{-2}\ hr^{-1}$	$mmhos\ cm^{-2}$
K^+ influx	4*	0.04±0.01	1.63±0.07	2.12±0.09
K^+ outflux	2	0.05	1.54	2.01
Ca^{++} influx	2	0.05	1.63	1.86

* One additional measurement was performed in *Bufo marinus*. The value obtained was 0.03 μeq cm^{-2} hr^{-1}.

TABLE VI

CHANGES IN BICARBONATE CONTENT (*Bufo arenarum*)

Observation No.	Changes in absolute CO_3H^- content		Short-circuit current
	Choroidal compartment	Epithelial compartment	
	$\Delta\mu eq\ cm^{-2}\ hr^{-1}$*	$\Delta\mu eq\ cm^{-2}\ hr^{-1}$	$\mu eq\ cm^{-2}\ hr^{-1}$
1	+0.09	−0.96	1.69
2	+0.08	−0.42	1.80
3	−0.05	−0.62	1.32
4	+0.01	−0.60	1.41
5	+0.07	−0.54	1.41
Mean	+0.04±0.03	−0.63±0.09	1.52±0.09

* Bicarbonate changes are expressed as a function of the surface of the membrane.

Cyanide (10^{-3} M) abolished the short-circuit current and electrical potential very rapidly. Anoxia (N_2) had a comparable effect but only after several minutes of latency; this was a reversible effect. Sodium azide (10^{-3} M) induced a transitory rise in short-circuit current which lasted about 10 min, followed by a rapid fall. Iodoacetate (5×10^{-3} M) did not have a marked effect; it only accelerated the usual rate of decline of the short-circuit current. Ouabain (10^{-5} M) had no appreciable effect.

Acetazoleamide (10^{-3} M) decreased the short-circuit current by about 20%;

this effect was achieved in 15 min. The unidirectional chloride fluxes were not changed by acetazoleamide (5 outflux and 3 influx determinations) and the net chloride flux still failed to account for all the short-circuit current.

DISCUSSION

An electrical potential difference of about 20 to 30 mv was shown to exist across the membrane formed by the isolated pigment layers of the toad eye, the pigment epithelium surface being positive with respect to the choroid. The precise site of origin of this potential was not determined, but available evidence points to the pigment epithelium of the retina as the electrogenic layer since, as noted before it is the only continuous cellular stratum within the membrane. Nevertheless, microelectrode studies similar to those performed in other biological membranes (6, 7, 25) will probably be needed to validate this assumption.

The recorded potential has the same polarity as the resting potential of the retina. Therefore, these results support Noell's (18) hypothesis that the pigment epithelium is the major source of that transretinal dc potential. The possibility that the neural retina contributes to the resting potential is not eliminated. However, if the neural retina of the toad is mounted in a lucite chamber similar to the one presently used for the pigment layers, a dc potential of only 0.6 to 0.8 mv, receptor side negative, is found in the dark-adapted state and no steady potential is observed in the light (unpublished observations). Nevertheless, a normal electroretinogram can be elicited during a period of more than 1 hr under these conditions (14).

The measurements of radioisotopic fluxes across the isolated pigment layers of the toad eye indicate a net transfer of chloride from epithelial surface to choroid which accounts for a large part of the mean short-circuit current. It is now generally accepted that asymmetric ion movement in the absence of an electrochemical potential difference is indicative of an active transport[1] of the particular ion involved (23, 24). It seems, therefore, that the pigment layers of the toad eye can be incorporated into the group of tissues in which active transport of chloride has been reported (4, 9, 10, 27).

On the other hand a question is posed by the finding of asymmetric sodium fluxes in *Bufo marinus*. The significance of this observation seems somewhat uncertain in view of the small magnitude of the net influx in relation to the unidirectional fluxes. A comparable net sodium flux was not observed in

[1] The magnitude of the water fluxes across the membrane is ignored and therefore it is not known whether convection might be a significant factor contributing to asymmetrical ion fluxes. It is to be noted, however, that equivalent asymmetries in the fluxes of ions other than chloride were not observed.

Bufo arenarum. However, due to the larger unidirectional sodium fluxes observed in this species, a small net flux would be still more difficult to detect. Clearly, the measurement of unidirectional fluxes is not an efficient way to deal with this problem.

About one-third of the mean short-circuit current is not accounted for by the measurements of the fluxes of Cl^-, Na^+, K^+, and Ca^{++}. This would appear to leave only bicarbonate as the probable carrier for that fraction of the current. The results of the manometric determinations do not lend support to this view. The reduction in bicarbonate content in the epithelial compartment of the chamber does not appear to be due to release of acid from the pigment epithelium cells according to the pH measurements in bicarbonate-free solution. It seems therefore more likely that a process of bicarbonate uptake occurs at the pigment epithelium. Whether this process is in any way related to the short-circuit current is totally unknown at the present time.

The results here reported do not coincide with those obtained by Noell et al. (19, 20) in the rabbit eye in situ. In this case a net flux of potassium from the blood toward the cavity of the eye accounted for most of the short-circuit current. In interpreting the discrepancy, the substantial differences in experimental set-up have to receive consideration, since the route traversed by the radioisotopic tracers is not the same in the toad and rabbit preparations. Nevertheless, it is not unlikely that the real explanation rests on actual differences between the toad and rabbit eyes with respect to the functional aspects under consideration. This possibility receives support from the fact that although an active transport of sodium has been described in the cornea of the rabbit eye (5), a transcorneal transport of chloride is found in the frog (26).

The interest in designing a method to perform electrical measurements on the isolated pigment epithelium of the retina was prompted by the fact that pigment epithelium cells are the retinal counterpart of ependymal cells. Due to its anatomical location the pigment epithelium probably has an important role in regulating the movement of ions and metabolites between the retina and the choroidal vessels (18). As has already been shown (13, 15), the intercellular spaces in the nervous tissue of the retina provide effective diffusion pathways and therefore the pigment epithelium is the only major barrier interposed between retinal neurons and choroidal blood. Consequently, it is hoped that data provided by the pigment epithelium and choroid preparation may prove relevant to such problems as the functional properties of glial cells and the mechanisms of regulation of the inner milieu in the central nervous system. In addition, this preparation may represent a useful incorporation into the list of tissues in which the basic features of membrane transport can be analyzed.

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