dent of the transmission characteristics of the ocular media. It may provide an efficient tool to separate patients with electrically normal retinas from those with diseased retinas.

From the Eye Research Laboratories, Department of Ophthalmology. The University of Chicago, Chicago, Ill. This investigation was supported in part by the Public Health Service Grants EY-00523 and FR-55 from the National Eye Institute, National Institutes of Health. Submitted for publication Feb. 2, 1977. Reprint requests: David Schanzlin, M.D., Department of Ophthalmology, The University of Chicago, 950 East 59 St., Chicago, Ill. 60637.

Key words: Electroretinography, transscleral, sclera, vitrectomy, opaque media, ERG.

REFERENCES

1. Fuller, D. G., Knighton, R., and Machemer, R.: Bright flash electroretinography for evaluation of eyes with opaque vitreous, Am. J. Ophthalmol. 80:214, 1975.
2. Krill, A.: Hereditary Retinal and Choroidal Diseases, New York, 1972, Harper & Row, Publishers, vol. 1, p. 243.
3. Smith, R. S., and Stein, M.: Ocular hazards of transscleral laser radiation, Am. J. Ophthalmol. 66:21, 1968.

Effect of prostaglandins on the blood-aqueous barrier of the perfused cat eye.

G. W. H. M. VAN ALPHEN* AND PETER WILHELM.

With a technique of direct visualization of the arterially perfused cat eye, the pressure head may be chosen so that fluorescein added to the perfusate just barely stains the ciliary processes. After addition of PGE_1, PGE_2, $PGF_{2\alpha}$, arachidonic acid, or indomethacin, with or without PG's, no more dye emerged from the processes. The addition of acetylcholine in the eserinized eye floods the processes with dye, apparently affecting the pore size of the blood-aqueous barrier; PG's may slightly inhibit, rather than facilitate, the emergence of fluorescein from the processes. It is speculated that in the cat PG's elevate pressure and protein content by backflow from the circle of Hovius, which is the equivalent of Schlemm's canal.

Topical, intracameral, or intravenous administration of prostaglandins (PG's) in rabbits, cats, and monkeys may induce miosis and may elevate the intraocular pressure (IOP) with a concomitant rise in aqueous protein.[1-6] It was therefore suggested that PG's disrupt the blood-aqueous barrier.[2, 3] This has been confirmed electron microscopically, with the help of tracers, in rabbit and monkey.[12-14] In a previous paper we have reported the effect of drugs on the blood-aqueous barrier in the isolated, arterially perfused cat eye by direct visualization of the ciliary processes and observation of the entrance of fluorescein.[7] In similar experiments we have perfused 60 enucleated cat eyes with PGE_1, PGE_2, and $PGF_{2\alpha}$, using fluorescein as a marker and acetylcholine as a control.

Materials and methods. The method was essentially the same as the one described previously.[7] Eyes were enucleated from adult cats anesthetized intraperitoneally with 30 mg/kg pentobarbital sodium. The cats had been heparinized 1 hr before enucleation. The enucleated eye was placed in a dish at 37° on moist gauze. One of the long posterior ciliary arteries was cannulated intrasclerally with tapered No. 10 polyethylene tubing and was perfused with oxygenated Krebs-Ringer solution under a pressure head of 140 cm of water. Two parallel incisions, 5 mm apart, were made in the sclera on each side of the long posterior ciliary arteries. With the use of blunt scissors the sclera was removed from the choroid in the area posterior to the equator, with the exception of two strips which contained the long posterior ciliary arteries. The cornea was removed at the limbus. The choroid was incised in two perpendicular meridians in the main direction of the blood vessels, and the four quadrants were reflected back over the sclera of the anterior segment. In this manner, the posterior surface of the iris and the ciliary processes could be visualized through vitreous and lens. The noncannulated long posterior ciliary artery was ligated.

The perfusion bottles contained Krebs-Ringer solution with and without PG or eserine (Es). The solutions were oxygenated prior to use with 95% O_2–5% CO_2 for 10 min. They were then maintained under this gaseous atmosphere during the perfusion period. The head pressure in all the bottles was the same, and flow could be obtained from any one bottle by means of stopcocks. This system allowed for perfusion at constant pressure, the perfusion of a measured amount of sodium fluorescein (0.25 ml of 0.05%), and the constant or intermittent administration of pharmacological agents. Final concentrations of PG's were 10 or exceptionally 100 μg/ml; Es, 1 μg/ml; and acetylcholine (Ach), 1 μg/ml. The appearance and outflow of fluorescein at the tips and valleys of the ciliary processes were observed through an operating microscope and were graded from 0 to ++++. The behavior of the pupil was seen through the lens.

Results

Effects of fluorescein. With a pressure head of 140 cm of water the administration of 0.25 ml of 0.05% fluorescein to the perfusion tubing produced only faint staining of the tips of the processes and less frequently of the walls. Occasionally slight outflow was noted in the valleys. When the dye was no longer seen in the tubing, it would also disappear from the processes. After 20 min of perfusion, fluorescein was entirely washed away, and further observations were possible by addition of another bolus of dye. Also, washing could be done by perfusate that contained predetermined amounts of pharmacological agents; the effects of fluorescein could then be tested again, and so on.

Effect of Es-Ach. After the eye had been perfused for 20 min with Es (1μg/ml.), fluorescein would only faintly stain the processes. However, the addition of 1 μg of Ach would produce an intense staining, with the dye emerging from tips and walls and filling the valleys of the processes. It would then flow over the root of the iris and gradually enter the anterior chamber. It required 20 to 60 min to wash out the fluorescein. This effect was referred to earlier as the Es-Ach effect, and its parameters were studied in the majority of over 200 cat eyes.[7]

Effect of PGE_1, PGE_2, and $PGF_{2\alpha}$. Sixteen cat eyes were perfused with PGE_1, PGE_2, or $PGF_{2\alpha}$. In 11 experiments these agents produced no more staining and no more outflow than fluorescein alone. In five experiments more fluorescein appeared at the tips or was seen in the valleys; in two eyes there was also slight outflow of the dye.

Effect of PGE_1, PGE_2, and $PGF_{2\alpha}$ followed by Es-Ach. In 21 cat eyes fluorescein alone was perfused first, PG + fluorescein second, and Es-Ach + fluorescein third. In 12 experiments there was no outflow of fluorescein at all, either after PG or after Es-Ach. In six of the nine remaining experiments the effect of PG's was no greater than with fluorescein alone; in three experiments a slight amount of outflow occurred after PG's. However, in *all* nine experiments a subsequent perfusion with Es-Ach alone produced a massive outflow of fluorescein from the tips and from the valleys, flowing over the root of the iris and entering the anterior chamber.

Effect of PG's after Es-Ach. In 11 out of 16 experiments, perfusion with Es-Ach was followed by massive outflow of fluorescein. In these 11 positive experiments subsequent perfusion with PG's produced less outflow of fluorescein than did Es-Ach. In three experiments in which outflow considerably decreased on infusion of PG's, the perfusion with Es-Ach was repeated. Again a massive outflow of fluorescein was observed. It would seem therefore that PG's might inhibit rather than facilitate the appearance of fluorescein from the ciliary processes.

Table I. Number of irides responding

	Constriction	*Dilatation*
PGE_1	2	2
PGE_2	4	1
$PGF_{2\alpha}$	2	18

Effect of arachidonic acid (AA), PGA, and PGB. In four experiments 10 μg/ml AA produced no more staining than did fluorescein alone. In two experiments, eyes were perfused with 10 μg/ml of PGA_2 or PGB_2, but no more fluorescein emerged than without PG's.

Effect of indomethacin. To exclude possible effects of PG precursors by endogenous PG synthesis, five eyes were perfused with indomethacin for 15 min; then PGE_1, or PGE_2, or $PGF_{2\alpha}$ was added to the indomethacin-containing perfusate. No effect was seen on fluorescein appearance from indomethacin alone or in combination with PG's.

Effect on pupil. The addition of PG's to the perfusate affects the pupil. The results are given in Table I.

The perfusion of PGE was sometimes followed by PGF and vice versa. Only definite effects are represented in the table. PGE sometimes followed Es-Ach. Since Es-Ach frequently produced pupillary constriction, PGE effects were possibly masked. The table shows that $PGF_{2\alpha}$ has a high frequency of pupil dilatation. In six experiments L-epinephrine, 1 μg/ml, was added after PG's or Es-Ach. It produced maximal dilatation in all six cases.

Discussion. The finding that perfusion of the cat eye with PGE_1 or $F_{2\alpha}$ did not obviously affect the blood-aqueous barrier was quite unexpected. Perfusion with PG's would only occasionally produce outflow of fluorescein, whereas in many experiments subsequent administration of Es-Ach would flood the eye with dye, pouring out of the tips and valleys of the ciliary processes but, incidentally, never emerging from the back of the iris. The effect of Es-Ach on the permeability of the blood-aqueous barrier as observed with fluorescein is indeed a striking demonstration of aqueous filtration, since it is pressure dependent. Other parameters which may affect the response to Es-Ach

were amply discussed in earlier studies.[7] This effect of Es-Ach has been repeatedly confirmed by more sophisticated tracer studies of Macri and collaborators.[8, 9] No such effect has been observed with PG's in concentrations of up to 100 μg/ml, although in a few experiments more dye emerged after PGE or PGF than with fluorescein alone.

The data presented here suggest an inhibiting effect of PG's on the appearance of fluorescein. In some experiments the barrier was first opened by Es-Ach; however, less fluorescein appeared after perfusion with PGE or PGF. After washing, Es-Ach would again produce maximal staining. It is possible that vasoconstriction by PG's might reduce the filtration pressure, thus producing less fluorescein. Finally neither AA nor indomethacin (with or without PG's) facilitated filtration. This again does not support an effect of PG's on permeability.

The question then arises as to how PG's elevate IOP and aqueous protein in the cat. There is a possibility of backflow from Schlemm's canal as suggested by tracer studies of Raviola[10] after paracentesis of the monkey eye; however, Okisaka[11] claimed that tracer also penetrates the anterior part of the ciliary processes. Moreover, subconjunctival injection of PGE_1 affected mainly the processes but left Schlemm's canal relatively unaffected.[12]

In the rabbit the main site of entrance of proteins is presumably the iridial process[13–15]; less protein enters through the ciliary processes. However, the exact morphological changes in the epithelial layers are partly controversial and not completely understood. With our perfusion technique the fluorescein molecule, having a low molecular weight and not bound to serum proteins, should have emerged if PG's affected the ciliary processes. Since it did not, we will have to conclude that the mechanism of the elevation of IOP in the rabbit differs from that in the cat; also, the dose required to elevate the pressure in cats is 2 magnitudes higher than in rabbits.[2] Obviously, only tracer studies of the cat eye may settle the question, but no such studies are available.

Finally, we do not know why $PGF_{2\alpha}$ dilates the pupil in the perfused cat eye in contrast to PGE_2. Both PG's produce miosis in vivo.[1, 2] On the other hand, both PG's produce sphincter *and* dilator contraction in vitro[16]; and although calculations indicate that in the cat eye, PG-induced sphincter and dilator antagonism is slightly in favor of miosis, they do not explain the frequent dilatation by $PGF_{2\alpha}$ in the perfused eye.

The prostaglandins were a gift from The Upjohn Co. and Dr. J. Pike, and indomethacin from Merck, Sharp & Dohme and Dr. C. Stone.

From the Department of Ophthalmology, The University of Michigan, Ann Arbor. *Present address: Ophthalmic Research Institute, Amsterdam, The Netherlands. This work was supported by the Rackham foundation. Submitted for publication June 26, 1977. Reprint requests: G. W. van Alphen, M.D., I.O.I., Wilhelmina Gasthuis, Eerste Helmersstraat 104, Amsterdam 1013, The Netherlands.

Key words: prostaglandins, arachidonic acid, indomethacin, blood-aqueous barrier, cat, Schlemm's canal, fluorescein

REFERENCES

1. Waitzman, M. B., and King, C. D.: Prostaglandin influences on intraocular pressure and pupil size, Am. J. Physiol. 212:329, 1967.
2. Beitch, B. R., and Eakins, K. E.: The effects of prostaglandins on the intraocular pressure of the rabbit, Br. J. Pharmacol. 37:158, 1969.
3. Eakins, K. E.: Increased intraocular pressure produced by prostaglandins E_1 and E_2 in the cat eye, Exp. Eye Res. 10:87, 1970.
4. Kelly, G. M., and Starr, M. S.: Effects of prostaglandins and a prostaglandin antagonist on intraocular pressure and protein in the monkey eye, Can. J. Ophthalmol. 6:205, 1971.
5. Bethel, R. A., and Eakins, K. E.: The mechanism of the antagonism of experimentally induced ocular hypertension by polyphloretin phosphate, Exp. Eye Res. 13:83, 1971.
6. Kass, M. A., Podos, S. M., Moses, R. A., et al.: Prostaglandin E_1 and aqueous humor dynamics, INVEST. OPHTHALMOL. 11:1022, 1972.
7. van Alphen, G. W. H. M., and Macri, F. J.: Entrance of fluorescein into aqueous humor of cat eye, Arch. Ophthalmol. 75:247, 1966.
8. Macri, F. J., and Cevario, S. J.: The induction of aqueous humor formation by the use of Ach + eserine, INVEST. OPHTHALMOL. 12:910, 1973.
9. Macri, F. J., Cevario, S. J., and Ballentyne, E. J.: The arterial pressure dependency of the increased aqueous humor formation induced by Ach + eserine, INVEST. OPHTHALMOL. 13:153, 1974.
10. Raviola, G.: Effects of paracentesis on the blood-aqueous barrier: an electron microscopic study on *Macaca mulatta* using horseradish peroxidase as a tracer, INVEST. OPHTHALMOL. 13:828, 1974.
11. Okisaka, S.: Effects of paracentesis on the blood-aqueous barrier: a light and electron microscopic study on cynomolgus monkey, INVEST. OPHTHALMOL. 15:824, 1976.
12. Okisaka, S.: The effects of prostaglandin E_1 on the ciliary epithelium and the drainage angle of

cynomolgus monkeys: a light- and electron-microscopic study, Exp. Eye Res. 22:141, 1976.
13. Tamura, T.: Electron microscopy of the ciliary body following local administration of prostaglandins E_1 and E_2 in rabbits, Folia Ophthalmol. Jpn. 25:1112, 1974 (quoted from Excerpta Medica 30:1592, 1976).
14. Oyvind Pedersen, O.: Electron microscopic studies on the blood aqueous barrier of prostaglandin treated rabbit eyes. I. Iridial and ciliary processes, Acta Ophthalmol. 53:685, 1975.
15. Ohtsuki, K., and Sears, M. L.: Disruption of the blood aqueous barrier demonstrated by histofluorescence microscopy, Acta Ophthalmol. Jpn. 79:663, 1975 (Japanese) (quoted from Excerpta Medica 30:2343, 1976).
16. van Alphen, G. W. H. M., and Angel, M. A.: Activity of prostaglandin E, F, A and B on sphincter, dilator and ciliary muscle preparations of the cat eye, Prostaglandins 9:157, 1975.

Collagen crosslinking in keratoconus.

DONALD J. CANNON* AND C. STEPHEN FOSTER**

The examination of reducible collagen crosslinks in keratoconus cornea revealed the presence of lysinonorleucine in amounts far greater than in normal age-matched corneas. There was no indication of decreased hydroxylysine levels in keratoconus, and there were no clinical indications of a generalized connective tissue disorder. The abnormal levels of dehydrolysinonorleucine in the tissue may represent a change in hydroxylation of selected lysyl residues of normal collagen or the synthesis of abnormal collagen, perhaps an unusual type.

Keratoconus is a central noninflammatory (usually bilateral) ectasia of the cornea, of unknown etiology. Histopathologic studies have demonstrated decreased numbers of collagen fibrils which appeared morphologically normal, keratocytes with membrane anomalies, fragmentation of the epithelial basement membrane, fibrillation and disintegrations of Bowman's membrane, and (at the electron microscopic level) degenerative changes of the basal epithelial cells.[1, 2] Biochemical studies[3] have revealed decreased levels of glucose-6-phosphate dehydrogenase in keratoconus corneas, and Robert et al.[4] have found relative decreases in hydroxylation of lysine and glycosylation of hydroxylysine, decreased total collagen, and relatively increased structural glycoprotein.

Collagen is the major macromolecular constituent of corneal stroma and is arranged in lamellae of uniform-diameter fibrils. Collagen molecules are secreted from connective tissue cells as procollagen,[5] a biosynthetic precursor of collagen consisting of three α-chains with additional amino- and carboxy-terminal extensions (pro-α-chains). Fibrils are constructed from the aggregation of collagen molecules from which the procollagen peptides have been cleaved. Collagen is subject to a number of posttranslational modifications, one of which is the formation of crosslinks via the enzymatic oxidation of lysine and hydroxylysine residues to their respective aldehydes.[6] Through a series of condensations these aldehydes condense with other aldehydes or intact lysyl and hydroxylysyl residues to form intramolecular and intermolecular covalent crosslinks between collagen polypeptide chains. These crosslinks can be stabilized and reduced by sodium [^{3}H]-borohydride, and their identity can be established by ion-exchange chromatography following hydrolysis.

Table I. Distribution of incorporated tritium in native keratoconus cornea*

Peak	Age (yr) and sex					Normal†
	22M	38F	40M	44F	48M	
HLHNL	12.3	30.0	—	23.0	23.0	(23-36)
HLNL	13.5	9.5	—	12.7	13.0	(9-11)
LNL	17.6	17.6	67.1	8.9	32.0	(0-5)
HisHM	9.6	—	—	—	—	(0-4)

*Individual peaks are expressed as percent of total eluted radioactivity. The in vivo crosslinks were reduced to give the derivatives resolved as the following peaks: HLHNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; LNL lysinonorleucine; HisHM, histidinohydroxymerodesmosine.
†Range of values in normal human cornea, 15-64 years old.[8]

Table II. Tritium activity in reduced lysinonorleucine

Case,* age (yr), and sex	Lysine (cpm/μmol)
1. 22M	1,087
2. 38F	1,383
3. 44F	1,793
4. 34M	177

*Cases 1 to 3 keratoconus; case 4 normal cornea.

Methods. The present study examines the reducible crosslink content of human keratoconus cornea buttons, 7.5 mm, which were surgically removed in the course of penetrating keratoplasty. The corneal buttons had no scar tissue but were thinner than normal human corneas. The patients were in all respects normal with the exception of keratoconus. No hyperextensibility of the joints was present. Following excision the tissue was immediately frozen in sterile physiological saline. The tissue was finely minced, washed briefly in iced 0.02M EDTA, pH 7.0, and then reduced with ^{3}H-$NaBH_4$ as described previously.[7]