Review

Unconventional aqueous humor outflow: A review

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ABSTRACT

Aqueous humor flows out of the eye primarily through the conventional outflow pathway that includes the trabecular meshwork and Schlemm's canal. However, a fraction of aqueous humor passes through an alternative or 'unconventional' route that includes the ciliary muscle, supraciliary and suprachoroidal spaces. From there, unconventional outflow may drain through two pathways: a uveoscleral pathway where aqueous drains across the sclera to be resorbed by orbital vessels, and a uveovortex pathway where aqueous humor enters the choroid to drain through the vortex veins. We review the anatomy, physiology and pharmacology of these pathways. We also discuss methods to determine unconventional outflow rate, including direct techniques that use radioactive or fluorescent tracers recovered from tissues in the unconventional pathway and indirect methods that estimate unconventional outflow based on total outflow over a range of pressures. Indirect methods are subject to a number of assumptions and generally give poor agreement with tracer measurements. We review the variety of animal models that have been used to study conventional and unconventional outflow. The mouse appears to be a promising model because it captures several aspects of conventional and unconventional outflow dynamics common to humans, although questions remain regarding the magnitude of unconventional outflow in mice. Finally, we review future directions. There is a clear need to develop improved methods for measuring unconventional outflow in both animals and humans.

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1. Introduction

The pathway of aqueous humor drainage has long been of interest and is of great importance because it provides the fluid resistance that maintains a proper intraocular pressure (IOP). IOP that is too low can impair vision by distorting the retina, cornea, and lens; IOP that is too high can lead to glaucomatous optic neuropathy.

Aqueous humor drainage from the anterior chamber through the trabecular meshwork, Schlemm's canal, collector channels, aqueous veins, and into the episcleral veins was first proposed by Leber (1873, Schwalbe (1870), and Knies (1875), and finally demonstrated by Seidel (1921) and Ascher (1942). The latter's observations of clear fluid in aqueous veins established this path as the primary route of aqueous humor outflow from the eye. After exploring aqueous humor drainage in more detail by using tracer molecules, investigators eventually realized that aqueous humor also left the eye through another route, passing through the uvea, the ciliary body and muscle, and into the choroid and sclera. This pathway has been called the uveoscleral, uveovortex, or unconventional pathway to distinguish it from the trabecular pathway, and has been estimated to carry 3—82% of the total aqueous humor outflow in different species. This paper will review the historical basis of our understanding of unconventional outflow, its properties and characteristics, how it is measured, and its significance to glaucoma research.

2. Historical basis of unconventional outflow

Leber reported in 1903 that tracers introduced into the anterior chamber passed not only into the conventional trabecular outflow pathway but also were found in the suprachoroidal space (Nesterov, 1986). Other early investigators (Nuel and Benoit, 1900; Erdmann, 1907; Seidel, 1921; Kiss, 1943) reported that colloidal tracer accumulated outside the conventional outflow pathway, often deep within the peripheral ciliary body, posterior sclera, and choroid after perfusion with these tracers. These studies led investigators to infer the existence of a secondary aqueous humor outflow pathway (Fine, 1964) now known as the "unconventional" outflow pathway.

It was not until studies in monkeys by Anders Bill and colleagues in the 1960's that our functional understanding of unconventional outflow solidified. Bill explored this pathway quantitatively by perfusing radiolabeled molecules of various sizes through the anterior chamber and examining the different pathways by which the tracers left the eye (Bill, 1962, 1965, 1966a, 1966c, 1966d). By
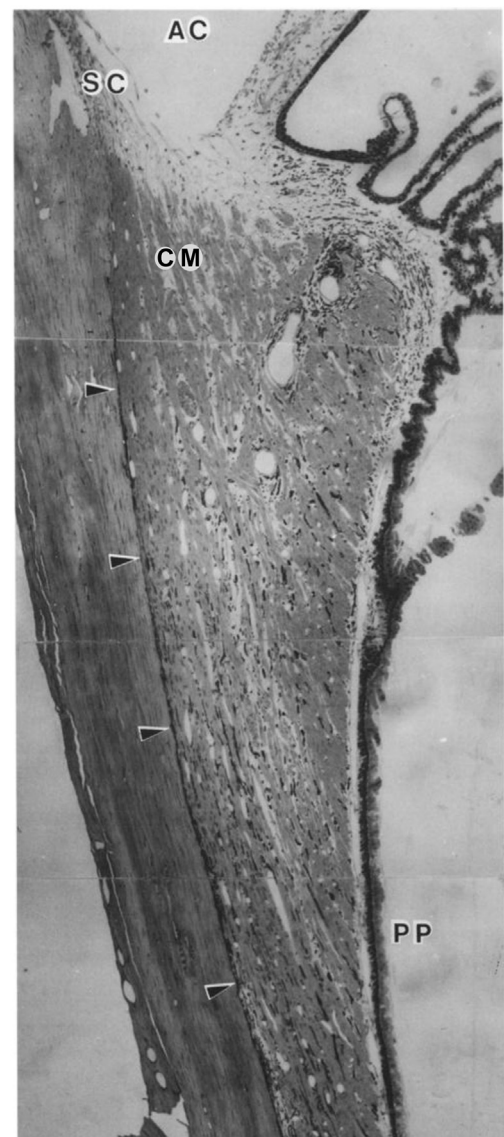
Fig. 1. Meridional section through the uveal tract of a *Macaca fascicularis*. Arrowheads show supraciliary and suprachoroidal space. Unconventional outflow passes from the anterior chamber (AC), through the most posterior aspects of the uveal meshwork, enters the open spaces between longitudinal aspects of the ciliary muscle (CM) and then enters the suprachoroidal space. SC — Schlemm's canal; PP — pars plana. Reprinted from (Wood et al., 1990) with permission from Springer.
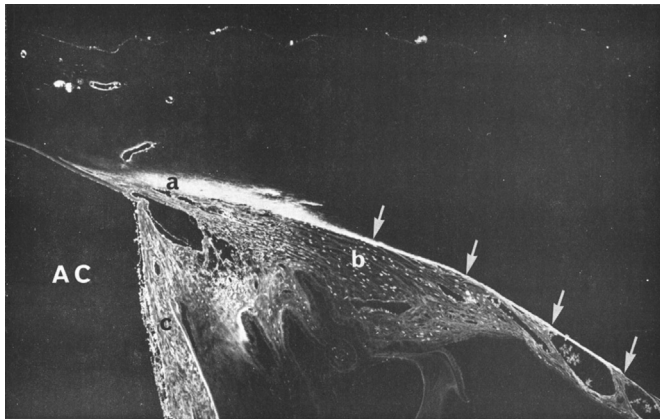
Fig. 2. Light micrograph of fluorescein dextran in the unconventional outflow pathway of a rabbit after intracameral perfusion (AC, anterior chamber). Fluorescence is visible in the conventional aqueous drainage pathway (a), comprising angular meshwork, angular aqueous plexus, and collector channels and aqueous veins, and in the unconventional outflow in the outer aspects of the ciliary body (b) and muscle, and in the suprachoroidal space (arrows). Tracer is also seen in the iris (c), which has been artefactually deflected posteriorly. Reprinted from (Tripathi, 1977) with permission from Elsevier.

collecting tracer in the blood, Bill could account for only about 80% of the tracer that left the eye, presumably through the trabecular outflow pathway. The remaining tracer accumulated in the ciliary body, choroid, and sclera. Because tracer accumulated at a constant rate in the sclera and other tissues and because this accumulation was not correlated with the tracer's diffusion coefficient, Bill concluded that there must be a flow from the anterior chamber through the uvea and into the sclera by way of the choroid and suprachoroid (Bill, 1965; Bill and Hellsing, 1965; Bill, 1966a). Bill referred to this as the "unconventional route" to distinguish it from the trabecular, or "conventional route".

The definition of unconventional outflow has been expanded to include any of the pathways through which aqueous humor might leave the eye other than through the trabecular pathway, including a corneal route, an iridial route (Fine, 1964) and a retinal route (Fowlks et al., 1963). Flow through the corneal and iridial routes has been shown to be negligible (Bill, 1977). Flow through the retinal route is generated by the pumping capacity of the retinal pigment epithelium, but is considered to be small as long as the retina remains attached (Pederson and Cantrill, 1984). This review will focus on the flow exiting the posterior aspect of the uveal meshwork, passing through the ciliary muscle, and entering the suprachoroidal space.

Bill (1966a,d) found that in cynomolgus and vervet monkeys, the rate of outflow through the unconventional pathway ranged from 25 to 40% of total aqueous humor production. He also noted that the magnitude of this flow changed little when IOP was increased, and concluded that this flow was relatively pressure-insensitive (Bill, 1966a). Since the time of these early experiments that demonstrated the unconventional pathway, much effort has been put into determining its flow rate in humans and animals under a variety of experimental conditions. The rate of unconventional outflow is clinically important because it partly determines IOP and it provides a means to reduce IOP for glaucoma therapy.

3. The anatomy of the unconventional outflow pathway

Because the interstitial spaces of the anterior uvea communicate with the intertrabecular spaces (Fig. 1), a fraction of the aqueous humor outflow that passes into the uveal meshwork can directly enter the interstitial spaces of the ciliary muscle (Henderson, 1950). Perfusion studies with microspheres (Inomata et al., 1972) and fluorescein dextran (Tripathi, 1977; Lindsey and Weinreb, 2002) have demonstrated flow of aqueous humor from this region through the interstitial spaces between the longitudinal ciliary muscle bundles and into the supraciliary and suprachoroidal spaces (Fig. 2). However, where the fluid travels from here has been debated. Bill (Bill, 1966a; Bill and Phillips, 1971; Bill, 1975) and Gabelt and Kaufman (1989) postulate that the fluid seeps through sclera and episclera, and passes into the orbit where it is absorbed by the orbital vasculature (the uveoscleral pathway). Bárány (1967) and others (Pederson et al., 1977; Sherman et al., 1978) have proposed that a significant fraction of this fluid is absorbed osmotically by the choroid and passes into the vortex veins (the uveovortex pathway). As a third potential route, recent studies have proposed drainage into lymphatic vessels located within the ciliary body (the so-called 'uveolymphatic' pathway) (Yücel et al., 2009). However, the existence of lymphatic vessels in the eye under normal physiological conditions remains controversial (Schroedl et al., 2014). We will address the evidence for flow through each of these pathways later in this review.

Unconventional outflow, whether through the uveoscleral, uveovortex or uveolymphatic pathway, must pass through the interstitial spaces of the ciliary muscle. Pilocarpine, which causes ciliary muscle contraction and decreases the size of spaces between the muscle bundles, decreases unconventional outflow (Crawford and Kaufman, 1987) while atropine, a muscarinic antagonist, relaxes the ciliary muscle and increases unconventional outflow (Bill and Wålinder, 1966). This indicates that the ciliary muscle likely represents a significant site of flow resistance along this pathway, and hence ciliary muscle tone can modulate unconventional flow. $PGF_{2\alpha}$, which increases unconventional outflow, is thought to act by reducing the amount of extracellular matrix between ciliary muscle bundles (Lütjen-Drecoll and Tamm, 1988) allowing increased flow through these spaces.

4. Methods of measurement

The greatest challenge facing studies of unconventional aqueous humor outflow has been measurement of its flow rate. By definition, this pathway encompasses any outflow of aqueous humor that does not pass through the trabecular (conventional) pathway. Unlike the trabecular route, the path of egress is diffuse and difficult to trace, and this complicates measurement of flow through this pathway.

Measurements of unconventional outflow rate have been classified as: (i) direct, tracer-based methods, or (ii) indirect methods. Direct methods are used to estimate both conventional and unconventional outflow from the rate of accumulation of a tracer molecule in ocular tissues and in the blood after introducing the tracer in the anterior chamber. They are believed to be the most accurate, but because they are invasive and may require histologic analysis, they are generally not suitable for use in humans. Indirect methods are used to infer unconventional outflow from the difference between aqueous humor production and aqueous humor outflow through the trabecular pathway, each determined independently. Both methods of estimating unconventional flow will be explored here in more detail along with their underlying assumptions and limitations.

4.1. Direct measurements using tracers

Anders Bill introduced tracer-based methods to determine aqueous humor outflow through both the conventional and unconventional pathways in experimental animals and, to a limited

extent, in human eyes (Bill, 1962, 1965; Bill and Phillips, 1971). His methods still provide the most definitive measurements of aqueous humor outflow through both pathways.

Any tracer introduced into the anterior chamber will be carried away by aqueous humor drainage. However, two characteristics of the tracer can be used to distinguish outflow through the conventional versus unconventional pathway: (i) transit time out of the eye and (ii) filtration based on tracer size. Outflow through the conventional pathway is relatively fast (a minute or less) and fairly insensitive to tracer molecular size, such that the rate of tracer appearance in the blood can be used to determine the flow rate through the conventional outflow pathway. In contrast, tracers draining through the unconventional pathway are retarded or captured as they move through the unconventional outflow pathway such that their transit may take up to two hours depending on tracer size, animal species and dimensions of the eye. Tracer accumulation in the uveal tissues thereby provides a means to estimate the unconventional outflow rate as well as identifying the anatomical route of unconventional outflow (as discussed in Section 3).

4.1.1. Tracer-based estimates of conventional outflow: tracer accumulation in the general circulation

Tracers are used to estimate conventional outflow directly by measuring the rate of tracer accumulation in the blood while maintaining a constant tracer concentration in the anterior chamber. Macromolecules of roughly 40 kDa (Toris et al., 1987) or larger (Nilsson, 1997) pass quickly through the conventional outflow pathway but pass slowly through or are captured within the unconventional outflow pathway. Under these conditions, the rate of conventional outflow is directly related to the rate of appearance of tracer in the blood.

Tracer concentration in the anterior chamber is maintained constant by using an infusion-withdrawal system that continuously infuses tracer-containing solution (at concentration C_o) into the anterior chamber of an anesthetized animal while simultaneously withdrawing at the same rate (Gabelt and Kaufman, 1989). This system maintains a constant IOP and a constant tracer concentration in the anterior chamber despite production and drainage of aqueous humor during the perfusion (Bill, 1962, 1965, 1966a, 1966c, 1966d). When the rate of appearance of tracer in the blood, dC_b/dt, becomes relatively constant, then the conventional outflow rate Q_c can be estimated as:

$$Q_c = \frac{V_b}{C_0} \frac{dC_b}{dt} \qquad (1)$$

where V_b is the distribution volume of the tracer in the blood that accounts for tracer dilution in the blood after it leaves the eye. V_b can be estimated as M/C_b, after injecting a known quantity, M, of the tracer into the general circulation and measuring its concentration, C_b, in the blood after mixing. For ^{131}I-albumin tracer in cynomolgus monkeys, Bill found that, V_b was the volume equivalent of

approximately 7% of the total body weight (Bill, 1966a).

Gabelt and Kaufman (1990) used this technique to show that PGF$_{2\alpha}$ does not increase conventional outflow in cynomolgus monkeys despite increasing unconventional outflow in this species. Alternatively, this method may be combined with measurements of aqueous humor production (Q_{in}) to estimate unconventional outflow indirectly as the difference between Q_{in} and Q_c.

4.1.2. Tracer-based estimates of unconventional outflow: tracer accumulation in ocular tissues

This is the most common method of directly determining unconventional outflow and is considered definitive. The flow rate is calculated from the rate of accumulation of tracers in the tissues of the unconventional pathway after maintaining a constant concentration in the anterior chamber for a fixed time. Typically, a solution with tracer is exchanged with aqueous humor in the anterior chamber of an anesthetized animal and tracer concentration is maintained for 0.5–2 h at constant pressure using an infusion-withdrawal system such as described in Section 4.1.1. At the end of a fixed perfusion time, the anterior chamber is exchanged with a tracer-free solution, and perfused for a short time to remove tracer from the conventional outflow pathway.

The animal is then sacrificed, the eye enucleated and the tissues separated. The mass of the tracer in each tissue (typically, the sclera, choroid, ciliary body, iris and orbital tissue) is then determined by methods specific to the tracer used in the study. The volume of fluid V_i required to move the measured quantity of tracer mass m_i into each tissue is given by $V_i = m_i/C_{AC}$, where C_{AC} is the concentration of tracer in the anterior chamber and $i = 1$ through n, where n is the number of tissues examined (e.g. Table 1). The average unconventional outflow during a time T is then:
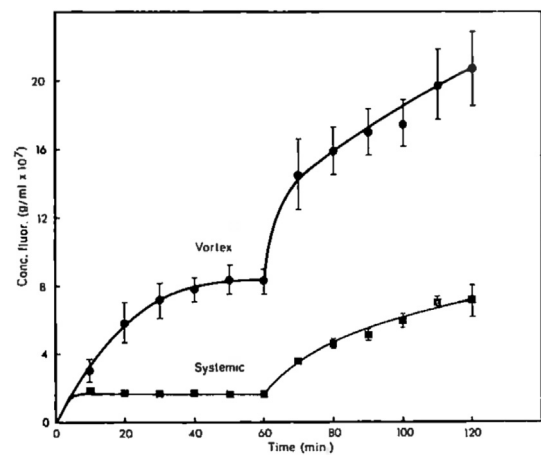
Fig. 3. Fluorescein concentration in the vortex veins and in general circulation in a rhesus monkey after introduction into the anterior chamber. IOP was held at 20 mmHg for 60 min and then increased to 32 mmHg. Reprinted from (Pederson et al., 1977) with permission from the Association for Research in Vision and Ophthalmology.

Table 1
Distribution of unconventional outflow in cynomolgus monkey at normal IOP (12 mm Hg) based on distribution of ^{131}I-albumin after 2 h of perfusion. Uncertainties are standard errors of the mean (Bill, 1966a).

Tissue	Estimated volume of AC fluid passing into each tissue (V_i : µl)
Anterior sclera	14.5 ± 2.4
Extraocular tissues	14.3 ± 5.5
Posterior sclera	10.8 ± 3.8
Choroid	5.1 ± 2.0
Ciliary body	4.3 ± 0.5
Iris	1.2 ± 0.2
Remainder of eye	5.1 ± 1.4

$$Q_u = \frac{\sum_{i=1}^{n} V_i}{T} = \frac{\sum_{i=1}^{n} m_i}{T\, C_{AC}} \qquad (2)$$

Tracers that have been used to study unconventional flow by this method include ^{131}I-γ-globulin (Bill, 1966a), ^{131}I-albumin (Bill and Phillips, 1971), FITC-dextran (Suguro et al., 1985), and ^{3}H-labeled dextran (Barrie et al., 1985). The tissue distribution of ^{131}I-albumin used to estimate unconventional flow in cynomologus monkeys by Bill (1966a) are given in Table 1. In this example, the total unconventional outflow was 0.46 µl/min, while conventional outflow measured by tracer accumulation in the blood was 0.71 ± 0.09 µl/min, yielding a total outflow rate of 1.17 ± 0.13 µl/min. Thus, roughly 40% of the aqueous outflow passed through the unconventional outflow pathway in this species, typical of other measurements in non-human primates.

The distribution of tracer recovered in the various tissues is thought to reflect the anatomical distribution of unconventional outflow. For example, the relatively high tracer content in the anterior sclera suggests that this was a preferential outflow route, as would be expected based on the organization of this tissue (see Figs. 1 and 2). Similarly, the high tracer content in the extraocular tissues suggests that a significant fraction of tracer passed across the sclera, consistent with uveoscleral outflow. In contrast, there was very little tracer in the iris, suggesting negligible iridial outflow. The retinal pathway was not separately assessed in these studies. However, while these measurements clearly indicate tracer accumulation in the various tissues, the tracer distribution does not necessarily reflect the distribution of aqueous humor drainage through those tissues. This is because filtration and diffusion of the tracer affect its transport and may thereby influence tracer accumulation in the various tissues of the unconventional outflow pathway. We will consider these issues further in Section 5.1.

4.1.3. Tracer-based estimates of unconventional outflow: tracer concentration in the vortex veins

Tracers introduced into the anterior chamber also have been used to study the component of unconventional outflow that passes through the suprachoroidal space into the choroidal circulation and vortex veins (the uveovortex pathway). According to this technique, a tracer small enough to pass relatively unimpeded through the vessel walls of the choriocapillaris (e.g. fluorescein) is introduced into the anterior chamber and maintained at constant concentration by the infusion-withdrawal system previously described. One of the four vortex veins is isolated and cannulated. The increased concentration of tracer in the vortex veins, C_{vv}, relative to that in the general circulation, C_b, is used to estimate the uveovortex outflow rate, Q_{uv} (Pederson et al., 1977):

$$Q_{uv} = 4Q\frac{C_{vv} - C_b}{C_{AC}} \qquad (3)$$

where Q is the rate of blood flow out of the cannulated vein and the factor of four accounts for the number of vortex veins. Pederson et al. (1977) found that uveovortex outflow could account for a significant fraction of unconventional outflow (Fig. 3).

The size of the tracer molecule is critical for measuring uveovortex outflow. In contrast to small molecular tracers, such as fluorescein, that are able to enter the choroidal vasculature with the aqueous humor flow, large tracers such as albumin are effectively excluded from passing into the choroid (Bill, 1962, 1964) and accumulate in the suprachoroidal space. From there, these large tracers diffuse across the sclera and into the orbit. Thus, the pathway taken by tracers the size of albumin or larger likely differs from the pathway taken by aqueous humor and small molecules.

4.1.4. Tracer-based estimates of uveolymphatic outflow: tracer accumulation in lymphatic vessels and lymph nodes

Traditionally, the eye has been considered to be devoid of lymphatics (Streilein, 2003). This view has recently been challenged with reports of vessels that express lymphatic markers within the ciliary body (Yücel et al., 2009) and choroid (Koina et al., 2015) of human eyes, although these reports have been met with considerable controversy (Schroedl et al., 2014; Chan-Ling et al., 2015; Heindl et al., 2015). If lymphatics are normally present within the eye, then these vessels may contribute to unconventional outflow.

To measure outflow via the putative uveolymphatic route, Yücel et al. (2009) administered radio-labeled albumin into the anterior chamber of sheep and measured its accumulation in lymph nodes and in cannulated lymphatic vessels (Kim et al., 2011). Tracer accumulated preferentially in the cervical lymph nodes (Yücel et al., 2009), with ~1–2% of tracer passing into the cervical and thoracic lymphatic vessels (Kim et al., 2011). Following intra-cameral injection, fluorescent tracer nanospheres were found within vessels of the ciliary body that expressed lymphatic markers, suggestive of drainage via these vessels (Yücel et al., 2009). In mice, quantum dots injected unilaterally into the anterior chamber accumulated in ipsilateral lymph nodes in the neck (Tam et al., 2011) and the rate of accumulation was increased by latanoprost (Tam et al., 2013).

When measuring uveolymphatic outflow, it should be recognized that not all tracer recovered within cervical lymphatics had necessarily originated from lymphatic vessels within the eye proper, as some tracer may have entered lymphatic vessels from within the periocular tissues. Albumin used to measure uveolymphatic outflow (Kim et al., 2011) will also follow a uveoscleral route (Bill, 1966a) and enter the conjunctiva where lymphatic vessels are plentiful. Consistent with this notion, radiolabelled albumin was measured in the periocular tissues following intracameral injection in sheep (Kim et al., 2011). Once within the periocular space, albumin may enter conjunctival lymphatics that drain into the cervical lymphatics. Quantum dots, while significantly larger than albumin, also accumulate in lymph nodes of mice following intracameral injection (Tam et al., 2011, 2013). However, quantum dots can diffuse through sclera as well (Amrite et al., 2008), although at a rate slower than smaller molecules the size of albumin (Pease et al., 2014).

Currently, there is no clear consensus on the presence of lymphatics in the eye under normal physiological conditions, although lymphatics may be recruited during inflammation or if the scleral border is compromised (Schroedl et al., 2014). In light of this lack of consensus and concerns regarding the methods used to measure uveolymphatic outflow, further work is necessary to demonstrate whether uveolymphatic outflow represents a physiologically significant outflow pathway.

4.1.5. Limitations of tracer-based methods

The use of tracers to measure unconventional outflow is based on two fundamental assumptions. First, all tracer that enters a tissue is retained or trapped within the tissue and that no tracer is lost before the measurement. Second, all tracer enters the tissue by the flow of aqueous humor (advection) and not by diffusion.

4.1.5.1. Retention of tracer. The first assumption will be satisfied if the tracer used is small enough to enter the tissue freely with the aqueous humor but too large to pass through the tissue during the time of the experiment. Toris et al. (1987) reported that dextrans of 40 kDa or larger were retained by the tissue whereas smaller dextrans (4 kDa) passed into the circulation or easily crossed the sclera and were lost in the orbit. Bill (1966a) found similarly that

albumin (65 kDa) crossed the sclera easily but left the orbit at a low rate, while γ-globulin (150 kDa) was significantly delayed passing through the sclera.

4.1.5.2. Flow vs diffusion. Tracers can enter a tissue by advection where they are transported at a rate dictated by the velocity of the fluid in which they are carried, or by diffusion where they are transported at a rate dictated by molecular Brownian motion, or by a combination of both advection and diffusion. Tracers that enter a tissue primarily by diffusion therefore cannot be used to make inferences regarding the quantity or velocity of flow. As diffusion becomes more significant for decreasing molecular size, smaller tracers tend to overestimate unconventional outflow based on tracer accumulation within tissue.

Over time t, a tracer will diffuse a distance of roughly $\sqrt{Dt}$, where D is the diffusion coefficient of the tracer, whereas a tracer carried by advection will move an average distance of Vt, where V is the average velocity of the fluid. Several consequences of these relationships allow us to evaluate the impact of tracer diffusion on the estimate of unconventional flow. First, because diffusional motion increases with the square root of time while advective motion increases linearly with time, the rate of accumulation of a tracer in a tissue can indicate whether diffusion or advection dominates the transport process. In Bill's study of cynomolgus monkeys, tracer accumulation in all unconventional outflow tissues examined after 30 min was equivalent to that contained in 13 μl of solution in the anterior chamber, while after 2 h, it was 55 μl (Bill, 1966a). Thus, tracer accumulated roughly linearly over time, which is consistent with an advection-dominated process.

Second, the influence of diffusion on estimating unconventional flow can be explored using tracers of different size that have different diffusion coefficients. If tracers are carried into a tissue primarily by advection, then their rate of accumulation in the tissue should be unaffected by the diffusion coefficient provided that their size is sufficiently small so as not to be excluded from this pathway. Bill (1966a) showed in cynomolgus monkey eyes that roughly the same unconventional outflow rate was found with albumin as with γ-globulin. Pederson and Toris (1987) reported similar findings using 40 and 150 kD dextran.

Third, the diffusion distance, estimated by $\sqrt{Dt}$, relative to the length of the unconventional pathway, can also indicate the importance of diffusion in tracer transport. In large animals, such as monkeys, the length of the unconventional pathway is on the order

of millimeters, and the estimated diffusion distance is relatively small, except for very long experiments. For example, the diffusion coefficient of albumin in sclera is $D = 1 \times 10^{-7}$ cm²/s (Anderson et al., 2008); in a half-hour experiment, a tracer would be expected to diffuse 130 μm, a distance significantly smaller than the length of the unconventional outflow pathway in most species. Pederson and Toris (1987) showed that in cynomolgus monkey eyes, the rate of diffusion was 200 times smaller than the observed rate of tracer movement. However, for smaller eyes, such as those of mice, the unconventional pathway is much shorter than in larger animals and may be comparable to tracer diffusion distances unless very short times are used for tracer delivery (see Section 6.6).

4.2. Indirect estimates of unconventional outflow

The tracer-based methods described in Section 4.1, while accurate, are invasive and thus not generally suitable for use in humans. For this reason, indirect methods have been developed to estimate unconventional outflow. The modified Goldmann equation (Brubaker, 2004) relates IOP to aqueous humor inflow (Q_{in}) under steady-state conditions:

$$Q_{in} = c\,(IOP - P_e) + U \tag{4}$$

where U is the unconventional outflow, c is the conventional outflow facility, and P_e is the episcleral venous pressure. Note that with this definition, $c\,(IOP - P_e)$ is an estimate of the conventional outflow rate and U is the pressure-independent outflow rate. Note also that U is not necessarily the same as the rate of outflow passing through the unconventional outflow pathway as there may be some pressure-dependence to unconventional outflow (see Section 5.2).

If both Q_{in} and c can be measured, then unconventional outflow can be indirectly estimated as the difference between inflow rate and conventional outflow:

$$U = Q_{in} - c\,(IOP - P_e) \tag{5}$$

Typically, Q_{in} is determined by measuring fluorescein clearance from the anterior chamber and cornea (Brubaker and McLaren, 1985). Outflow facility c has been measured by using tonography, in which a small weight is placed on the cornea to elevate IOP and the rate at which IOP returns to baseline is used to determine c (Grant, 1950). Alternatively, by measuring IOP and Q_{in} before and

Table 2
Studies that reported unconventional outflow measured by the direct method with tracers and by the indirect method. Mean ± standard errors. *Data corrected from manuscript per communication with authors. Young mice are between 2½—4½ months, while aged mice are between 10 and 12 months (Millar et al., 2015).

Reference	Drug	Animal	Unconventional outflow rate, μl/min (percent of total outflow)	
			Indirect method	Tracer method
Toris et al., 2000	None	Cynomolgus monkey	0.14 ± 0.3 (9%)	1.05 ± 0.26 (70%)
Toris et al., 1995c	Control	Cat	1.9 ± 0.8 (33%)	1.5 ± 0.2 (26%)
	PGA₂		4.0 ± 0.5 (59%)	2.3 ± 0.3 (34%)
Wang et al., 1999	Control	Cat	2.70 ± 0.75 (42%)	1.42 ± 0.48 (22%)
	Epinephrine		2.04 ± 0.59 (42%)	1.23 ± 0.4 (26%)
Zhan et al., 1998	Control	Rabbit	0.31 ± 0.12 (13%)	0.22 ± 0.03 (9%)
	Bunazosin		1.04 ± 0.17 (43%)	0.31 ± 0.03 (13%)
Millar et al., 2011	None	Mouse male BALB/cJ	0.029 ± 0.005 (21%)	0.012 ± 0.003 (9%)
Millar et al., 2015	None	Young mouse BALB/cJ	0.066 ± 0.022 (37%)	0.051 ± 0.018* (36%)
Millar et al., 2015	None	Aged mouse BALB/cJ	0.011 ± 0.007 (8%)	0.01 ± 0.007* (7%)
Millar et al., 2015	None	Young mouse A/J	0.12 ± 0.048 (54%)	0.055 ± 0.13 (28%)
Millar et al., 2015	None	Aged mouse A/J	0.036 ± 0.031 (25%)	0.009 ± 0.002 (9%)
Millar et al., 2015	None	Young mouse C3H/HeJ	0.109 ± 0.028 (71%)	0.054 ± 0.02* (30%)
Millar et al., 2015	None	Aged mouse C3H/HeJ	0.024 ± 0.009 (34%)	0.006 ± 0.003* (9%)
Millar et al., 2015	None	Young mouse C57-BL/6J	0.08 ± 022 (53%)	0.044 ± 0.012 (42%)
Millar et al., 2015	None	Aged mouse C57-BL/6J	0.012 ± 0.008 (14%)	0.006 ± 0.003 (9%)

after administering a drug (e.g. acetazolamide) that suppresses aqueous humor production, c can be determined by solving Equation (4) at two flow rates and the corresponding IOPs (Yablonski et al., 1985; Hayashi et al., 1989). A value of episcleral venous pressure, P_e, is required to calculate U. This measurement is difficult and values between 8 and 10 mmHg are typically chosen, and the same value is used for all subjects in a trial. More recent evidence suggests that the mean episcleral venous pressure is 6.3 mm Hg in normal subjects (Sit et al., 2011). Importantly, the lower the value used for P_e, the smaller the estimate of U.

Estimates of unconventional flow determined by using the indirect method have varied considerably and, in general, show poor agreement with unconventional outflow measured by direct methods that used tracers. Even in the same study, Toris et al. (2000) reported an unconventional outflow rate in cynomolgus monkeys of 0.14 ± 1.2 μl/min by using indirect methods, but 1.05 ± 0.6 μl/min by using tracers in this same population, almost an order of magnitude greater. Toris et al. also noted a lack of correlation between unconventional outflow estimated indirectly and those measured with tracers. Similarly, studies in mice (Millar et al., 2011, 2015) showed poor agreement between tracer-based and indirect estimates of unconventional outflow. In general, measurements with the indirect method are considerably higher than those measured using tracers (Table 2), although measurements by Toris et al. (2000) represent a significant exception. While both direct and indirect methods have been shown to detect changes in response to drug treatment, the absence of any quantitative agreement between the two approaches is troubling and raises questions as to what specifically is being measured by the indirect method.

4.2.1. Limitations of indirect estimates of unconventional outflow

The conventional outflow rate is similar to the aqueous humor production rate, and the difference between these (Equation (5)) is, under most circumstances, significantly less than either.
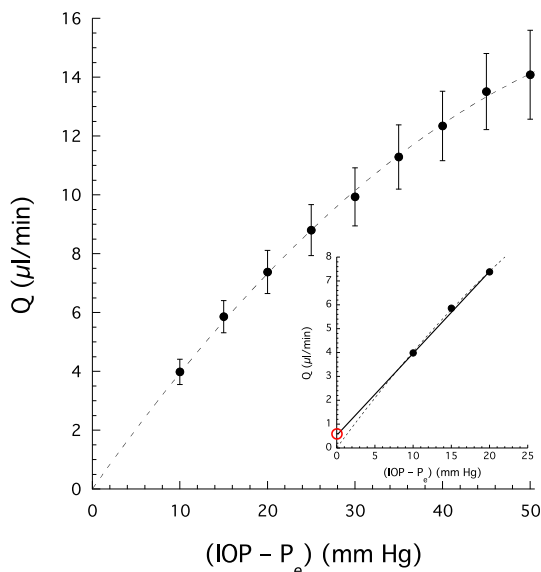
Fig. 4. Flow measured in enucleated human eyes ($P_e = 0$) as a function of IOP (uncertainties are standard errors) (Brubaker, 1975). The dashed curve is the best fit of $Q = b_1 \, IOP + b_2 \, IOP^2$ to the data. Inset shows flow at low perfusion pressure. The solid line is from Equation (4) with flow measured at pressures of 10 and 20 mmHg. The red circle indicates the apparent unconventional outflow (U) erroneously estimated by using Equation (5). This error arises from the modest pressure-dependence of outflow facility that imposes a non-linearity in the flow-pressure relationship (dashed curved) and is not captured by Equations (4) and (5).

Consequently, a relatively small error in either will result in a relatively large error in the estimated unconventional flow. For example, a 10% error in the estimated aqueous humor production rate or conventional outflow rate could result in a 50–100% error in the calculated unconventional outflow.

These considerations indicate that random errors and more importantly, systematic errors, will be magnified by the use of Equation (5). Lim et al. (2008) looked at the effect on estimates of unconventional outflow of using different methods to measure outflow facility (Schiotz tonography, 2-min pneumatonography, 4-min pneumatonography and fluorophotometry) and assumptions of episcleral venous pressure between 8 and 11 mm Hg. They found that the unconventional outflow estimated in the same placebo-treated healthy subjects varied from −0.49 to 1.46 μl/min (0%–60% of inflow), depending on the method used. As noted by Brubaker (2004) and others (Becker and Neufeld, 2002; Bill, 2003; Camras, 2003; Kaufman, 2003; Yablonski, 2003), Equation (5) is subject to a number of assumptions, in particular that conventional outflow facility, unconventional outflow, and episcleral venous pressure are constants that do not change with IOP. These assumptions are, at best, approximations, and in under some conditions, introduce significant systematic errors into the estimate of unconventional outflow, particularly when drugs are applied to the eye. We detail below these systematic errors and their effects on the estimation of unconventional outflow using the indirect method.

4.2.1.1. Pressure-dependence of outflow facility.
Outflow facility (c) is expressed as a constant in the Goldmann equation, but in fact c decreases as IOP increases (Levene and Hyman, 1969; Brubaker, 1975), which has been attributed to collapse of Schlemm's canal (Moses, 1979; Van Buskirk, 1982). In enucleated eyes, Brubaker (1975) measured a non-linear relationship between outflow (Q) and IOP (Fig. 4) consistent with an IOP-dependent decrease in outflow facility, which is defined $Q/(IOP-P_e)$. Despite a relatively modest decrease in outflow facility with pressure (~1.2% decrease in facility per unit mmHg increase in IOP; Brubaker, 1975), non-linearity in the flow-pressure relationship may lead to significant errors in the estimate of unconventional outflow. This is demonstrated in Fig. 4, where the dashed curve represents the best second order polynomial fit to Brubaker's data and the solid line represents the result of applying Equation (5) at pressures of 10 and 20 mmHg. This line may be extrapolated to the ordinate to yield a non-zero intercept that, according to Equation (5), would be interpreted as U, the pressure-independent or unconventional outflow (with $P_e = 0$ in an enucleated eye). However, in this case, the non-zero intercept results entirely from the non-linearity of the flow-pressure relationship analyzed in terms of an inappropriate linear model prescribed by Equation (5), giving the false appearance of a pressure-independent outflow. For example, a different U would have been determined from a line fitted through Q at 5 and 15 mmHg. Furthermore, the magnitude of the apparent pressure-independent outflow in Fig. 4 (~0.5 μl/min) is similar to or larger than that measured in living human eyes (Bill and Phillips, 1971). Because a similar non-linear flow-pressure relationship is expected in living eyes, any indirect estimate of unconventional outflow would be subject to large uncertainties. Moreover, because outflow facility decreases with increasing pressure, any indirect estimate of the pressure-independent outflow will tend to overestimate its true value. This may partly explain why the indirect estimates of unconventional outflow are typically greater than those made by using direct tracer-based methods.

4.2.1.2. Pressure-insensitivity of unconventional outflow.
Bill and others noted a striking characteristic of unconventional outflow,

namely its relative insensitivity to the pressure difference that drives the flow (Section 5.2). As measured using the indirect method, unconventional outflow is assumed to be entirely independent of IOP. However, direct tracer-based measurements indicate that unconventional outflow is not entirely pressure-*independent*, although it is relatively pressure-*insensitive* (Bill, 1966a; Becker and Neufeld, 2002; Bill, 2003; Kaufman, 2003). Any pressure-dependent changes in unconventional outflow will be attributed to conventional outflow in Equation (5). This will lead to a moderate underestimate of unconventional outflow and overestimate of conventional outflow as measured using indirect techniques. Kaufman (2003) suggests that the unconventional outflow facility is approximately 5% of total outflow facility in non-human primates. While this may seem small, this is roughly equivalent to 20—50% of total unconventional outflow at physiological IOP, and therefore the pressure-dependent contribution to unconventional outflow cannot be ignored.

This problem may be a particular concern for drug studies if the compounds used increase the pressure dependence of unconventional outflow (e.g. $PGF_{2\alpha}$ (Gabelt and Kaufman, 1990; Kaufman, 2003)). The assumption of a purely pressure-independent unconventional outflow can greatly confuse interpretation of data from such experiments and may explain why some studies have found that prostaglandins lower IOP primarily by increasing unconventional outflow (Gabelt and Kaufman, 1990; Toris et al., 1993; Stjernschantz, 2001; Woodward et al., 2010), while other studies suggest that these agents also have a significant effect on conventional outflow (Christiansen et al., 2004; Wan et al., 2007; Lim et al., 2008).

4.2.1.3. Effect of episcleral venous pressure. The indirect method of estimating unconventional outflow requires an accurate estimate of episcleral venous pressure (Christiansen et al., 2004) and requires that the episcleral pressure be independent of IOP. Studies in monkeys support the latter assumption (Mäepea and Bill, 1989), but accurately measuring episcleral venous pressure proves to be very difficult.

Mean reported episcleral venous pressures have ranged from 6.3 mmHg to 11.4 mmHg depending on the method of measurement (Sit et al., 2011; Sit and McLaren, 2011). Because of difficulty in its measurement, some investigators have assumed a single value for episcleral venous pressure for all subjects when calculating unconventional outflow (Toris et al., 2000; Zhao et al., 2010). Errors in episcleral venous pressure directly impact the estimate of unconventional outflow determined using Equation (5). For example, a 1 mmHg error in episcleral venous pressure leads to an error of approximately 0.3 μl/min in unconventional outflow (assuming c = 0.3 μl/min/mmHg), which is 25—100% of the typical unconventional outflow. Assuming too low a value of episcleral venous pressure in this calculation (Equation (5)) would lead to underestimated unconventional outflow whereas assuming too high a value of episcleral venous pressure would lead to overestimated unconventional outflow.

The assumptions inherent in Equations (4) and (5), combined with measurement inaccuracies and systematic errors, lead to considerable uncertainties in indirect estimates of unconventional outflow. Note that while both the direct and indirect methods of calculating unconventional outflow are subject to considerable experimental variability, the systematic errors inherent in the direct method are significantly smaller than those inherent to the indirect method. But unfortunately, there is currently no other method to assess unconventional outflow in humans other than by the indirect methods, and thus they are used despite their limitations. In animals, where unconventional outflow can be directly measured, the lack of agreement between direct and indirect measures casts doubt on the utility of the indirect methods. These issues apply a fortiori in studies on the effects of drugs on unconventional outflow, because drugs could directly affect the pressure dependence of unconventional flow and could alter episcleral venous pressure, thereby leading to erroneous conclusions regarding the effects of these drugs on aqueous humor dynamics. Indirect estimates of unconventional outflow must therefore be interpreted with caution.

5. Physiological characteristics of unconventional outflow

Several features of unconventional outflow are particularly interesting from a physiological perspective. These features include the route of aqueous humor drainage along this pathway, the insensitivity to changes in intraocular pressure, the role of the ciliary muscle, and changes in this pathway after death.

5.1. The final route of unconventional outflow: uveoscleral vs uveovortex flow

Unconventional outflow passes through the ciliary muscle and into the suprachoroidal space, but the pathway taken from the suprachoroidal space to exit the eye has been debated. Bill identified this path by tracing the route of radioactive-labeled proteins and other large molecules as they left the eye. These tracers moved through the ciliary muscle, into the suprachoroid space, and accumulated in the sclera and orbit, a route he termed the "uveoscleral" pathway (Bill, 1965). This suggested fluid movement across the sclera where it could be collected and drained by the extraocular lymph vessels. Bill calculated the flow rate through this route based on tracer accumulation in these tissues, but he recognized that this method would only give an accurate estimate of unconventional flow if the tracer followed the same path as the aqueous humor and also was not washed away into the general circulation during the course of the experiment.

The use of smaller tracers suggested a somewhat different route. Pederson et al. (1977) perfused the anterior chamber of monkeys with fluorescein, a small molecule, and found that a significant fraction of this tracer entered the vortex veins (Fig. 3). The fluorescein concentration in these vessels was somewhat dependent on perfusion pressure, although it was not as pressure-dependent as was conventional outflow. The sensitivity to pressure suggested that movement through this pathway required ultrafiltration of water and small molecules into the uveal capillaries. They
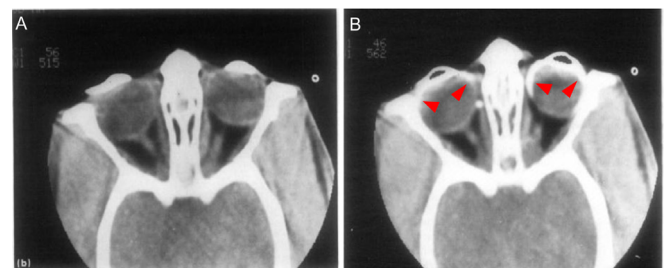
Fig. 5. Computed tomography oblique sections through the orbital region of an adult rhesus monkey perfused with radio-opaque contrast medium in the anterior chamber. (A) After 1 h of continuous perfusion while the monkey was alive, contrast medium was confined to the anterior chamber and there was no posterior motion of the medium. Similar images were recorded for up to 8 h. (B) In the same monkey 35 min after death by pentobarbital overdose, contrast medium migrated posteriorly (red arrows). Black voids within the anterior chamber are an imaging artifact. Both images were from the same section plane in the same monkey. The right eye was set to 20 mmHg and left eye to 40 mmHg by using a fluid column. Reprinted from (Butler et al., 1984) with permission from Elsevier.

Table 3
Unconventional flow in different species determined by recovering labeled tracers (either collection of tracers in ocular tissues, or collection of tracer in general circulation with a separate measurement of aqueous inflow).

Species	Unconventional flow (μl/min)	Fraction of inflow (%)	References
Cynomolgus monkey	0.48—1.07	27—65	(Bill (1966a, 1967, 1971)) (Bill and Wålinder, 1966) (Gabelt and Kaufman, 1989) (Nilsson et al. (1989, 2006)) (Toris et al., 2000) (Gabelt et al., 2005)
Vervet monkey	0.23—0.74	19—38	(Bill (1966d, 1969a, 1969b, 1970, 1971))
Rhesus monkey	0.63	37	(Gabelt et al., 2003)
Cat	0.36—1.5	3—26	(Bill, 1966b) (Toris et al., 1995c) (Wang et al., 1999) (Goh et al., 1994) (Toris, 2008)
Rabbit (albino)	0.11—0.53	3—22	(Bill, 1966c) (Cole and Monro, 1976) (Goh et al., 1989) (Poyer et al., 1992) (Takashima et al., 1996) (Zhan et al., 1998) (Kanno et al., 1998) (Sugiyama et al., 2001) (Honjo et al., 2001) (Zhan et al., 2002) (Oka et al., 2006)
Dog (beagle)	–	15	(Barrie et al., 1985)
Human	0.28	0—14	(Bill and Phillips, 1971)
Mouse (BALB/cJ)	0.01—0.051	7—36	(Millar et al., 2011) (Millar et al., 2015)
Mouse (A/J)	0.009—0.055	9—28	(Millar et al., 2011) (Millar et al., 2015)
Mouse (C3H/HeJ)	0.006—0.054	9—30	(Millar et al., 2011) (Millar et al., 2015)
Mouse (C57-BL/6J)	0.006—0.044	9—42	(Millar et al., 2011) (Millar et al., 2015)

suggested that Starling forces were responsible for fluid resorption into the uveal capillaries, with these forces arising from the higher colloidal osmotic pressure in these vessels relative to the uveal interstitial fluid, a possibility first raised by Bárány (1967).

Bill also found tracer in the vortex veins in his early studies of rabbits perfused through the anterior chamber with radio-labeled albumin, but he concluded that less than 1.2% of the tracer within the anterior chamber drained via this route (Bill, 1962). In making this conclusion, Bill assumed that albumin could freely enter the uveal vessels once within the suprachoroidal space, an assumption that is inconsistent with the relatively low protein concentration in suprachoroidal fluid compared to plasma (Emi et al., 1989; Toris et al., 1990). The large size of albumin (67 kDa), as compared to that of fluorescein (0.3 kDa), likely excluded albumin from entering the choriocapillaris and the uveal capillaries, such that albumin would accumulate in the suprachoroidal space, diffuse across the sclera and enter the orbit, thereby explaining Bill's experimental observations (Yablonski, 2002). Larger molecular tracers such as albumin therefore appear to travel along a separate pathway than does aqueous humor and smaller molecular tracers.

Visualization of unconventional outflow in living anesthetized rhesus monkeys by using small tracers has provided indirect evidence for the role of these vessels in unconventional outflow. Butler et al. (1984) perfused the anterior chamber with a small radio-opaque contrast agent (789 Da) and created images of this agent by X-ray computed tomography. Despite perfusion for up to 8 h while the animal was alive, tracer was restricted to the anterior chamber, but immediately after death, tracer was seen migrating from the anterior chamber towards the posterior pole (Fig. 5). This experiment suggests that small molecular tracers that permeate the unconventional pathway are rapidly swept away by circulating blood, but when blood flow ceases soon after death, these tracers accumulate within the tissue.

Yablonski (2003) suggested that flow through the uveovortex pathway could be increased by chronic prostaglandin exposure. Prostaglandins have been shown to modify the extracellular matrix of the uvea, and if these modifications also increased the hydraulic permeability of these vessels, then flow through this route would also increase.

Based on the concentrations of fluorescein in the vortex veins, Pederson et al. (1977) estimated that the uveovortex pathway could account for roughly 10% of aqueous humor outflow in the non-human primate eye. This estimate was similar to that of McMaster and Macri (1968), who found 8% of aqueous outflow to pass through the uveovortex pathway in an arterially-perfused enucleated rabbit eye. These estimates of outflow through the uveovortex pathway are significantly less than the total unconventional flow that has been measured using direct tracer-based techniques in animals (Table 3). In the living eye, unconventional outflow therefore likely drains through both the uveoscleral and uveovortex routes, where the distribution of outflow through each route likely depends on IOP, ciliary muscle tone, and other factors
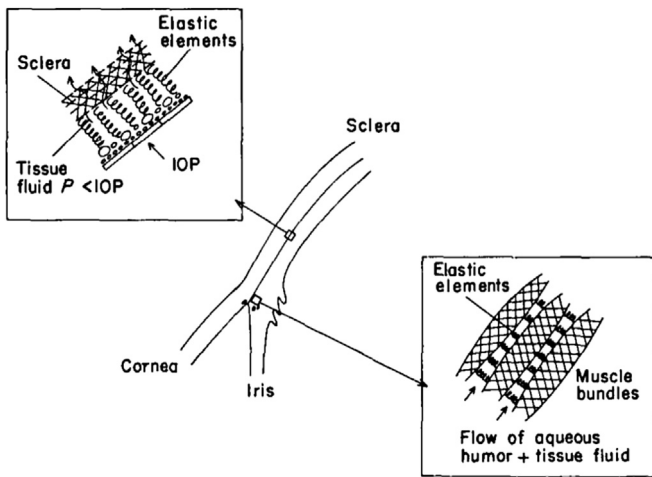
Fig. 6. Aqueous humor from the anterior chamber communicates with tissue fluid between the muscle bundles of the ciliary muscle, the choroid, and the suprachoroidal space. Schematic shows how elastic elements in the ciliary muscle, choroid, and suprachoroid help to keep the interstitial spaces open. Reprinted from (Bill, 1977) with permission from Elsevier.

such as pharmacological agents.

Because of these two parallel pathways, the term *uveoscleral flow*, which has been used to mean extra-trabecular flow, is misleading and refers to only the portion of unconventional flow that leaves the eye through the sclera. Some have used the term *unconventional outflow* to refer to the portion of outflow that passes through the uveoscleral route, uveovortex route, or both, and we have used this term in this review, although the term *non-trabecular outflow* would perhaps be more informative.

5.2. Pressure insensitivity of unconventional outflow

Bill and others found that as IOP increased between 4 mmHg and 35 mmHg, unconventional flow either remained constant or increased at a much slower rate than did conventional outflow. For example, using tracer-based measurements of unconventional outflow in monkeys, unconventional outflow was 0.44 μl/min at an IOP of 11 mm Hg, but when IOP was increased to 22 mm Hg, unconventional outflow increased only to 0.63 μl/min, less than a 50% increase (Bill, 1966a). In contrast, over the same pressure range, conventional outflow increased 5-fold from 0.8 μl/min to 4.18 μl/min (Bill, 1966a). Pederson et al. (1977) and Suguro et al. (1985) also found unconventional outflow to be relatively pressure insensitive. It is important to note that in these studies, unconventional flow was not found to be pressure-independent, only pressure insensitive.

The pressure insensitivity of the unconventional outflow rate seems counterintuitive, since fluid flow requires work be done to overcome viscous drag generated as the fluid passes fixed structures. The energy necessary to do this work is supplied by a gradient in either the hydrostatic pressure or the osmotic pressure of the fluid, or both, as the fluid passes through the tissue. If the hydraulic resistance of the tissue is constant, increasing the pressure gradient should lead to a proportional increase in flow. However, if the resistance is not constant, but increases with pressure, then the flow may appear to be relatively pressure insensitive.

Bill (1977) suggested that the sizes of the interstitial spaces that carry unconventional outflow through the ciliary muscle and suprachoroidal spaces are determined by a balance between IOP, that tends to collapse these spaces, and elastic elements and interstitial pressure within the spaces themselves, that tend to keep

them open (Fig. 6). As IOP increases, these spaces become smaller, and hence their resistance to flow increases. Bill (1977) referred to this as an "elastic sponge model" because of its similarity to channels in a sponge.

After passing through the ciliary muscle and suprachoroidal space, uveoscleral outflow must then traverse the sclera, and transscleral flow is pressure-dependent as it increases in linear proportion to the transscleral pressure gradient (Kleinstein and Fatt, 1977). Therefore, in order for the elastic sponge model to explain the pressure-insensitivity of uveoscleral outflow, the resistance to flow through the ciliary muscle and suprachoroidal space must be significantly greater than the resistance to flow across the sclera. However, a large resistance in the ciliary muscle is inconsistent with the observation that the resistance to flow through the sclera is comparable to total unconventional outflow resistance (Fatt and Hedbys, 1970; Kleinstein and Fatt, 1977; Jackson et al., 2008). Based on our current understanding of ocular physiology, it remains unclear how uveoscleral outflow can exhibit pressure-insensitivity.

Flow through the uveovortex pathway offers a second explanation for the pressure insensitivity of unconventional outflow. When IOP increases, pressure in the uveal capillaries also increases (Mäepea, 1992), and consequently, the pressure difference across the capillary wall increases by much less than does a change in IOP. Furthermore, the difference in pressure between IOP and that in the uveal capillaries is minor as compared to the difference in protein osmotic pressure between the aqueous humor and the blood (Pederson et al., 1977; Yablonski, 2003) that pulls fluid into the uvea. Thus, changes in IOP would have a minor effect on the total force driving fluid into the choroidal capillaries leaving uveovortex flow relatively insensitive to IOP changes (Johnson and Erickson, 2000).

The modified Goldmann equation (Equation (4)) treats unconventional outflow as a constant, *U*, that can change depending on the status of the tissues in the unconventional outflow pathways but is independent of IOP. Although this equation has been used for many years, a model that includes the driving forces and facility of outflow through these pathways might better describe aqueous humor dynamics. Modifications that recognize determinants of the unconventional outflows have been proposed by Becker and Neufeld (2002) and discussed by Bill (2003), Kaufman (2003), Yablonski (2003), and Camras (2003) in a series of letters. However, such a model would need to include the effects of pseudofacility (Kaufman, 2003), differing driving forces for the uveovortex and uveoscleral outflow pathways (Kaufman, 2003; Yablonski, 2003), and the increased pressure dependence of unconventional outflow at low IOP (Bill, 2003), leading to a somewhat unwieldy expression.

5.3. Role of the ciliary muscle: a principal site of unconventional outflow resistance

The ciliary muscle lies near the entrance of the unconventional outflow pathway, and it is not surprising that its tone greatly influences outflow through this route. Pharmacological or surgical alterations to the ciliary muscle tone cause changes in unconventional outflow. For example, contraction of the ciliary muscle by pilocarpine reduces unconventional outflow by 90% in cynomolgus monkeys (Bill and Wålinder, 1966), while relaxation of the ciliary muscle by atropine has the opposite effect (Bill, 1967). Prostaglandins tend to relax the ciliary muscle (Poyer et al., 1992), allowing an acute reduction in unconventional outflow resistance, but they also have a slower, more significant hypotensive effect by inducing remodeling of the extracellular matrix within the ciliary muscle (Lütjen-Drecoll and Tamm, 1988) (Section 7.1). Pre-treatment with

pilocarpine acutely abolishes the effect of prostaglandins on IOP and unconventional outflow in monkeys (Crawford and Kaufman, 1987; Nilsson et al., 1989).

Bill (1977) suggested that compression of the ciliary muscle with increasing IOP could increase its flow resistance and thereby contribute to pressure insensitivity of unconventional outflow (Fig. 6). When the ciliary muscle is removed by cyclodialysis, most of the resistance it offers is lost (Bill, 1966c) and unconventional outflow increases four-fold (Suguro et al., 1985) and becomes pressure dependent (Toris and Pederson, 1985). Experiments exploring the role of the ciliary muscle in unconventional outflow have been reviewed by Alm and Nilsson (2009).

5.4. Unconventional outflow in living and post-mortem eyes

Flow through the conventional outflow pathway is pressure-driven and thought to have largely the same characteristics in living and post-mortem eyes (Grant and Trotter, 1955; Grant, 1963), when corrected for the loss of episcleral venous pressure after death. The unconventional outflow pathway, however, is more complicated. Bill found that there was no correlation between rate of unconventional outflow in the eye of a living animal and that in the contralateral eye from the same animal after death (Bill, 1966a). Following death, unconventional outflow was approximately 150% higher than in eyes of living cynomolgus monkeys (Bill, 1966a), rabbits (Bill, 1966c), and cats (Bill, 1966b), and increased by nearly 400% in vervet monkeys (Bill, 1966d). In mice, however, there was no evidence for a difference in unconventional outflow between living and post-mortem (in situ) eyes (Millar et al., 2011). The pressure-insensitivity of unconventional flow in the physiological pressure range suggests that the increase in unconventional outflow after death was not likely caused by the loss of episcleral venous pressure. Unconventional outflow, however, may have increased after death due to loss of ciliary muscle tone and the associated decrease in outflow resistance offered by the ciliary muscle (Section 5.3).

6. Characteristics of unconventional flow in different species

Anatomical [see Henderson (1950) and Tripathi (1974)] or functional differences in the unconventional outflow pathway can affect the drainage of aqueous humor through this route and contribute to differences in unconventional outflow between species. Lower placentals, for example, exhibit a deep ciliary cleft with a relatively undeveloped ciliary muscle that allows open fluid communication to the posterior ciliary body. Primates, in contrast, have a well-developed ciliary muscle without a ciliary cleft that renders a strong dependence of unconventional outflow on ciliary muscle tone and accommodation.

Table 3 shows the unconventional outflow rate and its fraction of total outflow in a variety of species, as measured by tracer techniques. In general, non-human primates have the highest fraction of unconventional outflow. We explore the difference in unconventional outflow between species, focusing on primates and mice because of their utility as models for human eyes and because of considerable discrepancies in literature regarding unconventional flow in mice.

6.1. Human

Only one study (Bill and Phillips, 1971) has reported direct measurements of unconventional outflow in living human eyes by using tracer methods. In patients' eyes destined for enucleation, unconventional outflow was estimated from radioactive albumin tracer accumulation in the posterior scleral shell after injection into the anterior chamber. In 3 eyes, unconventional outflow was estimated to be 0%, 4% and 14% of total outflow from the anterior chamber (an absolute value of unconventional outflow was

Table 4
Unconventional outflow in humans as determined by using the indirect method. Q_u is unconventional outflow (μl/min) and Q_{in} is inflow rate. All values are population averages. "Fluorophotometric" or "tonographic" refers to the method used to measure aqueous humor outflow facility (LTP − laser trabeculoplasty).

Condition of subjects (method)	Q_u	% Q_{in}	Reference	Notes
Normal (tonographic)	0.8	36	(Townsend and Brubaker, 1980)	
Normal (tonographic)	1.06	41	(Coakes and Siah, 1984)	Control (Placebo)
Young normal [21–23 y] (tonographic)	−0.38	0	(Mishima et al., 1997)	Daytime
	1.23	85		Nighttime
Young normal [21–23 y] (fluorophotometric)	0.57	25		Daytime
	0.78	54		Nighttime
Normals (tonographic)	0.85–1.52	34–62	(Brubaker et al., 2001)	Control (Placebo)
Normal (fluorophotometric)	1.5	45	(Wang et al., 2002)	Control (Baseline)
	1.1	36		Control (Placebo)
	1.1	39		Experimental (Baseline)
Normal (fluorophotometric)	1.09		(Toris et al., 2002b)	
Normal [47–76 y] (tonographic)	0.94	38	(Nau et al., 2013)	Daytime
	0.07	6		Nighttime
Glaucoma patients before LTP (fluorophotometric)	1.1	78	(Yablonski et al., 1985)	Control (Baseline)
	0.51	40		Control (Placebo)
	0.96	64		Experimental (Baseline)
Ocular hypertension (fluorophotometric)	0.60	30	(Hayashi et al., 1989)	
Ocular hypertension (fluorophotometric)	0.66		(Toris et al., 2002b)	
Ocular hypertension (fluorophotometric)	1.14	46	(Toris et al., 2004)	Control (Baseline)
	0.99	48		Control (Placebo 1)
	0.86	37		Control (Placebo 2)
	1.24	52		Experimental (Baseline)
Ocular hypertension (fluorophotometric)	0.67	25	(Toris et al., 1995b)	Control (Baseline)
	0.62	24		Control (Placebo)
	0.58	23		Experimental (Baseline)
Ocular hypertension (fluorophotometric)	0.35	14	(Toris et al., 1995a)	Control (Baseline)
	0.5	22		Control (Placebo)
	0.12	4.8		Experimental (Baseline)
Ocular hypertension or glaucoma (tonographic)	0.64–0.96	25–40	(Christiansen et al., 2004)	Varying assumptions on episcleral venous press.

reported for only one eye, as 0.28 μl/min). As in monkeys, unconventional outflow in humans was reduced by pilocarpine (0%–3% of total outflow in 3 eyes) and increased by atropine (4%–27% in 6 eyes; flow rates in 2 eyes were reported as 0.43 and 0.62 μl/min), consistent with the notion developed from studies of monkeys that ciliary muscle tone modulates unconventional flow between muscle bundles (Section 5.3). In histologic samples, albumin tracer was often found in the perivascular space surrounding intrascleral vessels, suggesting that these spaces function as an outflow route, at least for the tracer used.

Indirect estimates (Section 4.2) of unconventional outflow in human have been reported in a number of studies (Table 4). Toris et al. (1999) found that unconventional outflow decreased with age in humans, from 1.52 μl/min (54% of total flow) in young adults to 1.1 μl/min (46%) in seniors, and also found that unconventional outflow was lower in glaucomatous eyes (Toris et al., 2002b), consistent with values in Table 4.

Considerable variability is seen in the results listed in Table 4, both for normal eyes (0–85% of total outflow was unconventional) as well as for eyes with ocular hypertension or glaucoma (5–78%). Because participants in these studies were untreated controls, such variation is difficult to explain other than as variability in the technique itself. Even within a single study, larger variations in unconventional outflow (5–22%) were reported in the same population (Toris et al., 1995a). Furthermore, most indirect estimates of unconventional outflow are much higher than tracer-based estimates of unconventional outflow (Bill and Phillips, 1971), similar to the discrepancies noted in animal studies as described in Section 4.2 (Tables 2 and 3).

6.2. Monkeys

Anders Bill and co-workers were the first to measure unconventional outflow in monkeys (Bill, 1965; Bill and Hellsing, 1965). They demonstrated that unlike the human eye, which has a relatively small fraction of unconventional outflow, in monkeys a significant fraction of aqueous humor passed through the unconventional pathway. In cynomolgus, vervet, and rhesus monkeys, roughly one quarter to one half of aqueous humor outflow passed through the unconventional outflow pathway, while in other species, less than a quarter passed this route (Table 3).

Alm and Nilsson suggested that an age-dependent reduction in unconventional flow may explain the difference between nonhuman primate and human eyes (Alm and Nilsson, 2009). Indeed Gabelt et al. (2003) and Gabelt and Kaufman (2005) reported that unconventional flow in young (3–10 years of age) and middle-aged rhesus monkeys (ages 19–23 years) was twice as high as in older animals (ages 25–29), and they associated this age-related decrease with extracellular matrix accumulation in the ciliary muscle. However, based on tracer studies, the rate they reported for unconventional flow in older monkeys (19.8% of outflow) was still a higher fraction of total outflow than that reported in humans. This also does not explain why the fraction of unconventional flow is much higher in monkeys compared to non-primates, considering that the ciliary musculature is better developed in primates and would seem to pose greater resistance to unconventional outflow relative to that in non-primates.

As noted in Section 5.3, pilocarpine has a large effect on unconventional flow in primates. By contracting the longitudinal fibers of the ciliary muscle, pilocarpine almost completely eliminates unconventional outflow (Fig. 7). In contrast, relaxing the ciliary muscle with atropine increases unconventional outflow in both cynomolgus (Bill, 1967) and vervet monkeys (Bill, 1969b). The increase in unconventional outflow was relatively modest (3%) in cynomolgus monkeys [from 39 to 42% (Bill, 1967)] and larger (22%)
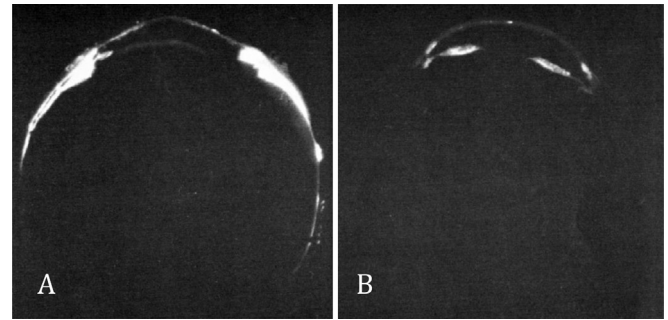
Fig. 7. Effect of atropine on unconventional outflow in cynomolgus monkey eyes with pilocarpine-induced tone in the ciliary muscle. In eyes with atropine and pilocarpine in the anterior chamber (A), radioactively-labeled albumin entered the uvea and sclera to a larger extent than in eyes with pilocarpine only (B). Reprinted from (Bill, 1967) with permission from Elsevier.

in vervet monkeys [31–53% (Bill, 1969b)] despite similar dosages. These differences may be attributable to differences in ciliary muscle tone or the reduction in tone caused by general anesthesia or both. For example, if in cynomolgus monkeys, the ciliary muscle is partly contracted by a subthreshold dose of pilocarpine, then atropine has a larger effect on unconventional outflow, increasing from 19 to 56% (Bill, 1967). Alternatively, the differences between monkey species may be related to the near absence of a scleral spur in vervet monkeys (Rohen et al., 1967; Bárány, 1979).

Much of our knowledge about the unconventional outflow comes from these pioneering studies in non-human primates. However, the higher fraction of outflow through this pathway in monkeys than in humans may limit the use of monkeys as a model for evaluating drugs for use in humans. While pilocarpine lowers IOP in humans and has been used as a treatment for glaucoma, its reported effects in monkeys depend on the concentrations used and perhaps also, the anesthetic chosen. In early studies with cynomolgus monkeys anesthetized using pentobarbital (which causes low resting IOP), pilocarpine reduced conventional outflow resistance but paradoxically increased IOP because of its effect on unconventional outflow (Bárány, 1962, 1966; Bill and Wålinder, 1966). However, in animals anesthetized by ketamine (which has less effect on resting IOP), pilocarpine decreased IOP, as it does in human eyes (Crawford and Kaufman, 1987).

6.3. Rabbit

Unconventional outflow is significantly lower in rabbits as compared to non-human primates (Table 3). This is somewhat puzzling because rabbits possess a deep ciliary cleft and a relatively undeveloped ciliary muscle that should provide a lower resistance to unconventional outflow as compared to primates (Tripathi, 1977).

As in primates, unconventional flow in rabbits increases after cyclodialysis (Bill, 1966c) or treatment with $PGF_{2\alpha}$ (Goh et al., 1989; Poyer et al., 1992), but not after prostaglandin D_2. The decrease in IOP after $PGF_{2\alpha}$ can be inhibited by pilocarpine (Goh et al., 1989). Other studies suggest that unconventional outflow in the rabbit may be increased by rho-kinase inhibitors such as Y-27632 (Honjo et al., 2001) and α_1-adrenergic antagonists such as bunazosin (Zhan et al., 1998) and nipradilol (Kanno et al., 1998), the latter possibly involving nitric oxide (Sugiyama et al., 2001).

6.4. Dog

Unconventional outflow in dogs has been documented by perfusion with fluorescent dextran that accumulated in the
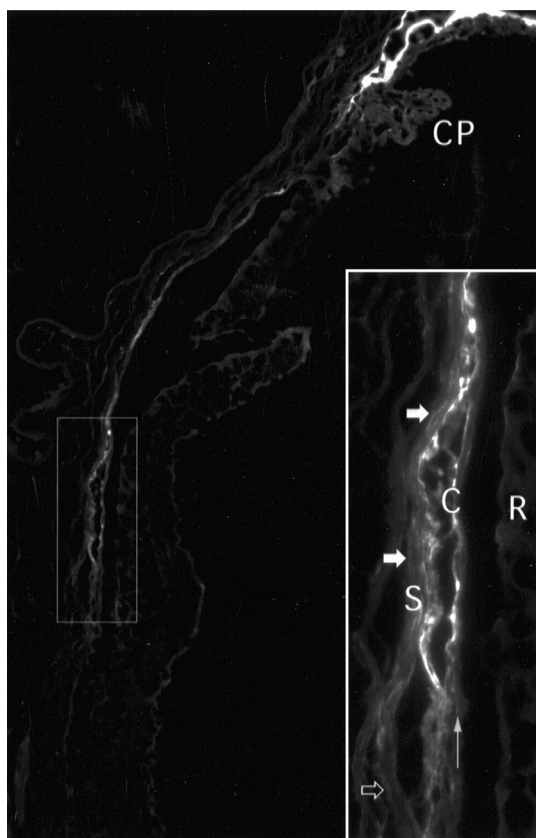
Fig. 8. Tracer decoration of the mouse unconventional outflow pathway 60 min after injection of 70 kDa dextran into the anterior chamber. Fluorescence lined the suprachoroidal space (ciliary processes, CP; choroid, C; sclera, S). High-magnification inset shows some regions of sclera near the suprachoroidal space contained substantial tracer (closed arrows) and other scleral regions contained minimal tracer (open arrow). Retinal pigment epithelium is indicated by a vertical arrow. Tracer did not enter the retina (R). Reprinted from (Lindsey and Weinreb, 2002) with permission from the Association for Research in Vision and Ophthalmology.

supraciliary, suprachoroidal, and scleral tissues (Gelatt et al., 1979). As in most other species, pilocarpine reduced unconventional outflow, as evidenced by a reduction in fluorescent dextran labeling posterior to the ciliary body. Perfusion with radiolabelled tracers provided a direct estimate of unconventional outflow that was 15% of total outflow in normotensive beagles but was reduced to 3% in beagles with advanced glaucoma (Barrie et al., 1985), consistent with the reduction in unconventional outflow reported in ocular hypertensive humans (Toris et al., 2002b). Beagles with advanced glaucoma had an accumulation of melanophores and elastic-rich extracellular matrix within the ciliary body musculature visible on histologic examination, and these deposits may have contributed to the elevated unconventional outflow resistance and consequent reduction in unconventional outflow in these animals (Samuelson and Streit, 2012). Unconventional outflow increased as IOP was increased from 20 to 50 mmHg, and the pathway became less permeable to microspheres larger than 1 μm at pressures above 20 mmHg (Samuelson et al., 1985).

6.5. Cat

Bill (1966a,b,c,d) reported an unconventional outflow of no more than 3% of total outflow in domestic cats, as measured by accumulation of radiolabelled albumin in ocular tissues. However, much higher estimates of unconventional outflow (up to 26%) have been reported by Wang et al. (1999) and Toris et al. (1995c) who

measured accumulation of fluorescent dextran in the uvea, sclera and retina. The reason for this large discrepancy remains unclear. Unconventional outflow was increased by prostaglandin A_2 in cats (Toris et al., 1995c) but remained relatively unaffected by epinephrine (Wang et al., 1999) based on direct tracer and indirect fluorophotometry methods.

6.6. Mouse

Mice are a useful animal model because of their relatively low cost, ease of use, and short lifespan that makes them ideal for genetic studies. Fluorescent dextran introduced into the anterior chamber of NIH Swiss mice appears in the supraciliary space and choroid within 60 min (Lindsey and Weinreb, 2002; Bernd et al., 2004) confirming the existence of unconventional outflow in mice (Fig. 8). Using tracers, Millar et al. (2011) directly measured[1] unconventional outflow in BALB/cJ mice aged between 30 and 42 weeks to be 0.012 μl/min, or approximately 9% of total outflow. With increasing age, however, there appears to be a significant reduction in unconventional outflow in mice (Table 2) (Millar et al., 2015), consistent with the age-related decline in unconventional outflow in humans (Toris et al., 1999) and monkeys (Gabelt et al., 2003 and Gabelt and Kaufman, 2005).

Estimates of unconventional outflow using indirect techniques (Section 4.2) in mice have yielded much larger values than those measured using tracers, ranging from 0.029 to 0.157 μl/min, corresponding to 21—83% of total outflow in NIH Swiss White and C57BL/6 mice (Aihara et al., 2003; Crowston et al., 2004; Zhang et al., 2009; Lee et al., 2011; Lei et al., 2011; Millar et al., 2011; Stamer et al., 2011; Boussommier-Calleja et al., 2012). While differences in age or strain may account for some of this variability (Millar et al., 2015), such differences cannot explain why there is such a large discrepancy between direct and indirect estimates of unconventional outflow in mice. Even when measured within the same study, Millar et al. (2011, 2015) reported a roughly 2-fold difference in unconventional outflow as measured by tracers versus that estimated by using the indirect technique. This discrepancy raises concerns regarding the accuracy of indirect estimates of unconventional outflow in mice, particularly considering the difficulty in the measurement itself in this small animal.

The small size of the mouse eye compounds the difficulty of determining unconventional outflow for two reasons. First, the magnitude of the flow rate in mouse eyes is so incredibly small as to push the limits of accuracy of most perfusion systems. Leaks and inaccuracies in the perfusion equipment could introduce significant inaccuracies in the measurement of outflow facility, raising concerns regarding the indirect estimates of unconventional outflow that are based on such measurements. Evaporation from the exposed surface of the perfused eye, for example, could artificially inflate the apparent unconventional outflow rate in indirect studies. Boussommier-Calleja and Overby (2013) and Boussommier-Calleja et al. (2015) have shown that the apparent pressure-independent outflow as measured by perfusion is indistinguishable from zero when enucleated mouse eyes are submerged in a bath of isotonic saline, in contrast to prior studies by the same group who measured a non-zero pressure-independent outflow when the eyes were exposed to room air (Boussommier-Calleja et al., 2012; Stamer et al., 2011; Lei et al., 2011). While fluid would evaporate in any species if the cornea or sclera were not kept fully hydrated, the relative importance of

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[1] During these experiments, the mouse eye was perfused with tracer via the anterior chamber at a rate of 0.5 μl/min, at least 3-fold greater than typical inflow rates in mice.

evaporation is potentially much greater in mice because of the larger ocular surface area to volume ratio. While this phenomenon might be of less importance in live animal studies (due to the presence of the extraocular muscles and orbital fat surrounding the sclera), the eyelids are usually kept open during such measurements. Even with hydrating drops, evaporation would tend to increase the osmolarity at the surface of the cornea and draw water from the eye.

Second, whereas in larger eyes, such as that of primates, diffusion does not play a significant role in transporting tracer into tissues around the anterior chamber (Bill, 1965; Bill and Hellsing, 1965; Bill, 1966a; Pederson and Toris, 1987), diffusional transport of tracers might be expected to be more significant in smaller eyes such as those of mice and this leads to an overestimate of unconventional outflow when using direct methods. Nevertheless, the distribution of dextrans in the anterior segment of mice was not influenced by tracer molecular weight (Bernd et al., 2004), consistent with findings in primates (Section 4.1.5.2). Furthermore, the observed spread of tracer over time through the suprachoroidal space of mice was consistent with bulk flow (Lindsey and Weinreb, 2002). These considerations suggest that diffusional transport of tracer may not be a major concern in the measurement of unconventional flow in mice.

The large fraction of flow passing through the unconventional outflow pathway in mice, as estimated by indirect methods, could be seen as an argument against use of mice as a model for human aqueous humor dynamics, where the majority of outflow passes through the trabecular route. However, as discussed in Section 4.2.1, unconventional outflow may be overestimated by using indirect methods in mice (Table 2). Furthermore, the observation that

pilocarpine reduces IOP in young black Swiss (Avila et al. 2001) and CD1 mice by increasing outflow facility (Li et al., 2014; Overby et al., 2014) argues against the notion that a large quantity of outflow passes through the unconventional pathway in mice (which would be blocked by pilocarpine).

The age-related decline in unconventional outflow observed in multiple mouse strains suggests that older mice having a smaller fraction of unconventional outflow may provide an appropriate model of aqueous humor dynamics in older humans (Millar et al., 2015). If mice are to be accepted as a model for human aqueous humor dynamics, then like humans, the bulk of outflow in mice should pass through the conventional or trabecular outflow pathway. Thus, the accuracy of the reports of high unconventional outflow estimated by using the indirect method should be reexamined, and the apparent discrepancy between direct and indirect estimates should be resolved in order to better assess whether mice are an appropriate animal model for aqueous humor dynamics in humans.

## 7. Clinical significance of unconventional outflow

7.1. Drug effects

Cholinergics, adrenergics, and prostagladins can affect the flow of aqueous humor through the unconventional outflow pathway. The principal site of unconventional outflow resistance is the ciliary muscle. Contraction of the ciliary muscle by cholinergics such as pilocarpine decrease the rate of unconventional outflow (Bill and Wålinder, 1966) while cycloplegics such as atropine increase unconventional outflow (Bill, 1967, 1969b). However, cholinergics also interfere with accommodation and are no longer used clinically to lower IOP.

Adrenergic agents also affect outflow through the unconventional outflow pathway. Bill (1969a) showed that epinephrine increased unconventional outflow in vervet monkeys, although the mechanism of action was unclear. Alm and Nilsson (2009) suggested that the effect of epinephrine might be related to relaxation of the ciliary muscle.

The introduction of the prostaglandin $PGF_{2\alpha}$ (Camras et al., 1977) and $PGF_{2\alpha}$-analogues (e.g. latanoprost, bimatoprost, and travoprost) to the pharmacopeia of glaucoma treatments marked a major change in glaucoma management. Not only is their reduction of IOP additive to most other glaucoma treatments, but the magnitude of their effect made them valuable as ocular hypotensive agent in their own right (Nilsson, 1997; Bron et al., 2001). Daily treatment with $PGF_{2\alpha}$ over several days causes a larger decrease in IOP than that from a single application, and the decrease is more marked after multiple dosing (Nilsson, 1997). As noted in Section 4.2.1.2, $PGF_{2\alpha}$ increases the pressure-dependency of unconventional outflow (Gabelt and Kaufman, 1990; Kaufman, 2003). The major effect on unconventional outflow appears to be mediated by remodeling of the extracellular matrix in the ciliary muscle (Ocklind, 1998; Sagara et al., 1999; Gaton et al., 2001) that enlarges spaces between ciliary muscle bundles (Fig. 9) and thereby presumably reduces unconventional outflow resistance and increases its pressure-dependency (Lütjen-Drecoll and Tamm, 1988).

Prostaglandins have an effect somewhat contrary to that of cholinergics; while prostaglandins increase unconventional outflow, cholinergics decrease it while also increasing conventional outflow. For this reason, the combination of prostaglandins and cholinergics would not be expected to be additive in reducing IOP. However, while perhaps not fully additive, combination of the two drugs reduces IOP more effectively than either alone (Fristrom and Nilsson, 1993; Toris et al., 2002a). This has led to the suggestion that prostaglandins may also increase conventional outflow (Bahler et al., 2008).
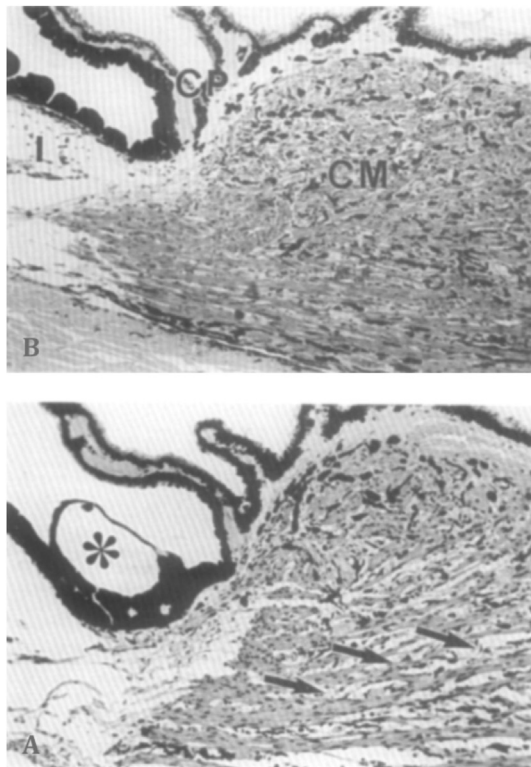
Fig. 9. Sagittal section through the anterior ciliary body. (A) Vehicle-treated control eye. CM, Ciliary muscle; I, iris. CP, ciliary processes. (B) After treatment with $PGF_{2\alpha}$, for 4 days. Note the enlarged spaces between the thin muscle fiber bundles in the prostaglandin-treated eye (arrows). Asterisk: Greeff's vesicle. Reprinted from (Lütjen-Drecoll and Tamm, 1988) with permission from Elsevier.

7.2. Surgery

In cyclodialysis, the attachment of the ciliary body to the scleral spur is disrupted, providing unimpeded access of aqueous humor to the suprachoroidal space, bypassing the ciliary muscle. Cyclodialysis is associated with hypotony caused by a decreased aqueous inflow (Chandler and Maumenee, 1961) and a greatly increased unconventional outflow (Bill, 1966c; Suguro et al., 1985). Cyclodialysis is usually the result of trauma or a surgical complication, although it can be created surgically in desperate circumstances. However, it is not routinely used to treat ocular hypertension because of the very low resulting pressures (7 mmHg or less) and the risk of acute IOP elevation should the cyclodialysis cleft spontaneously close.

While the cyclodialysis procedure is rarely used clinically, the benefits of greatly increased unconventional outflow without decreased aqueous production can be realized by using a suprachoroidal stent to bypass the ciliary muscle (Melamed et al., 2009; Figus et al., 2011; Hoeh et al., 2013; Oatts et al., 2013). These devices have been used successfully to lower IOP, although there have been reports of hypotony and of reactive fibrosis that blocks the bypass pathway (Brandao and Grieshaber, 2013). Nonetheless, they offer a potential means of achieving low IOPs that may be of particular benefit to cases of normal tension glaucoma.

7.3. Other clinical issues

The unconventional outflow pathway may be affected by pathologies of the anterior segment. In uveitis, accumulation of cells, protein and debris may block the conventional outflow pathway, and it has been suggested that the unconventional outflow pathway may have developed as an adaption to provide an alternate drainage route for aqueous humor under these circumstances (Alm and Nilsson, 2009). Indeed, in uveitis, along with a reduction of aqueous production to roughly one half of its normal rate, there is also a profound increase in unconventional drainage because of a four-fold increase in pressure-dependent unconventional outflow facility (Toris and Pederson, 1987; Nilsson, 1997; Alm and Nilsson, 2009). These changes produce hypotony until the uveitis subsides. It has been suggested that this effect is mediated by $PGF_{2\alpha}$ (Crawford and Kaufman, 1987).

Unconventional outflow may also play a role in the development of uveal effusion, an accumulation of fluid in the uvea that is thought to arise from impaired fluid drainage through the posterior segment and usually associated with abnormal thickening of the sclera, particularly in nanophthalmos (Ward et al., 1988; Jin and Anderson, 1990; Lam et al., 2005); uveal effusion can lead to detachment of choroid, ciliary body and retina. If a significant fraction of unconventional outflow passes into the vortex vein, then the proteins in the aqueous humor will be blocked from passing through these vessel walls, and they can only exit the eye by diffusion across the sclera (Section 5.1). Thickening of the sclera, such as occurs in nanophthalmos, would reduce this protein diffusion, potentially causing an accumulation of these proteins along the inner surface of the sclera. This in turn would lead to an increase in the local oncotic pressure causing fluid accumulation, as is seen in uveal effusion (Gass, 1983; Johnson and Erickson, 2000; Elagouz et al., 2010). This disease is treated by full-thickness sclerectomy to remove the barrier to protein transport.

8. Summary and future directions

Anders Bill was the first investigator to explore and characterize the physiologic processes of the unconventional outflow pathways. His pioneering measurement techniques are still the standard for identifying the pathway and estimating the amount of flow through this route. Originally this pathway was a scientific curiosity, but it became clinically important when Camras and Bito found that $PGF_{2\alpha}$ could profoundly redirect outflow through this pathway from the conventional pathway, reducing IOP. Our understanding of the unconventional outflow pathway has provided a new approach toward treating elevated IOP in glaucoma patients.

As unconventional outflow is pressure-insensitive under normal conditions, and because at least a fraction of this outflow passes through the uveovortex pathway, we would suggest that the terms "uveoscleral" and "pressure-independent flow" are misleading and can lead to incorrect conclusions about the nature of this route, particularly when considering the effects of drugs. Distinctions between pressure sensitive and anatomical pathways have important ramifications on the methods to be used for measuring and characterizing flow through this route. The more appropriate term for this route of outflow is "unconventional" or "non-trabecular".

Future animal studies of the unconventional outflow pathway should focus on species whose anatomic and physiologic properties of this route are close to those of humans. The similarity of the mouse outflow pathways to that of the human (Smith et al., 2001; Overby et al., 2014) and particularly the fraction of flow passing through the unconventional outflow pathway, make the mouse an attractive model. However, the method for accurate measurement of flow through the unconventional outflow pathway (the tracer method) is difficult in mice, cumbersome in larger animals, and virtually impossible in humans. Indirect methods to estimate unconventional outflow, while easily used, have serious flaws and the accuracy of such approaches are questionable. Thus, a more robust and accurate method to measure unconventional outflow, both in animals and in living humans, is necessary.

Development of a reliable non-invasive method for accurate measurement of unconventional flow would provide a great advance for characterizing the dynamics of the anterior segment and for developing clinical treatments of glaucoma. One possibility might make use of fluorophotometry. A large macromolecule that would be trapped in or pass very slowly through the unconventional pathway could be introduced into the anterior chamber through the cornea and tracked to the general circulation as an indicator of trabecular flow, while its dilution in the anterior chamber is simultaneously measured to determine aqueous humor inflow. The difference between trabecular outflow and inflow rates would be a direct measure of the unconventional outflow rate. Alternatively, tracers could be tracked through the outflow pathways by using high-resolution MRI or OCT.

Anders Bill developed the methods to establish the route and rate of unconventional outflow; our challenge now is to develop methods to measure this flow rate accurately and non-invasively in humans.

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