An in vitro investigation of ocular fibroblasts in relation to glaucoma filtration surgery.

by

JEREMY PAUL JOSEPH M.B.B.S., D.O., F.R.C.S., FCOphth.

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The Institute of Ophthalmology
Pathology Department
17-25 Cayton Street
London EC1V 9AT.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

The trabeculectomy is by far the most widely performed operation for chronic glaucoma. Studies of adult Caucasian patients undergoing this procedure report a success rate of about 85%. However, in children, Blacks, neovascular and aphakic glaucomas, and in patients who require re-operation, the success rate is far lower. The majority of trabeculectomies that fail, do so because of fibrosis which produces scarring at the operation site. It is unknown why certain patients are predisposed to surgical failure.

The migration of fibroblasts to the wound under the influence of locally produced chemicals, and their subsequent contraction, are important in the formation of scar tissue. Fibroblast migration and contraction have not previously been studied in the context of glaucoma surgery. In addition, the role of the aqueous humour in maintaining the patency of a trabeculectomy is poorly understood.

Rabbit and human ocular fibroblasts were established in tissue culture, and employed in migration assays using 48-well micro-chemotaxis chambers. Chemoattractants consisted of fibronectin, fibroblast conditioned medium, and aqueous humour. The aqueous was derived from normal rabbit and human eyes, and from patients undergoing glaucoma surgery. The fibroblasts were also investigated in contractile assays. A series of drugs were then evaluated as potential fibroblast inhibitors.

Rabbit and human aqueous humour was found to be strongly chemotactic to ocular fibroblasts. Samples derived from patients who had previously undergone failed glaucoma drainage surgery were of significantly greater activity than control specimens. The presence of high aqueous chemotactic activity pre-operatively may partially explain why some patients are prone to surgical failure. A number of the drugs evaluated markedly inhibited fibroblast migration and contraction. Further testing of these compounds, particularly taxol, is indicated in animal models of glaucoma drainage surgery as some of them may be beneficial in inhibiting fibrosis in patients undergoing trabeculectomy, thereby improving the success rate of surgery.

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Dr. Gaz Sheraidah measured the protein content of the aqueous humour samples, and performed osmolality measurements.
Dr. Chris Fry conducted the pH measurements of samples used in the chemotaxis experiments.
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PUBLICATIONS

The following papers have been published based on the work conducted in this thesis:

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Grierson I, Joseph J, Miller MH, et al.
Wound repair: the fibroblast and the inhibition of scar formation.
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Int Ophthalmol 1989;13:125-130.

Joseph JP, Miller MH, Hitchings RA.
Wound healing as a barrier to successful filtration surgery.
Eye 1988;2(Suppl):S113-S123.

CANDIDATE'S OWN RESEARCH AND OBSERVATIONS

The research and observations reported in this thesis have been carried out entirely by the candidate, with the exception of the following areas where some technical assistance was obtained:
Dr. Nicol McKechnie assisted in the preparation of specimens for immunofluorescence labelling.
Robin Howes and Lynn Millar assisted with electron microscopy.
Dr. Gaz Sheraidah measured the protein content of aqueous humour samples and performed the osmolality measurements.
Dr. Chris Fry measured the pH of the solutions in the wells of the micro-chemotaxis apparatus.

ORIGINALITY AND CONTRIBUTION TO MEDICAL SCIENCE

The thesis has evaluated the trabeculectomy in a manner not previously undertaken, since it has considered principally the failure of the operation, rather than its success. The role of aqueous humour in the outcome of glaucoma surgery remains poorly defined. My observation that aqueous humour has powerful activity in inducing fibroblast chemotaxis is original to this thesis. The further demonstration that aqueous humour taken from patients who have formerly undergone unsuccessful glaucoma drainage surgery, has significantly greater fibroblast chemotactic activity than normal aqueous may explain why surgery has a lower chance of success when performed in certain groups of patients. This observation may ultimately allow for the targeting of drugs to inhibit fibroblast accumulation at the site of glaucoma surgery, leading to a decrease in scarring and surgical failure.

Fibroblast chemotaxis and contraction have not been investigated in the context of glaucoma filtration surgery, although they have been studied in relation to wound healing at other sites in the body. The employment of Tenon's capsule fibroblasts in the micro-chemotaxis chamber, together with chemoattractants such as aqueous humour is therefore original to my thesis. The fibroblast contraction assay developed here to study ocular fibroblasts is also unique. Most of the drugs which I tested in the assays as inhibitors of fibroblast chemotaxis and contraction have not been previously assessed with regard to glaucoma surgery. My demonstration of in-vitro anti-fibroblast activity may indicate a potential for the use of some of these compounds in reducing fibrosis in human beings undergoing glaucoma surgery, thereby improving the success rate of the operation.

1.1. GLAUCOMA AND TRABECULECTOMY

The introduction of the operation of trabeculectomy in 1968 by Cairns (Cairns, 1968) has dramatically altered the management of patients with glaucoma. The hazards of the operative techniques available prior to this time meant that surgery at an early stage of the disease could generally not be contemplated. Surgery was therefore reserved as a last ditch manoeuvre in end stage glaucoma, when every type of medical therapy had failed, to attempt to preserve the small amount of vision remaining in a patient (Watson, 1981; Watson and Grierson, 1981). The trabeculectomy has so reduced the complications, and consequently the fear of surgery, that its implementation as a primary procedure for the management of glaucoma has been advocated by some (Cairns, 1982; Jay and Murray, 1988). In addition, where medical treatment has failed to control the disease, the decision to intervene operatively can now be made with little hesitation.

Although the trabeculectomy is generally a very successful operation (Watson and Grierson, 1981), in common with all other surgical procedures, a number of operations will inevitably fail, and invariably a proportion of patients will suffer adverse effects from surgery. In addition, in certain identifiable groups of patients, the success rates of surgery are disappointingly low. This thesis explores the reasons for the failure of the trabeculectomy and presents experimental studies directed at understanding and reducing this serious problem for patients undergoing glaucoma surgery.

1.1.1. Definition of glaucoma

The intra-ocular contents are normally subjected to a pressure up to 21 mmHg greater than the atmospheric pressure. The glaucomas are a diverse group of ophthalmological conditions with a number of manifestations. However, they have in common an intra-ocular pressure (IOP) which is believed to be too high for the intra-ocular tissues, particularly the retina and the optic nerve head, to withstand for a prolonged period without suffering irreversable damage. In most types of glaucoma the IOP is elevated beyond the normal range. However, the situation is complicated in normal or low tension glaucoma where seemingly normal IOP's still appear to cause visual damage. Therefore, except for extreme elevations of IOP, it has not yet been determined

whether the elevated pressure is a primary feature ie. causative in producing visual damage, or is merely a secondary manifestation of a disease resulting in both damage to the optic nerve and elevated pressure.

The brunt of the damage in glaucoma is borne by the ganglion cells of the retina (Quigley and Green, 1979). This is manifested by the progressive loss of axons in the optic nerve, which is seen clinically as cupping of the optic disc. Only when approximately 50% of the neurons are lost, do the characteristic field defects of glaucoma begin to be detected (Quigley, Addicks and Green, 1982).

The IOP is easily measured in a routine ophthalmological examination. The vast majority of treatment directed towards the glaucomas has therefore been aimed at reducing the IOP, based on the supposition that the pressure is a primary feature of the disease. This may well be untrue, at least in some forms of glaucoma, and may therefore have little relevance in controlling the disease progression. However, in the present state of ignorance, the lowering of the IOP remains a primary goal in the management of the glaucomas.

1.1.2. Pathophysiology

The IOP in engendered by the secretion and ultrafiltration of aqueous humour by the epithelium of the ciliary body. This blood derived transparent fluid circulates from the posterior chamber of the eye, where it is produced, through the pupil to the anterior chamber. The aqueous humour is absorbed back into the circulation via the structures in the angle of the anterior chamber of the eye. Most of the fluid percolates through the trabecular meshwork in the angle, encountering progressively increased resistance to its flow, until it finally reaches the canal of Schlemm. From here, the aqueous flows into collector channels, aqueous veins and ultimately into epi-scleral venous plexuses and thus to the general circulation. Between 4 and 14% of the aqueous percolates through the iris stroma and trabecular meshwork, into the ciliary body and sclera, and then is resorbed back into the choroidal circulation or drains directly through the scleral substance and via perivascular spaces, into the periocular connective tissues (Bill and Phillips, 1971). The importance of this non-conventional or uveo-scleral outflow of aqueous humour in health and in glaucoma is uncertain (Hitchings, 1987). The rate of production of

aqueous humour is believed to be constant at about 2 to 4 ul/minute (Cole, 1984). Consequently, the resorption of aqueous must match the secretion rate if the IOP is to be maintained. In glaucoma there is an increased resistance to the outflow of aqueous humour which is believed by the majority of authors to be situated in the inner wall of Schlemm's canal (Lee and Grierson, 1982). Overcoming this increased resistance necessitates an elevation of the IOP to once again achieve homeostasis where the inflow of aqueous humour into the eye balances its outflow.

1.1.3. Classification and epidemiology of the glaucomas

A number of classification systems of the glaucomas have been developed. One of the most useful systems clinically is described by Hitchings, (1987). Glaucoma that arises as a result of a developmental defect or a degenerative process occurring within the eye is termed Primary Glaucoma, while that resulting from another disease process occurring inside or outside the eye is referred to as Secondary Glaucoma. The two major groups of glaucoma can be further subdivided, based on the morphology of the irido-corneal angle, into Open angle and Closed angle types. Combined mechanism (mixed or narrow angle glaucoma) is a further category of the primary glaucomas.

There are estimated to be 125,000 cases of glaucoma in the United Kingdom of which one third are diagnosed. Primary Open Angle Glaucoma constitutes 35% of these; 17.5% are Low Tension Glaucoma; 30% are Secondary Glaucomas; Closed Angle Glaucoma accounts for 12.5%; while the remaining 5% are cases of Congenital Glaucoma. About 13,000 people who are registered blind have glaucoma, although other diseases may have contributed to their visual loss. This constitutes 14% of blind registrations. A similiar number of people, although not registrable as blind, have severe visual handicap resulting from glaucoma (Hitchings, 1987).

1.1.4. Treatment of glaucoma

The elevated IOP of the chronic glaucomas can be treated by two main methods (i.)Decreasing the inflow of aqueous humour and (ii.)Increasing its outflow. These can in turn be achieved by two basic means: medical or surgical.

The acute narrow angle glaucomas, which stem from an anatomical abnormality, are definitively treated by a surgical procedure designed to overcome the anatomical blockage. Thus by creating a permanent internal channel through the iris between the posterior and anterior chambers (iridectomy), the damming of aqueous in the posterior chamber of the eye is prevented. Iridectomy can be performed either as a formal surgical operation or as done more recently, by laser.

Until the development of the trabeculectomy, the initial management of patients with chronic glaucoma was generally medical. Medical treatments are of two basic types: drugs that diminish the inflow of aqueous humour into the eye, eg. carbonic anhydrase inhibitors; and drugs that enhance its outflow, eg. pilocarpine. A failure of maximum medical treatment with documented progression of damage to the visual system was necessary before proceeding to surgical treatment. In the United States of America this is still regarded as the only acceptable practice. Likewise, in many centres in the United Kingdom this conventional teaching is still practised.

Prior to trabeculectomy, a number of different operations had been developed to facilitate the outflow of aqueous humour. In general, surgery was directed at creating an alternative drainage pathway for aqueous humour out of the eye. This took the form of either a cyclodialysis where internal drainage or uveo-scleral drainage of aqueous humour was improved, or more commonly, a procedure was undertaken to facilitate the external drainage of aqueous humour by creating an artificial pathway from the anterior chamber. A channel through the sclera adjacent to the limbus of the eye, opened into the potential space deep to the conjunctiva (subconjunctival space) where the aqueous humour formed a blister-like elevation of the conjunctiva, known as a bleb. Although many of these procedures were successful in reducing the IOP, they were not directed at the site of the blockage of the drainage of aqueous humour, and they also suffered from a number of serious sight threatening complications.

Various surgical procedures were also developed to destroy portions of the ciliary body, thus diminishing the production of aqueous humour. Cyclo-destructive procedures are crude and lack precision so making it extremely difficult to titrate the amount of cyclo-destruction with the IOP drop required. These procedures have been refined somewhat and

now employ freezing (cyclocryotherapy) or laser (trans-scleral Neodymium-YAG cyclophotocoagulation). However, they are still reserved as a physical method of treatment in recalcitrant cases when all else has failed.

1.1.5. Trabeculectomy

Early techniques of drainage surgery suffered from a number of problems:
A full thickness drainage procedure provided poor control of the IOP in the post-operative period. Thus extreme hypotony might occur with loss of the anterior chamber, lens corneal contact, and the subsequent development of cataract. In addition, rapid, uncontrolled decompression could precipitate choroidal and supra-choroidal haemorrhage. The conjunctival blebs following full thickness drainage surgery were often thin and cystic and provided a life-long portal for the ingress of intra-ocular infection. Such infections were generally catastrophic (Katz, Cantor and Spaeth, 1985).

The trabeculectomy was developed in response to the need for a safe, predictable operation for the control of glaucoma. As with many revolutionary developments or inventions a number of people were simultaneously working on the same problem (Karyllos, 1967; Linnér, 1969; Phillips, 1969). Although Karyllos published his technique first, it appeared in Greek and was therefore largely ignored. Cairns published his work in the American Journal of Ophthalmology, received widespread acclaim and is generally credited with inventing the technique and the operation now bares his name. It is unfortunate that the other pioneers of the trabeculectomy have been largely overlooked.

The rationale of the operation was to remove a portion of the obstructed trabecular meshwork, which is thought to be the site of the problem in open angle glaucoma. This was performed under a partial thickness scleral flap, so that upon replacing the flap there would be no direct connection between the anterior chamber and the subconjunctival space. Rather, the excision of a portion of the meshwork would leave the two cut ends of Schlemm's canal opening into the anterior chamber allowing the direct ingress of aqueous humour. By providing internal drainage for aqueous humour in a "physiological" manner, it was hoped that the trabeculectomy would suffer from none of the disadvantages of full-thickness drainage procedures.

Although techniques of opening the canal of Schlemm into the anterior chamber had been devised by other authors eg. nylon filament trabeculotomy (Smith, 1960 and 1970), these operations had been technically difficult to perform and failed frequently, probably due to healing of the artificial opening into the anterior chamber. More recently, this concept has been revived with trabeculo-electropuncture (Hoffmann, Harnisch and Bill, 1977) and the experimental use of Neodymium-YAG lasers to form trabeculotomies (Lee, Dutton and Cameron, 1988), but remains to be clinically validated.

In the initial description of the trabeculectomy (Cairns, 1968), the operation was performed as follows (fig.1.1):
General anaesthesia was employed whenever possible and an operating microscope was used at all stages. The operation was performed at the 12 o'clock position, having previously inserted a superior rectus suture. A 6 mm limbus based conjunctival flap was dissected forwards, cleanly exposing the underlying sclera, limbus and cornea. A corneoscleral flap was marked out with shallow incisions demarcating three sides of a 5 mm square, with the free limbs extending backwards radially from the limbus. The other limb of the incision could be placed at the anterior border of the corneoscleral limbus, in which case the flap was turned backwards, or behind in the sclera, in which case the flap was turned forwards (fig.1.1a. and b.). Cairns expressed a preference for a fornix based flap in order to minimise bleeding from the scleral vessels. A paracentesis track was then made without loss of aqueous.

The incisions demarcating the corneoscleral flap were then deepened until over half the thickness of cornea and sclera had been cut. Preplaced sutures were inserted to close that part of the incision lying concentric with the corneoscleral limbus. If the flap was dissected forwards these sutures were omitted. The corneoscleral flap was then dissected up from the deeper layers (fig.1.1c. and d.).

At this stage the IOP was lowered by depressing the posterior lip of the paracentesis track. By this manoeuvre it was hoped to cause the Canal of Schlemm to fill with blood, and thus be more easily seen. A 4 mm incision was then made along the line of the scleral spur into the anterior chamber, with the attendant escape of aqueous humour. The anterior lip of the incision was grasped with a pair of Barraquer

toothed iris forceps, and using Vannas scissors the portion of the anterior lip grasped by the forceps was excised. It was intended that this excised portion would contain a length of the Canal of Schlemm (fig.1.1e.).

In the first ten cases reported by Cairns no iridectomy was performed. However, Cairns felt that it was probably safer to do an iridectomy to prevent prolapse into the area of excision, or even through the corneoscleral flap. The corneoscleral flap was sutured firmly back into place, the intention being to secure a watertight union (fig.1.1f.). The conjunctival flap was then returned to its bed and sutured (fig.1.1g. and h.). The paracentesis track was used to reform the anterior chamber if it appeared shallow. Polyfax ointment and 1% atropine drops were instilled and a pad and bandage applied. The eye was dressed after 24 hours, and received 0.5% atropine drops once daily for two weeks.

Since the original description by Cairns numerous modifications of the procedure of trabeculectomy have been described. These will be considered in section 1.6.

1.1.6. Mode of action of the trabeculectomy

The most comprehensive histological evaluation of microsurgical glaucoma techniques was performed by Spencer, (1972). He proposed that a successful trabeculectomy (or trabeculo-canalectomy) may permit increased aqueous outflow via several pathways (fig.1.2.):

1. The aqueous may gain direct access to the patent cut ends of Schlemm's canal.
2. Aqueous may leave via the the ostia of the intrascleral outflow channels or may permeate the thinned outer scleral lamellae.
3. It may filter externally along the course of the scleral scar, producing a bleb which may or may not be visible clinically.
4. The aqueous may filter internally via a surgically produced cleft between the sclera and ciliary body leading to enhanced uveo-scleral outflow.

Cairns devised the operation in order to allow direct access of aqueous humour into the cut ends of Schlemm's canal and consequently intended that the excised deep portion of the corneo-scleral limbus should contain a length of the canal. Of the original seventeen cases

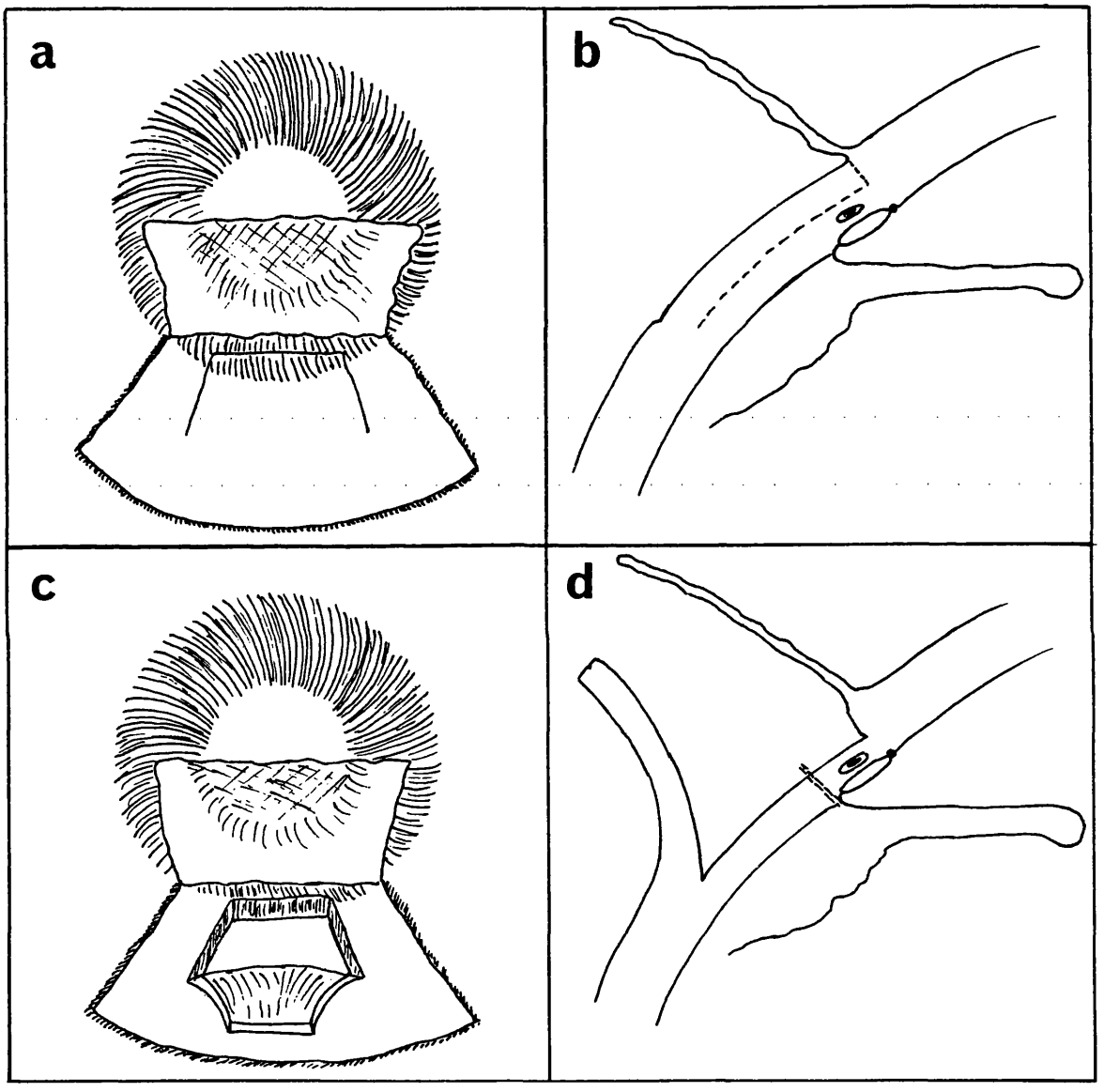


Fig.1.1. Diagrams demonstrating the Cairns Trabeculectomy.
a. and b.) A limbus based conjunctival flap is elevated. A half thickness 5 x 5 mm fornix based corneo-scleral flap is demarcated. Preplaced sutures are inserted along the limbal edge of the scleral flap but have been omitted for the sake of clarity. c. and d.) The half thickness scleral flap is reflected and a 4 mm incision made along the line of the scleral spur into the anterior chamber.

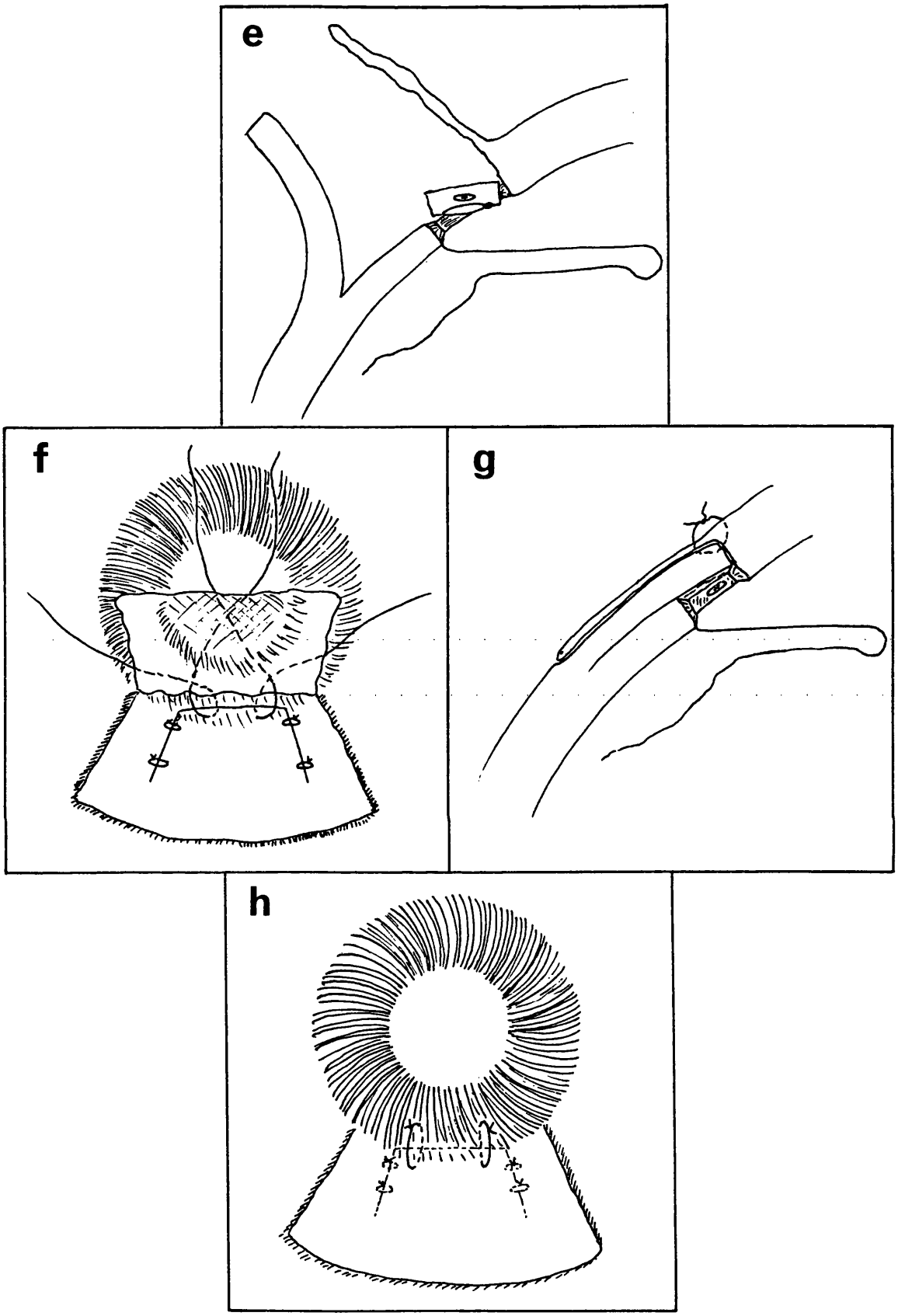


Fig.1.1.(Continued) Diagrams demonstrating the Cairns Trabeculectomy e.) The block containing the remaining corneo-scleral lamellae, Schlemm's canal and the trabeculum is excised. f. and g.) The corneo-scleral flap is replaced and sutured with virgin silk sutures with the intention to obtain a watertight seal. h.) The conjunctival flap is finally returned to its bed and sutured into place.

that he reported (Cairns, 1968), he considered that in eleven, normalisation of the IOP pressure was not due to subconjunctival drainage. In the remaining six cases a bleb appeared and he considered these to be a failure, in spite of normalisation of the IOP, because the effect intended was not produced. Histology performed on the excised specimens showed no trabecular tissue present in two cases. However in only one of these did a bleb develop. In four other cases the presence of trabecular tissue was doubtful, yet in only one of these did a bleb form.

Bárány, Linnér, Lütjen-Drecoll, et al., (1972) examined the structural and functional effects of trabeculectomy in 17 normal cynomolgus monkeys. A small trabeculectomy was performed in one or both eyes of the seventeen animals and follow-up ranged from 329 to 447 days. Outflow facility was determined by repeated anterior chamber perfusions before and after the operation. Of the 17 successfully operated animals, 4 died spontaneously so that the eyes were not suitable for histological studies. For the light microscopical study 18 eyes from 11 monkeys were analysed. A frank fistula between the canal of Schlemm and the anterior chamber was present in only 3 eyes of two animals. In 2 other eyes indirect communications appeared to be present between the anterior chamber and the cut ends of the canal. Of these 5 "successful" cases a lasting facility increase was seen. In the majority of cases, however, the cut ends of Schlemm's canal were closed by scar tissue and were completely lined by endothelium. In addition, no lasting effect on outflow facility was seen. The authors conclusion was that the patency of the cut ends of Schlemm's canal was important for the success of the surgery. They therefore suggested a modification of surgery consisting of placing a number of small holes in the meshwork, with each being sufficiently small so that no collapse of the canal occurred.

The mode of action of the trabeculectomy was also evaluated by Rich and McPherson, (1973) who found a different result to Barany et al., (1972). Rich and McPherson studied 10 adult Owl Monkeys (Aotus trivirgatus) on whom they performed a Cairns trabeculectomy (Cairns, 1968). In addition, after suturing the scleral flap with 7-0 chromic collagen sutures, the wound margins were sealed with a biological tissue adhesive, Bucrylate, to prevent the development of a filtering bleb. Histological preparations of excised specimens demonstrated the

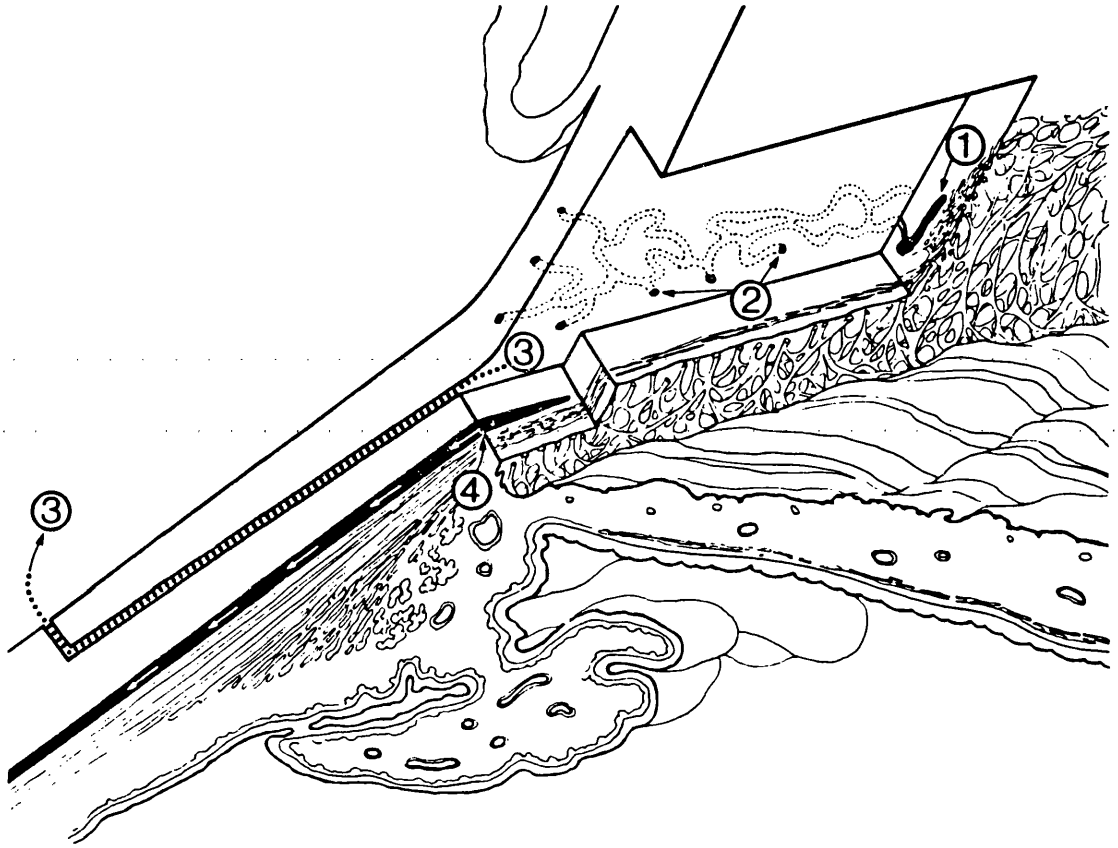


Fig.1.2. Diagram showing the possible routes for the outflow of aqueous humour after a trabeculectomy (Spencer, 1972).
1. Into the cut ends of Schlemm's canal if these remain patent;
2. Into intrascleral outflow channels if their ostia remain open, or through thinned scleral lamellae;
3. Along the course of the lamellar-scleral flap and into the subconjunctival tissues (dotted line);
4. Into the potential space between the ciliary body and sclera (white arrows) as in a cyclodialysis, provided that the resection has been performed behind the scleral spur (Watson trabeculectomy - see section 1.6.1.).

canal of Schlemm and trabecular meshwork in 9 of 10 specimens. In one monkey only corneal tissue was present. Monkeys were sacrificed at selected intervals up to one year. Consistently high outflow facility was maintained in only one monkey for the entire postoperative year. In the only animal in whom Schlemm's canal was absent in the excised tissue block, improved outflow was demonstrated until the 24th week postoperatively. Seven monkeys were sacrificed for histological study. In all animals the cut ends of the canal of Schlemm were closed and there was scar formation and fibroblastic activity in and around the margins of the resection. In two monkeys sacrificed when facility of outflow values were elevated, histologically, a cyclodialysis cleft was present. In the only animal who demonstrated consistently high outflows upto the end of one year, no cyclodialysis was found, there was no filtering bleb, and the canal margins at the operative site were firmly scarred. The mechanism of improved outflow in this animal was not determined. The authors concluded that it is very doubtful that improved outflow facility results from direct aqueous flow into the open resected ends of Schlemm's canal. They felt that a real possibility exists that filtration occurs directly into open collector channels and aqueous veins that have been transected in the operation, or perhaps that filtration occurs directly through the overlying scleral lamellae. However, they were unable to demonstrate this in their study.

The above two studies on monkey eyes can be criticised on the basis that normotensive and not glaucomatous animals were employed. This was done as unacceptable ocular damage is necessary to induce glaucoma in monkeys. When a trabeculectomy is performed in a normal eye, the aqueous which leaves the eye passes initially through both the trabeculectomy and the normal meshwork. By contrast, in a glaucomatous eye with a damaged trabecular meshwork, most of the aqueous will leave via the trabeculectomy. Therefore, a trabeculectomy may fail more readily in a previously normal eye, because of reduced aqueous flow through it, and the time course of the failure may be blurred as the flow of aqueous decreases through the trabeculectomy and increases through the normal meshwork. However, the situation is complicated by the finding that a chronically underperfused trabecular meshwork after surgery shows a build-up of deposits beneath the inner wall endothelium of Schlemm's canal, which may compromise the function of the meshwork (Lütjen-Drecoll and Bárány, 1974).

Human studies have also failed to consistently demonstrate filtration through the canal of Schlemm. Spencer, (1972) felt that the histological evidence gathered cast doubt upon the patency of the canal of Schlemm. He described the case of one trabeculectomy specimen that had been excised completely anterior to the trabecular meshwork and was covered on its inner surface by Descemet's membrane. The IOP of this patient had been 16 to 18 mmHg for two years postoperatively without visible evidence of external filtration. Spencer cited another patient with primary open angle glaucoma who underwent a successful trabeculectomy with control of the IOP without medication, and without evidence of a filtration bleb. Four months after the operation the patient died and the treated eye was enucleated and serial sections were prepared through the operation site. Both ends of the canal of Schlemm and the adjacent trabecular meshwork were covered by a connective tissue scar. No patent opening into the canal or to any of the intrascleral collector channels could be seen. The intrascleral surgical scar showed traces of pigment along its course and did not appear firmly healed, suggesting that external filtration along this route might have occurred despite the absence of a clinically visible bleb.

Galin, Boniuk and Robbins, (1975) concluded that the localisation of angle landmarks, such as Schlemm's canal, did not improve the clinical success of trabeculectomy. In addition, it did not appear to matter that trabecular tissue or Schlemm's canal be excised for the operation to be successful. On the contrary, in cases where a deliberate attempt was made not to excise these tissues, which was demonstrated histologically, clinical success was equally common. They therefore proposed that a trabeculectomy functions primarily as a fistulising operation carried out under a scleral flap, substituting a scleral-conjunctival barrier to aqueous flow rather than the conjunctival barrier alone provided by other glaucoma operations.

Taylor, (1976) reached a similiar conclusion after examining excised trabeculectomy specimens with conventional light and also scanning electron microscopy. He stated that the inclusion of particular anatomic structures in the trabecular block, confirmed histologically, did not correlate with postoperative success judged on the IOP or the formation of a filtering bleb. Thus, he deduced that trabeculectomy

works by filtration rather than by a more specific effect such as the opening of Schlemm's canal to the inflow of aqueous humour.

The question of the permeability of the outer layers of the limbus and anterior sclera to aqueous humour was investigated by Shields, Bradbury, Shelburne, et al., (1977). They performed an in vitro study on sixteen autopsy human eyes. A trabeculectomy was performed with a partial thickness 7 by 7 mm limbus based scleral flap and a 1 by 4 mm opening into the anterior chamber. The flap was repositioned with 5 sutures and the edges sealed with cyanoacrylate adhesive to prevent flow around its margins. The eye was perfused and the outflow facility from the eye determined. By varying the thickness of the scleral flap, it was determined that the increase in outflow caused by the trabeculectomy was inversely related to the thickness of the scleral flap. However, the number of eyes used in the study was too small to be statistically significant. Perfusion of the eyes with ferritin and India ink suggested that flow occurred through the outer layers of the limbus and anterior sclera in a trabeculectomy flap, and can significantly improve the outflow facility. This flow may be through vessels in the flap or through the collagen ground substance between individual fibrils. The clinical significance of these findings is uncertain because these were in vitro studies, which may not accurately depict the in vivo situation, particularly because healing processes are absent. The use of glue to seal the perimeter of the scleral flap may also have important effects on the surrounding sclera. The authors however, emphasised that this study did not prove that aqueous flow through the scleral flap is the main route of external filtration after trabeculectomy, but that it merely provided some support for the theory.

Some further evidence for the permeability of the outer layers of the sclera was shown clinically by David and Sachs, (1981) who performed "quantitative trabeculectomies". In accordance with the preoperative IOP, the thickness of the superficial flap was varied from 1/2 to 1/5th of the scleral thickness. The authors reported an excellent surgical result from their study of 35 eyes, however they provided no statistical correlation between the initial pressure, the postoperative pressure, and the flap thickness.

It is unlikely that the internal filtration of aqueous via a surgically produced cleft between the sclera and ciliary body (cyclodialysis) accounts for the increased filtration of aqueous humour after a trabeculectomy, since a pure cyclodialysis has a negligible effect on pressure (Cairns, 1986).

After considering the evidence from trabeculectomies performed over 18 years, Cairns, (1986) felt that trans-scleral passage of aqueous was likely to be the route operating in most cases. He felt that this accounts for the thick walled trabeculectomy bleb, centered exactly over the whole superficial flap.

Filtration of aqueous externally along the course of the external scar is also likely to be an important mechanism for the outflow of aqueous humour. This outflow tends to produce a bleb which may or may not be visible clinically. Although Cairns, (1968) considered that 6 of the 17 cases that he originally reported were failures because of the appearance of a bleb, it has come to be realised that there is a fairly close correlation between the presence of a bleb and success of the drainage procedure. This has prompted a number of investigators to regard a trabeculectomy merely as another filtering procedure, but with the added safeguard of a scleral flap intervening between the drainage channel and the outer surface of the eye (Watkins and Brubaker, 1978; Blondeau and Phelps, 1981).

In conclusion, the bulk of the evidence from both clinical and laboratory studies tends to favour as the mode of action of a trabeculectomy, both trans-scleral filtration of aqueous humour and filtration around the sides of the scleral flap. These two routes of aqueous outflow may co-exist in a particular patient or one may predominate, but clinically, it is not possible to determine this.

1.2. SUCCESS OF TRABECULECTOMY

A trabeculectomy can be successful based on a number of parameters. The most convincing index of success is preservation of the visual status of the patient undergoing surgery. This is naturally the primary aim of the surgical procedure. However, there are great difficulties in accurately measuring a patient's visual performance on a particular day let alone comparing the performance over a period of time. This is exemplified in the measurement of visual fields, where even with computerised automated perimetry, the best method of determining a significant change between two fields is yet to be defined. In addition to being relatively crude, the follow-up of patients on visual criteria is extremely time consuming and generally protracted studies are necessary before any meaningful result is obtained.

Hence most studies involving glaucoma surgery tend to have pressure control as the criterion for success. As mentioned in section 1.1.3. pressure control of glaucoma is not synonymous with disease control. Therefore patients with successful surgery on pressure criteria may actually be worse visually. For example, a patient who develops a cataract as a direct side effect of surgery, but attains adequate pressure control, would still be classified as a success in many studies of trabeculectomy. Measurements of intraocular pressure are easy to obtain and easy to evaluate statistically. However, the definition of adequate pressure control has not been universally agreed upon. This makes comparison between studies extremely difficult where different criteria have been used. In addition, many authors use different terms for success. Thus success for one author implies pressure control without the use of anti-glaucoma medications; other studies describe success as pressure control if necessary with the use of medications. Comparison is further complicated by various studies where visual parameters are also assessed in the determination of surgical success. Furthermore, varying follow-up times have been used in most of the published studies, thus making comparisons unrealistic. In general, short term studies tend to over estimate success.

Since the first description of the trabeculectomy 21 years ago, numerous studies have been published, and the success of the procedure in many different types of glaucoma has been described. The main

catergories of glaucoma that have been treated by trabeculectomy will be considered separately.

1.2.1. Primary open angle glaucoma

Cairns' original study (1968) described 17 patients with primary open angle glaucoma, all of whom achieved pressures less than 21 mmHg without medication. Follow-up ranged from 20 to 56 weeks. This 100% success rate of surgery, in terms of pressure control, with relatively minimal complications led to the extreme popularity of the procedure and his paper has served as the yard-stick for all subsequent investigations. However, the study is open to criticism due to the extremely short duration of follow-up and the small number of patients involved. Cairns presented an extension of this study including 80 eyes with a follow-up varying from under 1 year to about four years (Cairns, 1972). The paper does not stipulate how many patients were involved. IOP was considered controlled if it remained below 20 mmHg by applanation tonometry. No hypotensive medications were utilized. Pressure control after 1 operation was achieved in 97.5% of eyes, while reoperation was necessary to achieve control in 2.5% of eyes.

Watson, (1970) using a modification of the Cairns trabeculectomy, published a study on 44 glaucomatous eyes, 35 of which had primary open angle glaucoma. The maximum follow-up period was 30 months (average 15). Post-operatively 4 patients with primary open angle glaucoma required medication to keep the IOP below 20 mmHg and 1 required reoperation. The overall success rate for pressure control without medication was 86.4%.

The observations of Cairns and Watson were extended in a large review of 424 trabeculectomies (Watson and Grierson, 1981) of which 320 were performed on eyes with primary open angle glaucoma. The exact follow-up period is difficult to determine from the paper, but the minimum was 2 years, with the study including all trabeculectomies performed over a ten year period (1967-1977) at Addenbrooke's Hospital, Cambridge. Of the patients with primary open angle glaucoma, 86% were controlled without further medication, and 94% with surgery and further medication. Success was defined as an IOP of less than 21 mmHg on three successive readings with no further field loss. Thus, 6% of the trabeculectomies were failures.

Numerous other studies of trabeculectomy in primary open angle glaucoma have been published, confirming the excellent results of this surgical technique, although no study has attained the 100% success rate without medication first reported by Cairns, (1968). The major studies are summarised in Table 1.1., while some of the papers which report large numbers of patients or long term studies will now be reviewed briefly.

Wilson, (1977) presented data on 309 eyes undergoing trabeculectomy, studied over a seven year period, of which 126 suffered from primary open angle glaucoma. Successful control of the IOP under 21 mmHg was achieved in 87% of this group of eyes with a follow-up period ranging from 1 to 7 years. A breakdown of the subgroups of the different types of glaucoma is not provided, however, after 1 year 28.7% of all eyes required miotics to maintain a satisfactory IOP, but the exact level is not stated in the paper.

Jerndal and Lundström, (1977) reported on 330 trabeculectomies followed for 0.5 to 3 years. Of these, 281 eyes were available for follow-up for 3 to 5.5 years (Jerndal and Lundström, 1980). A high proportion of these eyes (157) suffered from late congenital glaucoma, which is a diagnosis frequently made in Sweden. In England the majority of these patients would probably have been classified as suffering from primary open angle glaucoma (Hitchings RA - personal communication). The paper does not report the results separately for the various types of glaucoma comprising the 281 eyes studied. However the overall success rate for eyes with an IOP less than or equal to 21 mmHg was 57% with a single trabeculectomy. A further 35% required post-operative medical treatment to control the IOP. Reoperation was performed in 8% of cases.

Rollins and Drance, (1981) presented a study involving a five year follow-up of 48 eyes that underwent a trabeculectomy as a first fistulising procedure. A sub-group of 31 eyes from 22 patients were available with adequate visual field examinations done on the Goldmann or Oculus perimeter. At the end of the first year, 52% had achieved an IOP less than 21 mmHg without medication, while a further 29% required medication. The remaining 19% failed to achieve an IOP less than or equal to 20 mmHg. At the end of the fifth year, 52% had achieved a pressure less than 21 mmHg without medication, while a further 27%

required additional medication. The remaining 21% had a pressure above 20 mmHg. Thus the overall pressure reduction to 20 mmHg or less was 81% at the end of one year and 79% at the end of five years. It is interesting to note that 29% of the patients with adequate perimetry showed progression of their visual field defect by the end of 5 years, while 71% showed no further progression. No difference could be observed in postoperative IOP between patients whose fields continued to progress and those whose fields did not. Studies such as this question the entire rationale for the current treatment of glaucoma.

Mills, (1981) published data on 444 cases of trabeculectomy, performed over a five year period, and followed-up for upto 7 years. This study excels in the clarity of its data. Two hundred and twenty of these operations were performed on patients with primary open angle glaucoma. 73.4% of eyes attained an IOP of less than 21 mmHg without medication, while a further 13.5% achieved this pressure with additional medication. Thus 13.1% had uncontrolled pressure despite medications.

Lamping, Bellows, Hutchinson, et al., (1986) presented a long-term study on 252 eyes that had initial glaucoma filtration surgery, including 71 trabeculectomies. Follow-up ranged from 2 to 14 years with a mean of 5 years. Rigid criteria of success were applied including an IOP less than or equal to 19 mmHg with or without medication, no further field loss or disc damage, and no glaucomatous aetiology for a decrease in visual acuity. The study also compared the results of trabeculectomy with that of full thickness drainage procedures. Thus of 199 eyes with chronic open angle glaucoma undergoing surgery, 61 underwent a trabeculectomy. Of these 26.2% were considered failures.

In all the studies described above trabeculectomy was performed only after a failure of medical treatment to control the IOP. In many studies it is not stated what the maximum medical treatment was, or how long this was persisted with. Thus Watson and Grierson, (1981) performed surgery after a failure of pilocarpine 2% and/or timolol to control the IOP, while other authors allowed three topical medications plus systemic carbonic anhydrase inhibitors before resorting to surgery. It is only in the primary treatment studies of Migdal and Hitchings, (1986) and Jay and Murray, (1988), described in table 1.1.,

Table 1.1. Major studies of trabeculectomy in primary open angle glaucoma

Authors	Date	Number of trabeculectomies	Number of eyes with POAG	Criteria for success	Success on pressure criteria Without drugs %	With drugs %	Failure %	Follow-up
Cairns (UK)	1968	17	17	IOP<21mmHg	100	0	0	20-56 weeks
Cairns (UK)	1969	49	49	IOP<21mmHg	73.5	98	2	<6-29 mths.
Watson (UK)	1970	44	35	IOP<21mmHg	88.6	97.1	2.9	<30 mths
Cairns (UK)	1972	80	80	IOP<20mmHg	97.5	0	2.5	<1-4 years
Ridgeway (UK)	1972	86	27	IOP<22mmHg	Approx. 93	97.6	2.4	3-18 mths.
Thyer (UK)	1972	55	29	Not stated	86.2 (IOP<21mmHg)	Not stated	13.8	upto 2 yrs.
Ridgeway (UK)	1974	203	69	IOP<22mmHg	71	87	13	1-3.5 yrs.
Schwartz (USA)	1974	85	26	IOP<21mmHg	Not stated	73	27	Mean 10mths
Watson (UK)	1975	90	66	IOP<20mmHg	Approx.84	98.5	1.5	1-6 yrs.
McPherson (USA)	1977	67	37	IOP<21mmHg	67.5	89	11	6 months
Warden (NZ)	1977	28	28	IOP<22mmHg	86	100	0	3-4 yrs.
Wilson (UK)	1977	309	126	IOP<21mmHg	Not stated for POAG	87	13	1-7 yrs.
D'Ermo (Italy)	1979	90	48	IOP<22mmHg	64.6	77.1	22.9	1-5 years
Murray (UK)	1979	108	62	IOP<20mmHg	45	83	17	1.5-3 yrs.
Stewart (USA)	1979	74	54	IOP<21mmHg	Approx. 55	66.6	33.4	1 year
Jerndal (Sweden)	1980	281	13+157	IOP<22mmHg + no further field loss	57	92	8	3-5.5 yrs.

Table 1.1. (Continued) Major studies of trabeculectomy in primary open angle glaucoma

Authors	Date	Number of trabeculectomies	Number of eyes with POAG	Criteria for success	Success on pressure criteria Without drugs %	With drugs %	Failure %	Follow-up
Zaida (UK)	1980	66	63	IOP<22mmHg	81.8	92.5	7.6	<4 years
Mills (UK)	1981	444	220	IOP<21mmHg	73.4	86.9	13.1	1-7 years
Rollins (Canada)	1981	48	48	IOP<21mmHg	52	79	21	5 years
			31	no further field loss	-	71	29	5 years
Watson (UK)	1981	424	320	IOP<21mmHg no further field loss	83.1	97.1	2.9	>2 years
Lamping (USA)	1986	71	61	IOP<20mmHg + no further field loss no further disc damage and no glaucomatous aetiology for decreased visual acuity	37.6	73.8	26.2	2-14 years (mean 5yrs)
Migdal (UK)	1986	57	57	IOP<23mmHg + no further field loss	98.2	0	1.8	7mths-3yrs
Jay (UK)	1988	46	46	IOP<23mmHg Field and disc assessment	76	97.8	2.2	1-5 years

Notes:

1.)Failures include all cases where the IOP failed to attain the prescribed level for success after the first trabeculectomy. Cases that subsequently underwent further successful surgery are still classified as failures in this table.

2.)The studies of Migdal and Hitchings (1986) and Jay and Murray (1988) are primary treatment trials ie. trabeculectomy was the first antiglaucoma treatment applied. In all other studies surgery was employed after a failure of medical treatment to control the glaucoma.

3.)The study of Jerndal and Lundström (1980) includes data on 157 trabeculectomies performed on patients with late congenital glaucoma.

4.)The study of Rollins and Drance (1981) includes detailed field assessment for 31 eyes of 22 patients followed for 5 years.

that surgery was used as the initial treatment for glaucoma. These primary treatment studies have been prospective, while most other studies have been retrospective, which may compromise the accuracy of data collection. Thus in comparing the results of different studies it is impossible to know whether one is comparing patients with a similiar severity or duration of glaucoma. Nevertheless, it is apparent from the large number of studies of trabeculectomy performed on patients with primary open angle glaucoma that something like 85% of average Caucasian glaucoma patients will achieve pressure control under 21 mmHg after a trabeculectomy. However, a variable proportion, which may be upto half of these may require additional hypotensive medication for pressure control if followed for a sufficiently long period. Many studies tend to underplay the rate of failure of trabeculectomy, but it is quite clear that a significant minority of patients fail to attain benefit from surgery. While some of these patients may attain pressure control after further attempts at trabeculectomy, undoubtedly a proportion will remain with uncontrolled IOP despite maximal medication and some will have been made worse by surgery.

1.2.2. Chronic Narrow Angle Glaucoma

The success rate of trabeculectomy performed for chronic narrow angle glaucoma is very similiar to that in primary open angle glaucoma, although there are fewer large published series.

Ridgeway, (1974) studied 203 trabeculectomy operations of which 59 were performed for chronic angle closure glaucoma. Pressure was maintained at or below 21 mmHg by surgery alone in 84.7% of cases and in a total of 91.5% with the addition of pilocarpine drops. Thus surgery failed in 8.5% of cases.

Wilson, (1977) reported an 80% success rate (IOP less than 21 mmHg) in 112 eyes with chronic angle closure glaucoma undergoing trabeculectomy, with follow-up upto 7 years.

Watson and Grierson, (1981) reported on 37 trabeculectomies performed on 29 patients with chronic closed angle glaucoma. Five eyes (13.5%) required further trabeculectomy or other procedures to control the IOP.

In Mills' study of 444 trabeculectomies (1981), 66 eyes had chronic narrow angle glaucoma (so called "compound" glaucoma). 68.2% of eyes were controlled by trabeculectomy with an IOP below 21 mmHg, while a total of 86.4% achieved control with additional medications. Thus 13.6% of operations were failures.

In summary, trabeculectomy achieves a similiar success rate in chronic narrow angle glaucoma as in primary open angle glaucoma. Similiar caveats apply when comparing various studies due to variations in the study populations, criteria for success, and variable periods of follow-up.

1.2.3. Blacks

The success rate for Black patients undergoing trabeculectomy is far from clear. This is partially attributable to using the generic term "Black" to describe all patients with dark pigmentation without much regard to their original place of origin. This makes it very difficult to compare the success rate of trabeculectomy performed for example in American Blacks with that of East African Blacks. The socio-economic situation in some countries may make long term studies impractical, and poor availability of anti-glaucoma medications in some places may further complicate the issue. It is also generally acknowledged that the clinical course of glaucoma tends to be more severe in Black patients and that Blacks are more predisposed to acquire the condition and develop it at an earlier age than White people (Cowan, Worthen, Mason, et al., 1988). Since glaucoma surgery performed in young people is generally less successful than in older patients (see section 1.2.5.), studies involving Blacks are likely to reflect this. In addition, Blacks have been attributed with a tendency to develop scar tissue more easily than light skinned people (Welsh, 1970). Thus, the failure of aqueous humour drainage in filtration operations in Blacks is thought to be mainly due to the overgrowth of the external scleral fistula by fibrous tissue derived from the sclera and Tenon's capsule (Welsh, 1970).

As a basis for comparison of trabeculectomy with older filtering operations, the studies of Berson, Zauberman, Landau, et al., (1969) are worthy of note. These results are summarised in Table 1.2. together with the other major studies of glaucoma surgery performed in Black patients. Berson, et al. studied 180 filtering operations in 119

Tanzanian Blacks. It is not stated whether the patients received anti-glaucoma therapy prior to surgery. The surgical techniques were corneoscleral trephination, filtering iridectomy (Scheie's procedure) and iridenclesis. Successful surgery was defined as an IOP less than or equal to 18 mmHg as measured by Schiotz tonometry. The overall success rate for the three types of operations was 30%. Iridenclesis fared worst with a 4% success rate, with corneoscleral trephination faring best at 39%. The success rate for patients over the age of 46 was better than that for patients under this age; 43% vs. 24%. Closure of the filtering bleb occurred mainly in the first six postoperative months and was thought to be due to an increased scarring tendency in Blacks.

By contrast, trabeculectomy performed in a similiar East African population in Kenya, reported far better results. Adala and Klauss, (1984) studied 203 cases of trabeculectomy performed for primary open angle glaucoma in 185 patients, of which 128 eyes (118 patients) were available for follow-up ranging from 6 to 40 months. Surgery was performed as the primary intervention for these patients. Surgical success was defined as an IOP less than or equal to 21 mmHg. In cases in which a filtration bleb was present, the IOP was controlled by surgery alone in 95.9% of cases. Medication controlled the remaining 4.1%. However in cases in which a filtration bleb was absent, surgery alone was successful in only 25.8% of cases, while additional medication controlled a further 61.3% of cases. Overall, the total number of cases controlled by surgery alone was 78.9%, while medication controlled a further 17.9%. Thus, surgery failed in only 3.1% of cases available for follow-up. However, it must be borne in mind that only 128 of 203 eyes were available for follow-up, and the success of surgery may have been dramatically altered if the absent data were to hand. In circumstances where surgery is performed as a primary treatment it may also be inappropriate to regard cases requiring supplementary medication as successful, since topical anti-glaucoma medication is unobtainable in many parts of Africa.

Welsh, (1972) studied trabeculectomy in South African Blacks, performing either a conventional Cairns trabeculectomy or a filtration trabeculectomy with excision of the distal portion of the superficial scleral flap, and replacement of this flap without suturing. One hundred and twenty eight operations on 96 patients were prospectively

followed up. However, only 77 trabeculectomies (59 patients) were available for follow-up for a minimum period of 3 months. Success was defined as an IOP of less than 20 mmHg (Schiotz); partial success as a pressure between 20 and 30 mmHg while on miotics and acetazolamide, while failure was regarded as a pressure above 30 mmHg. Trabeculectomy alone had a success rate of 14% without bleb formation. Those trabeculectomies in which an unintentional fistula with bleb formation developed gave rise to an additional 14% success. Even with the relatively high criteria of partial success, 50% of trabeculectomies were considered a total failure. In the case of filtration trabeculectomies (essentially a full thickness drainage procedure), the success rate was much improved with a rate of 65%, and only 21% considered a total failure. This study can be criticised as it is unclear whether surgery was performed as a primary procedure in these patients, or after a failure of medical treatment. The length of follow-up is not clearly stipulated, and the low proportion of patients followed (61%) may cause bias in the results.

In a similiar South African population undergoing trabeculectomy, entirely different results were found by David, Freedman and Luntz, (1977). They studied 61 trabeculectomies on 49 eyes of 34 patients undergoing surgery after a failure of medical treatment to control the disease. The follow-up period ranged from 6 to 30 months, with 70.5% of patients being followed for more than one year. The operation was considered successful if IOP was 20 mmHg or less with or without additional medication for the total follow-up period. As a first procedure, trabeculectomy performed either by the Cairns technique or the Watson modification (see section 1.6.1.) was successful in 73.3% of cases. Thirteen of the initial trabeculectomy procedures were failures. Of these 12 had a second trabeculectomy of which 8 were successful. The authors therefore claim an overall success rate for surgery of 91.6%. However 25 of the original 34 patients (73.5%) required addition medical treatment, which has not been clearly stipulated in the paper, to control the IOP. This study could therefore be regarded as somewhat over optimistic in its stated success rate and the authors' conclusion that trabeculectomy is a "good procedure in open angle glaucoma in Black South Africans" should be disputed, particularly since more than one operation is often needed and 73.5% of patients require additional hypotensive medication to control the IOP.

Trabeculectomy was examined in a West African population by Chatterjee and Ansari, (1972) who studied 24 Ghanaian cases, operated on after a failure of medical treatment to control the IOP, and followed for 2 to 12 months. Three patients were lost to follow-up. All but one of the remainder ultimately attained IOP's less than 21 mmHg. The remaining patient had a pressure of 20 mmHg on 6% Pilocarpine drops. The presence of a drainage bleb was not associated with surgical success in this paper, unlike the study of Adala and Klauss, (1984), where a drainage bleb strongly correlated with surgical success.

In an enormous study of primary glaucoma surgery performed in Nigerian eyes, Keitzman, (1976) reported results comparable with many studies of Caucasian patients. Over a five year period, 419 sclerectomies and 612 trabeculectomies were undertaken, of which 196 eyes and 221 eyes respectively, were followed-up for at least 4 months. Control of the IOP below 21 mmHg was achieved in 74% of the trabeculectomies and 84% of the sclerectomies. Fewer serious complications were encountered in the trabeculectomy group, suggesting that this was a superior operation overall, in spite of a slightly lower success rate. Keitzman, (1976) also felt that the excellent results described in his series were partially attributable to excising Tenon's capsule, which was performed routinely in all cases. This is in contrast to the other studies described above, where Tenon's capsule was not excised and where good results were still obtainable.

Sandford-Smith, (1978) studied 123 filtering operations in Nigerians, and compared the effectiveness of traditional full thickness filtering procedures with trabeculectomies. Thus 82 trabeculectomies and 41 sclerectomies were available for investigation. It is not stated in the paper whether surgery was performed as a primary procedure. Seventy eight of the 123 operations were available for follow-up for 3 months or more. The operation was considered successful if the final applanation pressure was 20 mmHg or less, unsuccessful if the pressure was 35 mmHg or more, and indeterminate if the pressure was 25 to 30 mmHg. 64.7% of all trabeculectomy cases were considered successful; 21.6% were indeterminate; 13.7% were failures. This compared with a 60% rate of success for full thickness sclerectomies, with a 26% outright failure rate. As a complicating feature of this study, Onchocerciasis was evident in 21% of cases and in those who failed surgery it was present in 43%. In many other West African studies,

this disease is likely to complicate the response of eyes undergoing surgery, but little attention has been paid to it in the published reports. In addition the average age of the patients in Sandford-Smith's study was 38, while the average age of the patients on whom the operation failed was 32. This is considerably younger than most Caucasian patients undergoing trabeculectomy, making comparison of these results with other studies very difficult.

Even better results were reported in a similiar Nigerian population by Thommy and Bhar, (1979) who studied 139 trabeculectomies performed on 87 patients between 36 and 72 years. One hundred and eleven eyes were followed for a minimum of 6 months and a maximum of 19 months. The average duration of preoperative topical hypotensive medication was 1 month. A standard Watson type trabeculectomy (see section 1.6.1.) was performed and Tenon's capsule was not excised. The criterion of success was maintainance of the IOP below 20 mmHg. This was achieved in the period of observation in 106 eyes (95.4%). The visual status of patients was also maintained in status quo or a line or part of a line less than pre-op. In one eye with well maintained IOP no bleb was seen; a bleb was present in all other successful cases. This extremely high success rate was achieved without the use of post-operative steroids and using conventional surgery, indicating that, at least in this study, trabeculectomy functions at least as well in African patients with glaucoma as in White races.

These results have not been confirmed in a Black West Indian population (Shingleton, Distler and Baker, 1987) where full thickness posterior lip sclerectomies were compared with trabeculectomies. Eighty filtration procedures were performed on patients with primary open angle glaucoma, uncontrolled on maximal medical treatment. Thirty nine trabeculectomies and 41 full thickness procedures were evaluated. The average postoperative IOP of patients who underwent full thickness procedures was significantly less than patients who underwent trabeculectomy ($p < 0.0001$; 10 mmHg vs. 17 mmHg). In total 69.2% of the trabeculectomies attained a pressure less than 20 mmHg (although 20.5% required additional medication), while 95.1% of the full thickness group attained a similiar pressure (only 2.4% required additional medication to achieve this pressure), ($p < 0.001$). These authors therefore considered that the full thickness sclerectomy

should be considered as the surgical procedure of choice in Black patients.

There are a number of studies available of trabeculectomy in American Blacks. Freedman, Shen and Ahrens, (1976) performed trabeculectomies on 64 eyes of 51 Black patients in New York after medical treatment had failed to control the condition. In 60% of cases the superficial scleral flap was sutured back into position; in the remainder it was left unsutured. The average follow-up period was 19 months with success taken as an IOP consistently below 20 mmHg over the whole follow-up period. Surgery was successful in 82% of cases but 25% required medication to keep the IOP below 20 mmHg. Success did not depend on suturing the scleral flap. In 12.5% of cases the IOP was not controlled by further surgery or medication. A conjunctival drainage bleb was found in 85% of the controlled eyes. The recommendation of this study for trabeculectomy as the operation of choice in uncontrolled glaucoma in Black patients is in contrast to that of the former study, where a full thickness sclerectomy was advocated.

Similiar optimistic results were reported by Ferguson and Macdonald, (1977) on 50 trabeculectomies performed on 46 Black patients in Virginia, USA, after a failure of medical treatment to contain the glaucoma. The minimal follow-up time was two years. Patients received pre-operative systemic steroids - 40 mg Prednisolone by mouth on the day prior to surgery and on the day of surgery. Subconjunctival steroids were also administered at the end of surgery. Systemic prednisolone 40 mg per day was continued for upto 2 weeks post-operatively. Success was defined as an IOP equal to or less than 21 mmHg whether antiglaucoma medications were required or not. 84% of eyes were considered controlled with only 8% requiring additional medication.

The results of Miller and Barber, (1981) were less encouraging in a study of 122 trabeculectomies performed in 86 Black patients in Texas. All patients were operated on after a failure of medical treatment. The minimum follow-up period was 12 months with a range upto 8 years. Success was defined as an IOP below 21 mmHg. Patients over the age of 60 had the highest rate of success and no patient under 20 years was classified as a success. Almost all patients who had an IOP under 21 mmHg without medication had a filtering bleb and no patient with a

filtering bleb failed to have pressure under control. Thirty nine percent of eyes had the IOP successfully controlled without medication and a further 18% were successful with additional medication. Thus 43% were classified as failures. The primary cause of failure was scarring of the bleb or blocked filtration.

In conclusion, while excellent results from trabeculectomy can be attained in Black patients using unmodified surgery and without post-operative topical corticosteroids, most of the studies tend to show that the results are inferior to those seen in Whites. Thus, failure can be expected in anything from 5 to 50% of trabeculectomies performed on Blacks. This indicates that modifications of glaucoma surgery or methods of reducing scar formation are urgently required in some populations of Black patients in order to make surgery successful more frequently. In addition, further work is required on the role of preoperative topical medications which may have some bearing on the surgical outcome, and may in part explain the differences between the success rates found in Africa, where in general surgery was performed as a primary procedure, and the United States of America, where surgery was performed after a failure of medication to control the glaucoma.

1.2.4. The Japanese

As with glaucoma surgery performed in Blacks, the results of trabeculectomy in the Japanese are generally inferior to most of those reported in Caucasians.

The cumulative results of treating various types of glaucoma by trabeculectomy at 10 institutions in Japan were reported by Inaba, (1982). This study also elegantly addressed the problem of variable periods of follow-up post-operatively, which had not previously been achieved in most of the Western publications. Trabeculectomy was performed in 427 eyes, with follow-up periods from 3 months to 5 years. One hundred and fifty one of these eyes undergoing surgery had primary open angle glaucoma. All surgery was performed on Japanese patients. The data was subjected both to simple statistical analysis and to life table analysis so that the final failure probability was calculated on the assumption that the eyes with short follow-up are subject to failure at the same probability as the eyes followed to the end of the follow-up periods. Failure was defined as the mean IOP of

Table 1.2. Major studies of glaucoma drainage surgery in Black patients with primary open angle glaucoma

Authors	Date	Area	Type of surgery	No. of operations	Criteria for success	Success on pressure criteria Without drugs %	With drugs %	Failure %	Follow-up
Berson	1969	Tanzania	Sclero-corneal trephination	88	IOP<19mmHg	39	-	61	2 years
			Filtering iridectomy (Scheie)	68		26	-	74	
			Iridenclesis	24		4	-	96	
Chatterjee	1972	Ghana	Trabeculectomy	21	IOP<21mmHg	95	100	0	2-12 mths.
Welsh	1972	South Africa	Trabeculectomy	33	IOP<20mmHg	28	Not stated	50	>3 months
			Filtration trabeculectomy	26		65	Not stated	21	
Freedman	1976	USA	Trabeculectomy	64	IOP<20mmHg	57	82	17	Av. 19mths
Keitzman	1976	Nigeria	Sclerectomy	196	IOP<21mmHg	84	-	16	>4 months
			Trabeculectomy	221		74	-	26	
David	1977	South Africa	Trabeculectomy	49	IOP<21mmHg	Not stated	73.4	26.6	6-30 mths.
Ferguson	1977	USA	Trabeculectomy	50	IOP<22mmHg	76	84	16	24-33 mths
BenEzra	1978	Malawi	Trabeculectomy	100	IOP<21mmHg	79	-	21	2-12 mths.
Sandford-Smith	1978	Nigeria	Trabeculectomy	51	IOP<21mmHg	65	-	35	> 3 months
			Sclerectomy	27		60	-	40	
Thommy	1979	Nigeria	Trabeculectomy	111	IOP<20mmHg	95.4	-	4.6	6-19 mths.
Miller	1981	USA	Trabeculectomy	122	IOP<21mmHg	39	57	43	1-8 years
Adala	1984	Kenya	Trabeculectomy	128	IOP<22mmHg	78.9	96.8	3.1	6-40 mths

Table 1.2. (Continued) Major studies of glaucoma drainage surgery in Black patients with primary open angle glaucoma

Authors	Date	Area	Type of surgery	No. of operations	Criteria for success	Success on pressure criteria Without drugs %	With drugs %	Failure %	Follow-up
Shingleton	1987	W.Indies	Trabeculectomy	39	IOP<20mmHg	48.7	69.2	30.8	4-10 mths.
			Posterior lip Sclerectomy	41	IOP<20mmHg	92.6	95.1	4.9	5-10 mths.

Notes:

1.)All studies involve patients with primary open angle glaucoma except those of Freedman, et al., (1976) and Miller, et al., (1981), where the type of glaucoma has not been stated in the paper.

2.)In the paper of Welsh, (1972) failure refers to cases with an IOP greater than 30 mmHg.

3.)In the study of Adala and Klauss (1984) only 63% of cases included in the initial study were available for follow-up.

the observation period exceeding 20 mmHg despite the use of medications. Simple statistics showed an overall success rate of 75% in controlling the IOP below 21 mmHg, but this was only achieved in 25% of 427 eyes without the use of hypotensive medication. For the group with primary open angle glaucoma alone, the final failure probability was 0.42+/-0.07 (SE) which is higher than the rate of failure calculated from simple statistics, which was 22.5%. The failure probability calculated projects the results to the end of the 5 year period of follow-up which probably provides a more realistic assessment of the long term success of the procedure.

Of the 427 trabeculectomies in the study of Inaba, (1982) 147 were performed for primary angle closure glaucoma. However, it is not possible to determine from the paper whether these patients had acute or chronic angle closure. By conventional statistical analysis the success rate of surgery was 81%. However, life table analysis showed a cumulative failure probability of 0.48+/-0.14 (SE) projected to the end of 5 years. These amalgamated results from 10 centres therefore indicate that the success rate for surgery both with and without additional medication is far lower in the Japanese than in Caucasian populations. This study can however be criticised because composite results may blur differences between individual centres.

Nevertheless, similiar results were demonstrated by Shirato, Kitazawa and Mishima, (1982), who presented data from a single institution where trabeculectomy was performed 145 times on 113 eyes of 100 patients. Simple statistics showed control of the IOP below 21 mmHg in 70% of eyes, but this was achieved without medications in only 36% of eyes. When the 67 eyes of patients with primary open angle glaucoma were considered, the final failure probability was 0.43+/-0.09 after 2.5 years of follow-up. When these operations were followed up for 5 years, similiar trends were demonstrated by Yamashita, Eguchi, Yamamoto, et al., (1985).

The low rate of success of trabeculectomy in controlling the IOP in the Japanese, particularly without the use of hypotensive medications, has prompted the investigation of adjuvant treatments to reduce wound healing at the operation site. This has involved the use of 5-Fluorouracil (Kitazawa, Taniguchi, Nakano, et al., 1987), (See section 1.7.4.).

1.2.5. Young people

Amongst a number of factors, the prognosis for the control of the IOP following filtering surgery is generally believed to be related to the age of the patient. Thus, the younger the patient, the less the chance of success, and correspondingly, with age over about 40, the chance of success is thought to improve. While this is a generally held to be true, the are relatively few studies which have examined this subject in detail.

Schwartz and Anderson, (1974) investigated the effect of age on the success of trabeculectomy. Thirty nine adult phakic eyes undergoing trabeculectomy were assessed. In patients under 60, success (IOP < 21 mmHg) was achieved in 9 cases out of 15 (60%) while in those over 60, 20 of 24 operations were successful (83.3%). These results were statistically significant ($p < 0.05$). The authors believed that the poor result in the younger patients was due to their better healing capacity, which results in scarring down of the scleral and conjunctival flaps.

Beauchamp and Parks, (1979) evaluated trabeculectomy in advanced paediatric glaucoma. Twenty-six trabeculectomies were performed on 25 eyes in 16 patients. Only 22 of the 25 eyes were followed-up sufficiently to be included in the final statistical analysis. Eleven of the eyes suffered from primary congenital glaucoma, while aniridia (5 eyes) was the second most common clinical pathology. Successful surgery was defined as an IOP less than or equal to 24 mmHg. The patients ages ranged from 4 months to 19 years at the time of surgery. The follow-up ranged from 2 to 39 months with a mean of 18 months. Fifty percent of the trabeculectomies were successful and the mean post-operative visual acuity was 14/200, with the best visual result only 20/200. Complications occurred in 20% of operations. The authors felt that the barriers to success in this group of patients could be ascribed to the complexity of the associated pathology, anatomical and physiological factors, the management of the patients, and the previous surgery that many of them had undergone. The more rapid healing processes in children than in adults were partially responsible for increased scarring at the operation site, and thus surgical failure (Beauchamp and Parkes, 1979).

Stewart, et al., (1979) arbitrarily divided 74 patients undergoing trabeculectomy into two groups: one less than 40 and the other greater than 40 years. Only 9 trabeculectomies were performed on patients less than 40 and of these eyes IOP was less than or equal to 20 mmHg in 66.6% at six months and in only 33.3% at 1 year after surgery. In the remaining 65 trabeculectomies performed on patients over 40 years of age the success rate was 76.9% at 6 months; success at one year is not quoted in the paper. It appears that young patients did not do well in Stewart's study, although the results have not been evaluated statistically.

Shalash, el Hoshy and el Aziz Ali, (1981) performed 20 trabeculectomies on 14 patients suffering from congenital glaucoma, with an age range between 3 months and 6 years. Successful surgery was defined as an IOP below 21 mmHg. However, follow-up was only for 6 to 10 weeks. Success was obtained in 80% of cases using these criteria. Histology of the excised specimens failed to demonstrate Schlemm's canal in most cases, and no relation was found between the nature of the excised tissues and the lowering effect of the operation on the IOP. Although these results are excellent they must be regarded with some scepticism due to the short duration of follow-up.

A far more accurate idea of the effect of age on the success of trabeculectomy is provided by the study of 427 Japanese eyes by Inaba, (1982). The follow-up in this study ranges from 3 months to 5 years, but 344 of these eyes were followed for longer than one year. For congenital glaucoma (67 eyes), orthodox statistical analysis showed a 58% success rate, ie. IOP less than 21 mmHg with or without medication. Life table analysis for a projected 5 year period indicated an overall final failure probability of 0.65+/-0.08 (SE). However, when the cases were further subdivided according to the number of operations it was apparent that the final failure probability was 0.55+/-0.10 after the first trabeculectomy and 0.80+/-0.03 after multiple interventions. Among the total of 351 eyes which underwent single trabeculectomy, there were 206 eyes younger and 145 eyes older than 60 years of age. The final failure probability was 0.35+/-0.10 in the older age group and 0.58+/-0.10 in the younger, which was significantly different at the 95% confidence level. This result may have been achieved due to a bias due to the inclusion of a considerable number of cases of congenital glaucoma in the younger age

group. Therefore similiar analyses were carried out for the two age groups with primary open angle glaucoma. The final failure probability was 0.34+/-0.11 in the older age group and 0.45+/-0.10 in the younger age group. Similiar results were found when the final failure probability was calculated for the patients with primary angle closure glaucoma. However, these differences were not statistically significant due to the small number of cases studied. Nevertheless the same trend towards a high probability of failure is found in both the primary open angle and the primary angle closure glaucomas in young patients.

Inaba, (1982) felt that in view of the poor results of trabeculectomy in Japanese patients with congenital glaucoma, it was not the operation of choice and should be reserved for cases where goniotomy is impossible to perform or had previously failed. The higher failure rate of trabeculectomy in younger patients could be attributed to a thicker Tenon's capsule and subconjunctival tissues providing a greater capacity for scar formation. Although definite proof was lacking, Inaba proposed that this may explain, at least in part, some of the age difference in surgical results.

Gressel, Heuer and Parrish, (1984) performed 117 trabeculectomies on 98 American patients under the age of 50 years. A complete surgical success was defined as an IOP less than or equal to 21 mmHg without medication; a qualified success as IOP 22 to 25 mmHg without medication or an IOP less than or equal to 21 mmHg with medication; a qualified failure as IOP greater than 25 mmHg without medication, while a complete failure was defined as further glaucoma surgery (or its recommendation) or loss of light perception. Only trabeculectomies with follow-up of at least one year were included in the study, this comprising 106 eyes (91%). The success rate (the sum of complete and qualified successes) in primary glaucomas (29 of 39 cases, 74%) was considerably higher than in secondary glaucomas (24 of 50 cases, 48%) or in developmental glaucomas (6 of 17 cases, 35%). Trabeculectomies for secondary glaucomas were significantly more successful in eyes that had not undergone previous surgery. Forty five trabeculectomies were performed on patients aged 10 to 29 years of which 17 (38%) were successful. Sixty six trabeculectomies were performed on patients between 30 and 49 years; of these 43 (65%) were successful. The authors concluded that age as an isolated factor may have its greatest

influence on surgical outcome in patients under the age of 30 years. Only 1 of 11 trabeculectomies performed for neovascular glaucoma was successful. Overall the success rate for the study was 51% which is much lower than the commonly cited success rates for primary glaucomas in older patients.

Gressel, et al., (1984) felt that youth could be a determinant of surgical outcome because of anatomic factors such as a greater thickness of Tenon's capsule which may impede filtration. Since in most cases the failure of filtering operations is related to wound healing and wounds seem to heal more quickly in youth than later on in life (Walter and Israel, 1979), this could be another factor predisposing to surgical failure. The type of glaucoma that occurs in young people is also an important determinant of surgical outcome, and many patients are more predisposed to complications because of the nature of their disease.

Further evidence of the effect of age on the outcome of glaucoma surgery is provided by the study of Lamping, et al., (1986), in which 252 eyes were evaluated. Trabeculectomy was performed in 71 cases with the remainder undergoing posterior lip sclerectomy or trephination. Successful surgery was defined as an IOP less than or equal to 19 mmHg with or without antiglaucoma medication, no evidence of progressive disc damage or no further visual field loss, and no glaucomatous aetiology for a decrease in visual acuity. With these stringent criteria, the highest failure rate of 26% was seen in the age group 0 to 39 years; 15% failed in the 40 to 59 years group; while in those over 60 years there was a 14% failure rate.

In conclusion, the general trend from the published studies suggests that a.)trabeculectomies have a poor success rate in congenital glaucomas, and b.)in acquired glaucomas, surgical success rates are lower the younger the age of the patients.

1.2.6. Aphakic Glaucoma

There are many mechanisms of glaucoma in aphakia, and the term "aphakic glaucoma" which implies a single aetiology is misleading. Bellows and Johnstone, (1983) have separated aphakic glaucoma into early and late forms which can be further subdivided as follows:

A. Early (1-4 weeks after cataract surgery)
   1. Acute transient elevation due to Healon, a tight wound or alpha-chymotrypsin.
   2. Acute angle closure due to pupillary block or malignant glaucoma.
   3. Inflammatory
   4. Debris - blood or lens material

B. Late (5 weeks or longer)
   1. Sustained chronic open angle
   2. Chronic angle closure
   3. Inflammatory
   4. Neovascular
   5. Epithelial downgrowth
   6. Steroid induced

Identification of the cause of glaucoma will naturally identify the appropriate treatment. While medical treatment may control the IOP in some patients with aphakic glaucoma, treatment not infrequently fails and many patients require surgical intervention. The surgical management of these patients with uncontrolled intraocular pressure in association with aphakia remains one of the most difficult problems encountered in patients with glaucoma. The problem is compounded by the large number of cataract extractions performed in an increasingly large elderly population, and in the increased frequency of intraocular lens implantation in eyes with glaucoma.

The work of Herschler, Litinsky, Shaffer, et al., (1978) is of particular interest in aphakic glaucoma. Thirty four patients with aphakic glaucoma of unspecified aetiology, who had very severe disease, uncontrolled on medical treatment, and having previously undergone multiple glaucoma procedures that had failed, were included in the study. Thirty one patients were followed for a minimum of six months. The average age of the patients in the series was 47 and ranged from 5 to 95. Approximately two thirds of the patients had undergone intracapsular cataract extractions and one third extracapsular extractions. Of the latter group, none had intact posterior capsules at the time of surgery. All patients underwent trabeculectomy. Only two of the patients were controlled following surgery without medication, and two patients were controlled with mild

miotics alone. All other patients who were considered controlled in the study required maximal medical treatment. At 6 months 32% of the patients had pressures under 21 mmHg, and 55% had pressures under 25 mmHg. Similiar percentages were seen at one year. These results were obviously inferior to most other studies in phakic patients. However, the authors felt that trabeculectomy achieved a much higher success rate with fewer postoperative complications than cyclodialysis (one of the treatments used prior to the introduction of trabeculectomy) in the treatment of glaucoma in aphakic patients (Herschler, et al., 1978). Thus, although the failure rate for trabeculectomy in aphakic glaucoma was high, Herschler, et al., felt that this was the safest available technique in their hands.

Herschler, (1981) based on his studies in tissue culture, hypothesized an altered state of the aqueous humour in patients with aphakia. Changes in the aqueous could occur due to greater mixing of the aqueous and vitreous due to the absence of the crystalline lens. A stimulatory substance might be present in the vitreous and overcome the normal inhibitory properties present in the aqueous humour. Alternatively the crystalline lens might play an active role and add an inhibitor to the aqueous or detoxify a stimulator already present. In order to test these hypotheses, Herschler performed vitrectomies on aphakic patients undergoing filtration surgery with medically uncontrolled glaucoma. A total of 41 patients underwent vitrectomy-filtration surgery, performed on 47 occasions. Nineteen of these filtration operations were carried out on patients in whom vitrectomy had been performed previously. Five of the 41 patients undergoing filtration surgery required a revision of the operation and one patient had two revisions performed. Thus 47 operations were done and patients received either a trabeculectomy or a posterior lip sclerectomy, but it is not clear from the paper what the proportions of the operations were. Thirteen patients had neovascular glaucoma, 16 angle closure glaucoma and 12 suffered from chronic open angle glaucoma. Success was defined as an IOP less than or equal to 21 mmHg at the 6 month postoperative visit. The success rate for surgery was affected by the type of glaucoma. Thus 53% of procedures performed on aphakic eyes with neovascular glaucoma were successful, 72% were successful in eyes with angle closure glaucoma, while 83% were successful in aphakic eyes with primary open angle glaucoma. Of the 32 patients successfully controlled, 24 showed evidence of a filtration

bleb. Two patients developed per-operative retinal detachments. These results were very different from Herschler, et al., (1978) where no vitrectomy was performed. However, the complications of vitreous surgery provide a reason for caution. Herschler felt that removing 95% of the vitreous at surgery withdraws the majority of the biochemical reservoir that may be releasing a stimulant to fibroblast growth into the aqueous humour, although this theory remains to be confirmed by other authors. Nevertheless, the discovery that total vitrectomy improves bleb formation is important as it indicates that the biology of the aqueous humour may be a key to improving surgical treatment for glaucoma.

Bellows and Johnstone, (1983) studied trabeculectomy in 21 eyes of 21 patients with aphakic glaucoma, all of whom had uncontrolled intraocular pressure and further threat to the pre-existing optic nerve damage and field loss while on maximum tolerated medical therapy. Three patients had chronic open angle glaucoma, two had chronic uveitis, one had neovascular glaucoma, and 18 had surgically induced peripheral anterior synechiae. Follow-up was from 5 to 72 months, with an average of 26 months. The average preoperative intraocular pressure was 38 mmHg (Range 28-64) and the postoperative pressure was 21 mmHg (Range 5-38). Sixty two percent of the eyes maintained a pressure of 21 mmHg or below for the period of the study. There was no correlation clinically between the presence of a filtering bleb and adequate pressure control.

Heuer, Gressel, Parrish, et al., (1984) reviewed 127 trabeculectomies performed in aphakic eyes. Eighty two of these cases were suitable for follow-up in the study, of which 78% had angle closure glaucoma (predominantly secondary), 15% had primary open angle glaucoma, 4% had developmental glaucoma, while 4% could not be diagnostically classified. Surgery was completely successful (IOP less than or equal to 21 mmHg) in 16% of cases, and partially successful in a further 23% (IOP less than 21 mmHg with medication or less than or equal to 25 mmHg without medication). Thus 61% of cases were failures. The time interval until unequivocal failure was documented was 3 months or less in 88% and 6 months or less in 92%. As a group, the patients who failed surgery were significantly ($p < 0.002$) younger than the patients in the successful categories, with only 1 of 20 patients younger than 50 years categorised as a success. The authors felt that

the effect of age may be related to the fact that younger patients generally show more vigorous wound healing, or to the fact that younger patients with aphakia have a greater prevalence of secondary glaucomas. Since other trabeculectomies generally fail due to scarring at the episcleral and subconjunctival interface, the authors concluded that, in spite of it being unclear why trabeculectomies fail frequently in aphakic eyes, agents that inhibit wound healing might improve the success rate in this type of filtering surgery.

It is apparent that the surgical management of patients with aphakic glaucoma is far from satisfactory. This has led to the use in some centres of adjuvant anti-wound healing agents, particularly 5-Fluorouracil, to attempt to improve the surgical success rate in this unfortunate group of patients (See section 1.7.4.).

1.2.7. Neovascular glaucoma

Neovascular glaucoma is a serious disease with a relentless course and usually with a poor prognosis (Madsen, 1973). It is most commonly associated with proliferative diabetic retinopathy and ischaemic central retinal vein occlusion. Conventional filtering surgery has been minimally successful in rubeotic eyes because of the tendency to form scar tissue at the operation site (Katz and Spaeth, 1987). For example, in Mills' study (1981) of 444 trabeculectomies, 8 of the operations were performed on rubeotic eyes. Of these, intraocular pressure was controlled in only one case by a conventional trabeculectomy.

The poor success rate of filtration surgery for neovascular glaucoma has led to the adoption of surgical modifications, particularly the use of pre-operative panretinal ablation (Katz and Spaeth, 1987). Allen, Bellows, Hutchinson, et al., (1982) reported a study on 26 filtration operations in 24 eyes with neovascular glaucoma. The follow-up ranged from 6 months to 7 years with a mean of 22.8 months. In this study neovascular glaucoma was defined as increased IOP associated with neovascularization of the entire filtration meshwork with 360^{o} of peripheral anterior synechiae and all or most of the angle completely closed. Seventeen of the eyes underwent preoperative panretinal photocoagulation in order to decrease fibrovascular activity, and surgery was delayed 2 to 3 weeks in order to maximize this response. In all cases vigorous treatment with steroids and

atropine, as well as pressure lowering agents was applied. Thirteen of the drainage procedures were trephinations or posterior lip sclerostomies and were regarded as full thickness procedures; 13 of the operations were trabeculectomies, while one was a pars plana procedure performed 4 mm posterior to the limbus. Fourteen of 24 eyes (58%) attained an IOP less than 21 mmHg. Two further eyes attained pressures in the mid-20s but did not require medical therapy as it was felt that the optic nerves withstood the elevated pressure adequately. The overall success rate was thus 67%. Of the 16 eyes that had successful outcomes with filtration surgery alone, seven had trabeculectomies and eight had full thickness procedures. Conjunctival scarring in the 33% of unsuccessful cases was thought to be the main cause of failure of these operations.

Dissatisfaction with the results of conventional drainage surgery in recalcitrant glaucomas such as neovascular glaucoma led to the development of implantable tubes to overcome the problems of conjunctival scarring (Molteno, Straughan and Ancker, 1976a). A number of appliances similiar to the Molteno tube have been designed. The aim of these devices is to shunt aqueous humour via a tube from the anterior chamber, to a large subconjunctival sac, demarcated by an implanted plate or strap. Although scar tissue, which may inhibit the passage of aqueous humour, is formed around the plate or strap, the surface area of the wall of the sac is sufficiently large to allow adequate amounts of aqueous humour resorption to maintain a low intraocular pressure. Schocket, Lakhanpal and Richards, (1982) implanted an anterior chamber tube shunt to an encircling no.20 silicone band in 19 eyes with neovascular glaucoma. Eyes with severe rubeosis iridis received panretinal cryotherapy to reduce the risk of haemorrhage. After surgery 18 of the 19 eyes (95%) followed for periods ranging from 5 to 26 months (mean: 59 weeks) had intraocular pressures less than 21 mmHg, and one eye had partial control with an IOP of 26 mmHg. Side effects were relatively minimal. However, subsequent studies with other types of implants have demonstrated that there can be considerable problems with their use (Sherwood, Joseph and Hitchings, 1987; Hitchings, Joseph, Sherwood, et al., 1987).

Other surgical strategies for the management of neovascular glaucoma involve goniophotocoagulation, panretinal cryotherapy, and cyclodestructive procedures using cryotherapy, photocoagulation or

partial cyclectomy (Katz and Spaeth, 1987). The number of procedures available is testimony to the fact that both the medical and the surgical management of neovascular glaucoma is far from satisfactory. If trabeculectomy alone is employed it will fail in at least a third of cases.

1.2.8. Reoperations

In most reported studies the fate of patients who fail to attain a reduction of IOP by trabeculectomy and maximal medical therapy is not described. If these patients are not to continue to suffer the deleterious effects of the elevated IOP, then a further attempt at drainage surgery is indicated. There are few studies that report in large numbers on reoperation performed after an initial failed trabeculectomy, although many papers mention these cases superficially. For example, 27 eyes out of 435 (6.2%) in the study of Mills, (1981) underwent further surgery but their fate is not described. A similiar criticism can be made of the study of 309 eyes undergoing trabeculectomy (Wilson, 1977), in which 8 required further surgery, but no mention is made of the result. The most informative papers are in the Japanese literature where detailed evaluation of cases undergoing reoperation has been undertaken.

Inaba, (1982) in reporting on 427 eyes undergoing trabeculectomy, included 76 eyes undergoing repeated surgery. Of these, 56 eyes underwent surgery twice and 20 eyes, more than twice. The results of repeated surgery were less successful than the first surgery, and the final failure probability after 5 years was 0.49+/-0.09 after the first surgery and 0.64+/-0.09 after multiple interventions.

Shirato, et al., (1982) carried out trabeculectomy 145 times on 113 eyes of 100 patients. Of the total number of operations 88 were performed for the first time, 29 for the second, and 28 were for the third and subsequent time. In eyes with POAG, the life-table analyses of the trabeculectomy results were carried out in 46 eyes operated upon once, in 19 eyes operated on twice, and in 24 eyes operated on 3 or more times. Most of the surgical failures occurred within one year. The final failure probability after a first trabeculectomy was 0.43+/-0.09 (SD) after 2.5 years follow-up; the failure probability of the second surgery was 0.63+/-0.18 after 2 years follow-up, but after 3 or more repeated operations the failure probability reached

0.90+/-0.07 after one year. The authors felt that their data justified repeating trabeculectomy on the same eye when the first surgery failed. However, the extremely high failure rate of 90% in third and subsequent surgery, suggested that trabeculectomy was not recommended in these circumstances.

Although the non-Japanese literature does not contain adequate data on the success rate of repeated trabeculectomies, the small number of cases included in other studies generally indicate a reduced success rate for repeated surgery (Skuta and Parrish, 1987; Simmons, 1986).

1.3. REASONS FOR THE SUCCESS OF GLAUCOMA SURGERY

1.3.1. Histological studies

There are few histological studies of eyes with functioning glaucoma drainage operations. This is due to the difficulty of obtaining satisfactory material, since if the operation works, further intervention is unnecessary and consequently the functioning bleb and sclerostomy cannot be examined pathologically. Instead the supply of suitable material is largely determined by chance and dependant upon eyes removed soon after death and satisfactorily fixed from people who happen to have had previously successful glaucoma surgery and adequately documented clinical histories. In some instances material has been derived from unusually large functioning blebs which have had to undergo surgical modification.

In the days before trabeculectomy, Teng, Chi, and Katzin, (1959) studied the histology and mechanism of six successful filtering operations, two after iridenclesis, two after trephining operations, one after sclerectomy with iris inclusion, and one after combined cataract extraction and sclerectomy with iris inclusion. They concluded that there were three routes of aqueous drainage which could be created by filtering operations: (1) the transconjunctival route; (2) the route through areas of perivascular degeneration; and (3) direct new recanalization caused by proliferating capillaries meeting the trabecular region. There was a definite loss of collagen fibres in the area of the drainage blebs. Fundamental to the creation of these drainage pathways was the effect of aqueous humour which they felt not only had a degenerating effect on mature collagen, but also worked to prevent fibroblasts from forming collagen (Teng, et al., 1959).

Addicks, Quigley, Green, et al., (1983) published the first report of an electron microscopic study of limbal tissues after filtering surgery, in which they were able to compare the clinical and histopathological appearances of drainage blebs. Specimens were obtained from eye bank eyes or by surgical excision of a portion of a bleb. These were either non-functioning, leaked or required surgical revision because they had dissected onto the cornea. Four failed and six functioning blebs were examined. However, it was not stated in the paper what type of drainage surgery had been performed on each eye examined. Beneath the epithelium there was an area of collagenous connective tissue in both types of bleb. However in the four failed

blebs examined this connective tissue was denser and thicker than normal throughout the entire bleb wall. Ultra-structurally this zone contained large amounts of collagen in which there were fibroblasts and blood vessels. In the four failed bleb specimens there was no cellular lining on the inner (anterior chamber) side of the bleb wall, nor was there an accumulation of any abnormal extracellular material at this site. Functioning blebs had looser, thinner subepithelial connective tissue. Electron microscopy demonstrated that this tissue contained scattered collagen fibrils with channel-like spaces throughout the stroma. Collagen fibrils in both functioning and failed blebs had a normal appearance and banding pattern. No specific signs of collagen fibril abnormalities were detected. Thus the electron microscopic observations by Addicks, et al., (1983) did not confirm the light microscopic studies of Teng, et al., (1959), in which degenerated, hydrated collagen was found to be important in the formation of a functioning filtration bleb. Addicks, et al. felt that the looser subepithelial connective tissue of functioning blebs would allow greater fluid movement through the tissue to its ultimate site of exit. This may be into blood vessels present in this tissue, or directly through the epithelium into the tears.

Addicks, et al., (1983) noticed clinically that functioning blebs contain small cystic spaces. The microcysts correspond histologically to the spaces in the subepithelial connective tissue, and are good evidence of the functioning of a filtering bleb. Addicks' study points to the importance of the fibroblast and its production of new extracellular matrix components in the response to filtering surgery, since eyes with less vigorous fibroblast proliferation or less extracellular matrix production would be more likely to achieve the appearance of functioning bleb tissue.

1.3.2. Timing of surgery

The timing of glaucoma surgery may have a crucial effect on its outcome. However, current knowledge on this aspect of surgery is extremely poor.

Most studies do not document the time elapsed from the diagnosis of glaucoma until its surgical treatment. The severity of the glaucoma is also generally not mentioned. Although it is at present impossible to determine, the duration of the disease may or may not be related to

its severity at diagnosis. It is well documented that the worse the initial condition of the eye, the lower the intra-ocular pressure appears to need to be to prevent further visual loss (Grant and Bunke, 1982). Drainage surgery may extinguish the last vestige of the central visual field in eyes with end-stage glaucoma (Aggarwal and Hendeles, 1986), but it is as yet unknown whether the severity of the glaucoma and its duration affects the healing response of the eye to drainage surgery.

Wright, (1980) has described the complications of topical drug therapy for glaucoma. All drops can cause conjunctival irritation and hyperaemia which may be accepted by the patient as an inevitable side effect of the therapy. Adrenaline causes a reactive hyperaemia in all patients in whom it is used. Sub-clinical uveitis accounting for the frequent finding of posterior synechiae is common in patients who have been on long term treatment with miotics. Guanethidine 5% drops have now been withdrawn because of the side effects noted on the conjunctiva which include fibrosis, fornix shallowing and epithelial metaplasia. In addition, nearly all eye drops contain preservatives, which are well known to cause allergic reactions in the conjunctiva (Wright, 1980).

Sherwood, Grierson, Millar, et al., (1989) have evaluated the effect of long-term topical antiglaucoma medication on the conjunctiva of patients undergoing trabeculectomy after a failure of medical treatment to control the IOP, and compared this with a group of patients undergoing trabeculectomy as a primary treatment for glaucoma. A statistically significant increase in the number of macrophages, lymphocytes, mast cells and fibroblasts in the conjunctiva and Tenon's capsule and a significant decrease in the number of epithelial goblet cells were seen in the group that received long-term medical therapy. Additional unpublished work from these authors indicated a significantly lower failure rate in those patients undergoing primary surgery (2%), compared to the topical treatment group (21%, mean follow-up 28 months). Sherwood, et al., suggested that since topical medical therapy increases the number of tissue inflammatory cells, the risk of filtration surgery failure may be worsened by its use.

The relatively minimal anti-glaucoma medications of pilocarpine 2% tid or timolol 0.5% bd or a combination of these, employed by Watson and Grierson, (1981) before proceeding to trabeculectomy may partially explain their overall success rate for surgery of 98% (including patients controlled with additional medications). The 98% success rate for trabeculectomy, without the need for additional medical treatment, in the trial of Migdal and Hitchings, (1986) may also be associated with the fact that all patients underwent primary surgery without previously having been subjected to hypotensive medications. Against this however, is the fact that Jay and Murray, (1988) found that delayed trabeculectomy after failed medical treatment, was just as effective as primary surgery in reducing intraocular pressure.

In summary, the timing of trabeculectomy may be important in determining the surgical outcome, but as yet the paucity of data does not allow a valid conclusion to be drawn.

1.4. REASONS FOR THE FAILURE OF GLAUCOMA SURGERY

The failure of filtering operations may be divided into intra-ocular, scleral, and extra-ocular causes (Maumenee, 1960). Intra-ocular complications are usually due to faulty surgical technique and include lens, iris, vitreous and ciliary body prolapse into the scleral opening. The main factor which prevents filtration at the level of the sclera is an inadequate opening into the anterior chamber. This may be due to leaving part of the sclera or Descemet's membrane remnants in the sclerostomy. Extra-ocular factors are, however, thought to account for the majority of failures after glaucoma filtration surgery. Maumenee, (1960) ascribed this to a condensation or compression of Tenon's capsule, rather than to fibroblastic proliferation. However, his evidence was based on clinical observation, both at the slit-lamp and when reoperating on patients with functioning and non-functioning blebs. He did not perform histological studies to confirm his clinical impression.

Hitchings and Grierson, (1983) performed a clinico-pathological correlation of eyes with failed fistulising surgery. They pointed out that clinically there may be little to differentiate functioning from non-functioning blebs, and that there may be little if any change in the appearance of a filtering bleb if it should fail years after the operation. On clinical grounds the authors felt that it was possible to separate eyes with failed filtration surgery into two groups - early (in the first few months following surgery) and late (those eyes which fail years following drainage surgery). Accordingly they studied histological and ultrastructural features of the conjunctiva and episclera in these two groups of patients. Specimens were obtained from patients who were undergoing reoperation who had previously sustained failed glaucoma drainage surgery and whose IOP's were uncontrolled despite hypotensive medications. Specimens of conjunctiva and episclera were removed from the region of the original bleb. Twenty two filtration blebs were examined, 9 from eyes undergoing reoperation within the first 6 months, and 13 from eyes undergoing surgery more than one year from the original glaucoma surgery.

Early onset failures (less than 6 months post surgery) were characterized by a marked inflammatory response in the subconjunctival tissues and in Tenon's capsule. The inflammatory infiltrate consisted

mainly of macrophages and lymphocytes. Activated spindle shaped fibroblasts were plentiful. Some of the fibroblasts had well developed rough endoplasmic reticulum in their cytoplasm, while a variable but significant proportion had their cytoplasm packed with microfilaments. The tissue was thickened by the deposition of new collagen, consisting mainly of thin fibrils, but there was a normal deposition of extracellular materials. Normal blood vessels and lymphatics were evident.

In late onset failures (over 9 months post surgery) the filtering bleb was lined with a thick layer of fibrin. The lining was predominantly acellular with only a few fibroblasts present. The bleb was encapsulated, in part, by hypocellular fibrous tissue, which consisted of irregular bundles of collagen, ground substance and variable amounts of elastic fibres. The capsule separated the bleb from what was relatively normal conjunctiva and Tenon's capsule. Subconjunctival vessels and lymphatics were normal and very few inflammatory cells were seen. Thus late failure appeared to be due in part to the bleb wall or scar preventing the aqueous from reaching the relatively normal conjunctiva beyond, where it could, if only it reached there, be absorbed.

The healing of glaucoma filtration operations has been studied in experimental animals. Seetner and Morin, (1979) performed trabeculectomies on 28 albino rabbits, 10 of which received subconjunctival methylprednisolone at the end of surgery. All but one animal developed functionally patent filtration, proven by flourescein injection, but by day 14, vigorous fibroblastic proliferation and collagen production from the subconjunctival tissue, uvea and to a lesser extent, the sclera combined to close off the fistula. The steroid treated eyes showed slightly delayed wound healing, but by day 30 there was little difference between treated and untreated groups.

Similiar but far more extensive studies were reported by Miller, (1988) who performed a modified thermal sclerostomy on albino rabbit eyes. Light and electron microscopy demonstrated a massive inflammatory response at the operation site with the production of granulation tissue. Bulk filling of the filtration bleb was produced by fibroblast proliferation and fibroblast migration predominantly from Tenon's capsule. Synthesising fibroblasts produced a loose

intercellular matrix. The bleb collapsed due to contraction of the scar by myofibroblasts (see section 1.8.3.). The fibrous scar tissue subsequently became organised and little different to the original sclera.

Peiffer, Lipper, Merrit, et al., (1981) investigated the healing of filtering wounds in rabbits, dogs and cats, although the number of animals studied was very small. They found that in sclerectomy wounds reparative tissue appeared to stream into the wounds from the episcleral tissue, while in trabeculectomy repair arose from adjacent corneal and limbal tissue. Ultrastructural examination of the healing wounds showed large numbers of fibroblasts, many of which had the features of myofibroblasts (see section 1.8.3.). The transient nature of this cell type was demonstrated in further studies of filtration surgery in cats by Reddick, Merrit, Ross, et al., (1985) who showed that while myofibroblasts constituted the predominant cell type in the reparative process of a filtration operation, appearing as early as the seventh post-operative day, they were virtually absent after day 14. Their histological data obtained from healthy cats suggested that contraction of the wound sites in the early post-operative period may cause scarring and thus failure of standard filtering operations.

Primate studies are likely to be more directly relevant to human glaucoma surgery. Desjardins, Parrish, Folberg, et al., (1986) reported on posterior lip sclerectomies performed in both eyes of 13 and in one eye of 3 normal owl monkeys. Tenonectomies were routinely performed. All drainage blebs failed by 10 to 14 days. Fibroblast migration was evident by the sixth post-operative day. Presumably the migrating fibroblasts originated from the adjacent episclera. During postoperative days 8 to 14, fibroblasts lined the walls of the fistula and proliferated to occlude the channel. Considerable contraction of the conjunctival wound with anterior migration towards the limbus was observed.

Cellular proliferation after glaucoma filtration surgery was studied in detail by Jampel, McGuigan, Dunkelberger, et al., (1988) who performed posterior lip sclerectomies on 4 experimentally glaucomatous cynomolgus monkeys. Light microscopic autoradiography demonstrated nuclear incorporation of tritiated thymidine as early as 24 hours following surgery. Peak incorporation occurred 5 days postoperatively

and had returned to baseline levels by day 11. This coincided with the clinical course of the failure of the filtration surgery. Transmission electron microscopy was used to characterize the proliferating cells identified by autoradiography. By two days after surgery, proliferating fibroblasts were identified at the margins of the sclerostomy. The major source of the fibroblasts that eventually occluded the wound was thought to be the episclera.

In summary, while the time course to the failure of filtering wounds in experimental animal eyes is far more rapid than in human eyes, the sequence of events seems similiar. The fibrosis produced by fibroblast migration, proliferation, synthesis of extracellular matrix, and subsequent contraction, plays a prominent role in the failure of human glaucoma drainage surgery (Skuta and Parrish, 1987). Factors likely to be important in surgical failure include the pre-operative state of the conjunctiva, Tenon's capsule, and episclera; the integrity of the blood-aqueous barrier and the nature of the aqueous humour; the trauma of the operation and the degree of post-operative inflammation (Hitchings and Grierson, 1983).

1.4.1. Time course of failure

The time course for the failure of trabeculectomies has been well documented in a number of large studies, and reasonably consistent trends have emerged.

Ridgeway, (1974) studied 203 trabeculectomies performed on 161 patients, of which 180 eyes were followed-up for 1 year. In patients operated on for both primary open angle glaucoma and chronic angle closure glaucoma, the bulk of the failures occurred within the first year, with most of these occurring within the first three months. A small proportion of cases were noted to fail upto 2 years of follow-up.

D'Ermo, et al., (1979), studied 90 trabeculectomies performed on 75 patients and followed them for 1 to 5 years. During the course of the first year the percentage of eyes with pressures less than or equal to 21 mmHg dropped markedly to approximately 75%. Thereafter the percentage of successful cases remained substantially constant, although only 22 eyes were followed for 5 years.

The maintainance of pressure control was confirmed by Rollins and Drance, (1981) who, in a 5 year follow-up of 48 eyes undergoing trabeculectomy found that 52% attained a pressure of 20 mmHg or less without medication at the end of one year, with a further 29% controlled with medication. At five years, 79% of eyes were still pressure controlled with or without medication. In their paper, however, the authors were careful to point out that pressure control cannot be equated with disease control since adequate control of IOP does not guarantee an arrest of the glaucomatous visual field progression.

A more detailed picture of the time course of failure of trabeculectomies within the first year is provided by Mills, (1981) reviewing 444 trabeculectomies. Eyes requiring postoperative medications were grouped in three monthly periods postoperatively according to the time when they were started on drugs. Within the first 3 months 16% of eyes required medications; in the next three, 3.4%; in the next three, 1.9%; and in the last 3 months of the first year a further 1%. During the subsequent year a further 3% required to be started on medication. Over the subsequent 5 years only a further 2.4% of patients required the commencement of anti-glaucoma medication.

The large Japanese study of Inaba, (1982) confirmed that the time course of failure of the IOP control after trabeculectomy is predominantly of the early failure type. By life table analysis the cumulative failure probability increased rapidly within the first year, particularly within 6 months, and subsequently showed a slow increase. The cumulative data of 427 eyes demonstrate that this slow increase in the failure probability was maintained up to the end of the follow-up period of 5 years.

Lamping, et al., (1986) found less encouraging long term results from trabeculectomy. While about 92% of operations were successful at one year, this had fallen to 70% at five years. Trabeculectomy compared disfavourably in this respect with full-thickness drainage procedures which maintained a greater pressure control rate for longer.

It can thus be seen that with most of the cases that do not attain satisfactory pressure control after trabeculectomy, failure occurs

within the first year, and the bulk of this is within the first 3 to 6 months. Failure is therefore predominantly of the early type (Hitchings and Grierson, 1983). Trabeculectomies may also fail after a number of years, but this is relatively rare. It would seem that attempts to improve the success rate of trabeculectomy should therefore be directed at reducing this early failure.

1.4.2. Consequences of surgical failure

As has been mentioned above the definition of surgical failure differs from one published paper to another, and in addition, most studies do not comment on what happens to patients who do not attain pressure control from surgery. The consequences of failure also depend on the initial reason for performing surgery.

In the Third World, anti-glaucoma medications may be unavailable, or their quality and safety may be open to question. Even if medications are available, social circumstances may make the frequent instillation of drops impossible. Facilities for the follow-up of patients can be extremely poor, distances to the hospital are often large and transport non-existent. Thus, surgery may be the patients' only hope of arresting their glaucoma. Surgical failure to control the IOP will be disasterous and further visual deterioration will ensue. This problem is compounded by the presentation of the majority of patients in these countries late in the course of their disease and for an increased tendency to wound healing at the site of glaucoma drainage surgery in heavily pigmented people. Once an operation such as a trabeculectomy has failed, the facilities for further more technically complicated surgery may be impossible in this environment.

Where a trabeculectomy is performed as a primary procedure to control glaucoma and where medication is available, long term anti-glaucoma drugs may adequately control the IOP. However, in some patients compliance with medication may be a considerable problem, (Davidson and Akingbehin, 1980) particularly if poor compliance prompted the initial decision to perform primary surgery. This may be exacerbated by the patient feeling that he has undergone unnecessary surgery which has failed in any case, and which may even have worsened his vision. It may thus be more realistic to classify partially successful surgery which requires supplementary medications to control the IOP as a failure.

Where surgery was performed after a failure of medical treatment to control the IOP and where post-operatively the pressure remains uncontrolled despite the reintroduction of these medications, there are two alternatives. Either further intervention with physical methods of treatment is undertaken, or the patient takes his chances and hopes that his remaining and presumably deteriorating vision exceeds his life expectancy.

The success rate from undertaking a second trabeculectomy has been shown by Shirato, et al., (1982) and others, to be less than the first procedure, but still probably worth undertaking. However, a third and subsequent operation had a success rate of about 10% only (Shirato, et al., 1982). Some surgeons would undertake further full-thickness drainage procedures as an alternative. In some centres the implantation of shunting devices (see section 1.2.7.) would be the procedure of choice. Cyclodestructive procedures in eyes with poor visual potential are a further treatment option, either performed with cryotherapy, therapeutic ultrasound or transcleral Nd:YAG cyclophotocoagulation (Reviewed by Heuer, 1988). None of these treatment options is particularly satisfactory and the periods of high IOP suffered before an alternative treatment is undertaken may do further harm to the patient's already compromised optic nerve.

It follows that methods to improve the success rate of trabeculectomy are urgently required.

1.5. COMPLICATIONS OF TRABECULECTOMY AND COMPARISON WITH OLDER TECHNIQUES OF GLAUCOMA SURGERY.

The side effects described by Cairns in his original description of trabeculectomy, (Cairns, 1968) were a relatively mild intra-ocular infection in one case, which responded well to antibiotics, iris prolapse in one case performed without an iridectomy, one persistently flat anterior chamber lasting for 3 days, 2 flat chambers for 2 days, and 4 for 1 day. All of the flat chambers reformed after treatment with acetazolamide. In most cases there was also a small hyphaema present for some postoperative days. In spite of the fact that his success rate of 100% has not been equalled in larger studies, and that more serious side effects have emerged after trabeculectomy, numerous comparisons have demonstrated that the operation compares extremely favourably with a number of older full thickness glaucoma drainage procedures.

Drance and Vargas, (1973) evaluated trabeculectomy and thermosclerectomy in a retrospective non-randomised study. Thirty six trabeculectomies were compared with 22 thermosclerectomies, with follow-up ranging from 3 months to 3 years. No statistical analysis of the results was performed, but the pressure control of the trabeculectomies was considered slightly superior compared to the thermosclerectomies. Thirty six percent of the thermosclerectomies lost over 2 lines of vision due to cataract, while for trabeculectomies the equivalent figure was 11%, or one third of the number of cataracts. None of the trabeculectomies had post-operative flat anterior chambers, and uveitis producing posterior synechiae occurred in only 2 out of 36 eyes. Thermosclerectomy was usually followed by a flat anterior chamber and uveitis was not uncommon. The only post-operative complication seen more frequently in the trabeculectomies was a small transient hyphaema.

Trabeculectomy was compared with Scheie's procedure (peripheral iridectomy with a thermal sclerostomy) by Spaeth, Joseph and Fernandes, (1975). A total of 71 cases were followed for 3 years, but the type of surgery was not randomised at the start of the study. Success was judged both in terms of pressure criteria and visual function. The authors suggested that the Scheie procedure reduced pressure to a lower level and for a longer duration than trabeculectomy. There were no differences in the long term visual

result between the two procedures, but trabeculectomy caused fewer flat anterior chambers than the Scheie procedure. Further data was provided by Spaeth, (1980) in a prospective, randomised controlled study comparing the above procedures. Fifteen patients requiring bilateral glaucoma drainage surgery were randomised to receive a Scheie procedure in one eye and a trabeculectomy in the other. All cases were followed for one year, and 13 from 5 to 8 years after surgery. IOP was lower in the eyes with the full thickness procedure (14 vs 16 mmHg). Control of the disease was more easily accomplished without requiring medications in the eyes treated with the Scheie procedure. The average postoperative visual loss was the same in both groups, with a mean of about 1.5 Snellen lines lost 5 years after surgery. Partially flat anterior chambers were more common in eyes treated with a Scheie procedure, with one fifth of the eyes treated with substantial iris-cornea contact. Cataract developed post-operatively with approximately equal incidence. In every cases except one, progression of cataract occurred only in those eyes with the presence of cataract preoperatively. The Scheie procedure resulted in the classic, thin walled cystic bleb in almost all cases, whereas only 2 trabeculectomy eyes developed such a cystic bleb. Spaeth is careful to point out that this was a small study with literally no serious complications, but that "it is now widely accepted that the incidence of complications following trabeculectomy is substantially lower than following a Scheie procedure".

Watkins and Brubaker, (1978) compared trabeculectomy with a full-thickness corneoscleral trephination procedure (as described by Sugar, 1970a). Seventy six eyes were studied, comprising 49 trabeculectomies and 27 full-thickness procedures. However, patients were not randomised to a particular treatment. The decision as to which operation to perform was made on the basis of the final pressure that it was hoped to attain. Thus patients for whom it was believed that low pressure was necessary for control, underwent the full-thickness procedure. Follow-up was for a minimum of one year. In the full thickness group there were significantly ($p < 0.001$) greater numbers of shallow and flat anterior chambers in the immediate postoperative period. During the early postoperative period there was a small but significant difference in IOP ($p < 0.05$), with the full-thickness group having slightly lower pressures. Significantly fewer ($p = 0.005$) filtration blebs were present in the partial thickness group and there

was no significant visual change in either group. The major postoperative problem was failure of the procedures to control the IOP at a satisfactory level which occurred in 10% in each group. After a year the small difference in IOP between the two groups persisted. The visual acuity remained unchanged. Approximately 20% of all patients were receiving anti-glaucoma medication. Although the incidence of complications was higher in the full thickness group, the difference between the two groups was not statistically significant.

Trabeculectomy was again compared with thermosclerostomy in a well organised prospective clinical trial by Blondeau and Phelps, (1981). All patients were phakic and were undergoing their first operation for open angle glaucoma. Ninety eight eyes from 64 patients were included in the study and patients were randomly assigned to surgical treatments. Where both eyes of a single patient were operated upon, they received different operations. Thus, 50 thermosclerostomies and 48 trabeculectomies were assessed. The mean follow-up period was 2.7 years. Postoperatively the thermosclerostomy group had a lower mean IOP than the trabeculectomy group. However, the average difference was not statistically significant except for the second year. The most noticeable difference between the two groups occurred on the first postoperative day in that thermosclerostomy usually caused a very low pressure. IOP was controlled (under 22 mmHg) with or without medications in 80 to 95% of eyes at each follow-up period no matter which operation was performed. Thermosclerostomy eyes had a higher proportion of thin blebs, and trabeculectomy eyes a higher proportion of thick blebs or no bleb at all. More than two thirds of the eyes in both groups lost acuity. In eyes that lost acuity, the cause was almost always cataract. Ten (20%) of the 50 eyes in the thermosclerostomy group required cataract extraction during the time of follow-up, while none of the trabeculectomy group had this complication. Some hyphaema was present in approximately 50% of both types of operation. A flat anterior chamber, in which lens or iris was in contact with the corneal endothelium occurred in 20% of the thermosclerostomies and 4% of the trabeculectomies. As late post-operative complications, 2 of the 50 thermosclerostomy blebs perforated and a further 2 became infected. None of the trabeculectomy blebs suffered this complication. When the follow-up was extended in a further study of 71 of these eyes to five years (Lewis and Phelps, 1984), essentially similiar results were seen. However 3 cataract

extractions were necessary in the trabeculectomy group. This led the authors to suggest that thermosclerostomy may have two cataractogenic effects: one that is immediate and related to the operative technique and a second that is more chronic and common to both types of filtering technique. The cataractogenic potential of filtering surgery had been studied in more detail some years earlier by Sugar, (1970b) who among other factors suggested that postoperative flattening of the anterior chamber may play a significant role in this unfortunate complication.

Other types of full thickness filtering procedures have been compared with trabeculectomy. Shields, (1980) reported on 26 trabeculectomies and 23 posterior lip sclerectomies in a retrospective study. It is not reported in the paper why a particular procedure was performed on a particular patient. Follow-up ranged from 3 months to 38 months. Success was defined as control of the IOP at a level that was sufficient to prevent further damage to the optic nerve head. For the trabeculectomies this pressure ranged from 7 to 28 mmHg, while for the posterior lip sclerectomies the range was 4 to 21 mmHg. Fifty four percent of the trabeculectomies required medication to be categorised as successful while only 35% of the full-thickness procedures required this. Cataracts causing a reduction of Snellen acuity by more than one line occurred in similiar proportions of patients (about 35%). Flat anterior chambers were more common in the posterior lip sclerectomy group. The study was weak in that no statistical comparisons were made. However the author concluded that a full thickness procedure provided slightly better pressure control than a trabeculectomy, but that there was no difference in the final visual result, nor the incidence of cataracts between the two surgical procedures. On this basis and on the evidence from a literature survey presented in the paper, Shields concluded that there was little evidence for the superiority of the trabeculectomy as a drainage procedure, and that he still preferred a full-thickness procedure in advanced cases where a very low IOP is essential.

It can thus be seen that many of the studies comparing trabeculectomy with full-thickness drainage procedures are methodologically poor. Even in those studies that are sound, the number of cases for comparison is low, therefore making it impossible to compare the relative risks of rare complications, eg. infected blebs

postoperatively, in a statistically meaningful way. Nevertheless, the trend from these studies indicates that the final IOP attained after a trabeculectomy is generally slightly higher than after a full thickness procedure. Full thickness procedures appear to function for longer periods than trabeculectomies. This is compensated for by a lower risk of complications after trabeculectomy, particularly flat anterior chambers, predisposing to cataract progression. The relative rarity of thin cystic blebs after trabeculectomy compared to their almost invariable presence after a full thickness procedure theoretically reduces the life-time risk of endophthalmitis due to an infected bleb. However, this potentially devastating complication has been reported after trabeculectomy (Freedman, Gupta and Bunke, 1978). In this particular study the incidence of endophthalmitis after trabeculectomy was 1.8%. After comparison with other studies performed by different authors, the conclusion of the Freedman study was that the incidence of endophthalmitis after trabeculectomy is similiar to Scheie's procedure but less than that after full-thickness trephination.

The true incidence of complications after any form of surgery can only be estimated once large numbers of patients have been operated upon. Thus the numerous small studies of trabeculectomy are unlikely to give an accurate picture of the expected side effects. Even in large studies involving over 400 patients, consistent trends are lacking, leading different authors to recommend different approaches to treatment. In addition, comparison with historical studies is likely to lead to bias since the recent use of the operating microscope in glaucoma surgery, and better suture materials may by themselves improve the results of surgery.

The retrospective study of Mills, (1981) of 444 trabeculectomies stated that the long-term side effects of trabeculectomy were more widespread and problematical than previous reports suggested. He found shallow or flat anterior chambers in 13% of cases; decreased visual acuity greater than 2 Snellen lines in 15% (mostly due cataract) and further glaucoma surgery necessary in 6% of eyes. Endophthalmitis occurred in 0.5% of eyes. Complications, however slight, occurred in 33.9% of eyes in his study.

By contrast, the 424 trabeculectomies reported by Watson and Grierson, (1981) indicated a lower rate of complications. Surgical intervention was therefore recommended by the authors in patients with glaucoma as soon as simple medication (ie. pilocarpine 2% tds and/or timolol 0.5% bd) fails to control the IOP, or at any time regardless of the IOP, that progressive field loss is confirmed. In their study although shallow anterior chambers occurred in 13% of cases, they were flat in only 3%. Uveitis was noted in 26% of the patients post-operatively, but this was only regarded as severe in 2%. However, 28% developed posterior synechiae following trabeculectomy. Transient hyphaemas occurred in 23% of cases, but persisted for longer than 7 days in 1%. Progression of cataract was noted in 23% of cases operated on using the Cairns approach (Cairns, 1968) while 6% of cataracts progressed using the Watson approach (see section 1.6.1.), (Watson, 1970). Some form of complication however slight was recorded in 28% of eyes, but was only regarded as significant in 16%. In a further study of these eyes it was felt that only 1% could be considered to be worse off because of a surgical complication (Watson and Grierson, 1984).

In conclusion, it can be seen that the trabeculectomy is a reasonably successful operation, but it is far from being the perfect operation for glaucoma. There is a significant rate of failure associated with the procedure as well as a significant rate of complications. The decision to proceed to surgery in a patient with glaucoma is a complicated one and is made on the basis of the surgeon's beliefs about the disease of glaucoma, the patient's wishes, the availability of medication, as well as on the patient's compliance with medical treatment. Many surgeons feel that the physiologically functioning eye should be maintained as long as possible, which can be achieved medically but not surgically. Nevertheless, in Britain there is a school of thought that advocates early surgery (Watson, 1981; Murray and Jay, 1979; Jay, 1983; Jay and Allan, 1989). This is seen by many ophthalmologists, particularly in the United States of America, to be a rather radical view, and trabeculectomy is still reserved for use after a failure of medical treatment to halt the progression of the disease.

1.6. SURGICAL MODIFICATIONS OF THE ORIGINAL CAIRNS TRABECULECTOMY

In an effort to improve the surgical success rate and to make the operation easier to perform, numerous modifications of the original Cairns trabeculectomy have been advocated, commencing with the technique of Watson, (1970) and continuing until the present time. Most surgeons, even when performing a "Cairns trabeculectomy" have subtle modifications of their own. The non-uniformity of surgical technique is likely to account for some of the variation in surgical results discussed in section 1.2. It is also a general truism of surgery that when there are numerous ways of doing the same operation, the ultimate way is yet to be found.

1.6.1. Watson's modification

Watson, (1970) described a modification of Cairns' procedure which he found technically easier to perform. The modification involved removing a larger deep scleral flap, extending further posteriorly than that described by Cairns, such that the ciliary body and underlying scleral spur were dissected free from the flap as it was extended forwards. This differed from Cairns' operation where the trabecular meshwork was dissected anterior to the scleral spur. Watson's modification had the effect of creating a small cyclodialysis. The procedure could be performed with either a limbus- or a fornix-based conjunctival flap. In a comparative study of 452 eyes after a five year follow-up, there was found to be no difference in the IOP control or the complication rates using either technique. (Watson and Grierson, 1984). However, there were considerably fewer cystic blebs produced when fornix-based flaps were used. Watson's modification of the trabeculectomy has become widely accepted and is used in many centres as the standard trabeculectomy.

1.6.2. Flap size

The effect of varying the size of the scleral flap and the corneal block was investigated by Starita, Fellman, Spaeth, et al., (1984). Patients were prospectively randomised into two groups based on the eye receiving surgery. Twenty eight eyes of 23 patients were followed-up for 10-18 months. Both groups of patients underwent a Cairns type trabeculectomy with a limbus based conjunctival flap. Only one aspect of the trabeculectomy technique was altered in the two groups and that was the size of the scleral flap and the block excised. In the first

group the scleral flap was 4x4x4 mm and the excised corneal block 3x3 mm. In the other group the scleral block was 2x2x2 mm and the excised corneal block 1x1 mm. No clinically significant difference was demonstrated between the two groups in final IOP, visual acuity or complication rates. This led the authors to recommend that smaller scleral flaps be used in trabeculectomy surgery in order to disturb the eye as little as possible and to leave as much virginal conjunctiva as possible, should further glaucoma surgery be necessary. The authors pointed out that this study did not solve the controversy as to the mode of action of a trabeculectomy, since adequate trans-scleral filtration could occur even with a scleral flap area reduced to 4 mm^2, or the majority of the filtration could occur around the margins of the flap. Further studies are needed to clarify this point, but as yet, are not available.

1.6.3. Triangular, square or circular flaps.

The effect of using a triangular or a square superficial scleral flap was investigated in 22 eyes of 11 consecutive patients by Kimborough, Stewart, Decker, et al., (1982). No statistically significant difference was demonstrable in the final intra-ocular pressures or complication rate between the two groups. However, since the authors found a triangular flap easier to fashion than a square one, they recommended the former as a simplified approach to the standard trabeculectomy procedure. This technique has been adopted by a number of different surgeons.

The use of trephined semi-circular superficial scleral flaps and circular deep corneo-sclerectomies was advocated by Dellaporta and Fahrenbruch, (1971) as being technically easier to perform than a Cairns trabeculectomy. A direct comparison of results has not been made with a standard trabeculectomy and the operation has not become popular as many surgeons find it technically more complicated than the standard procedure. Nevertheless, the operation appears to be as successful as a standard trabeculectomy and has similiar complications (Dellaporta, 1981).

1.6.4. Flap thickness

David and Sachs, (1981) investigated the effect of scleral flap thickness on the final IOP attained after trabeculectomy. This study has already been mentioned in section 1.1.5. Thirty five eyes with

diverse types of glaucoma and preoperative pressures up to 50 mmHg were included. In accordance with the preoperative IOP the thickness of the scleral flap was chosen between 1/2 and 1/5 of the sclera. Thus, the higher the pre-operative pressure the thinner the scleral flap. 91.4% of eyes attained an intra-ocular pressure less than 21 mmHg with or without medication. Two of the eyes required re-operation. The authors suggested that pre-operative planning of the flap thickness allowed a final IOP for a particular patient to be selected.

This observation must be regarded with some degree of scepticism as it has been clearly shown that in most patients undergoing a standard trabeculectomy, the IOP can be reduced to that of the episcleral tissue pressure, regardless of the pre-operative pressure (Watson and Grierson, 1981 and 1984). In other words, the higher the initial IOP the greater the drop in pressure produced by the operation. David and Sachs, (1981) have failed to show that in patients with equal pressures pre-operatively, that a thinner scleral flap will result in lower post-operative pressures. Nevertheless, although there is little hard data available, it is still the practice of some surgeons to fashion a very thin scleral flap in situations where a very low post-operative IOP is required.

1.6.5. Limbus- vs fornix-based conjunctival flaps

The use of the different types of conjunctival flaps was investigated in a prospective, randomised study by Shuster, Krupin, Kolker, et al., (1984). Eighteen eyes underwent a trabeculectomy under a limbus-based conjunctival flap, while 19 trabeculectomies were performed under fornix-based flaps. In both instances the scleral flap was triangular. The surgery was equally successful in both groups with a mean post-operative follow-up of 17.6 months (range, 9 to 37 months). Transient leakage of aqueous humour occurred from the flap margins in 4 cases after the fornix-based flaps and in none of the limbus-based flaps. The authors felt that there were a number of technical advantages in fashioning a fornix-based flap, and in particular it was easier to perform. Accordingly, they have adopted this technique as part of their surgical routine when performing trabeculectomies.

Their results were confirmed by a similiar study (Traverso, Tomey and Antonios, 1987). Trabeculectomies were performed on each eye of 20

patients, randomly assigned to receive a fornix- or a limbus-based conjunctival flap in a particular eye. The superficial scleral flaps were square in this study. Comparable results for the two types of surgery were obtained in terms of pressure control, the size and nature of the bleb, and the rate of complications over the follow-up period varying from 3 to 13 months.

Similiar results were shown by Reichert, Stewart and Shields, (1987) but they expressed a preference for limbus-based conjunctival flaps. Luntz (1980) by contrast has extoled the virtues of fornix-based flaps.

In summary, it would seem that there is little to choose in terms of final results between the two kinds of flaps, and the choice can safely be made on the particular preference and expertise of the surgeon.

1.6.6. Clear cornea trabeculectomy

The clear cornea trabeculectomy was devised by Cairns, (1985) in order to reduce the failure rate of conventional trabeculectomy. The rationale was to prevent trauma to the conjunctiva and Tenon's capsule, thus obviating scarring at this site, and therefore improving the success rate of surgery. Sixteen eyes in a state of advanced glaucoma and having suffered a previous failure of a conventional trabeculectomy were operated upon by this method (Cairns, 1986b). Success was achieved in only 10 of the 16 eyes.

A similiar poor success rate was confirmed by Keillor and Molteno, (1986) who achieved normalisation of the IOP in only 11 of 22 cases of clear-cornea trabeculectomy. Nevertheless, the authors concluded by saying that with appropriate instrumentation and experience the technique could become a remarkably safe and atraumatic technique for controlling open angle glaucoma. This promise has yet to be fulfilled.

1.6.7. Visco-elastic substances

Visco-elastic substances have been employed in a number of studies in order to reduce the complication of post-operative collapse of the anterior chamber after trabeculectomy.

Blondeau, (1984) used sodium hyaluronate 1% (Helon) in a retrospective study of 21 trabeculectomies in order to cushion the anterior chamber, thus endeavouring to prevent its post-operative collapse. Ten patients received 0.1 to 0.2 ml of the substance into the anterior chamber at the end of surgery, and were compared with historical non-randomised controls. On the first post-operative day sodium hyaluronate prevented ocular hypotony, but thereafter there was no statistically significant difference in mean IOP in the two groups. No other statistically significant difference could be demonstrated but there was a trend towards slightly better visual acuity in the treatment group. The retrospective and uncontrolled nature of this study must, however, prejudice its conclusions.

A 1.9% solution of sodium hyaluronate (PhEA 34c) was investigated by Teekhasaenee and Ritch, (1986) in a prospective randomised controlled study of 30 eyes undergoing trabeculectomy. Balanced salt solution was used as a control in 15 of the cases. PhEA 34c or balanced salt solution was injected both into the anterior chamber and under the conjunctival flap at the end of surgery. Even this sodium hyaluronate solution, which has three times the viscosity of Helon, did not prevent post-operative flat or shallow anterior chambers. No significant difference was demonstrated in the success rate of surgery between the two groups although PhEA 34c appeared to lead to loculation of the filtering bleb, which required reoperation in four cases in this study.

A study in favour of the use of Helon was reported by Raitta and Setala, (1987). The experimental group comprising 46 eyes of 38 patients, received Helon into the anterior chamber at the end of surgery, and were all operated upon by one surgeon. They were compared with a similar number of historical matched controls, operated upon by a number of different surgeons. The authors concluded that Helon is useful in trabeculectomy and prevents collapse of the anterior chamber, thus preventing cataract formation, but no statistics were performed in the study. The conclusion appears to be unjustified since the two groups of patients are clearly not comparable.

In summary, the viscoelastic substances, based on sodium hyaluronate, that have been investigated as an adjunct to trabeculectomy, have not

fulfilled their theoretical promise of maintaining the anterior chamber post-operatively.

1.6.8. Methods of rescuing failing trabeculectomies

Focal pressure has been advocated by Traverso, Greenidge, Spaeth, et al., (1984) in the first week after trabeculectomy, where evidence of closure of the scleral flap was shown by a rise of the IOP, with no apparent filtration and no other discernable cause for the rise of pressure. In their series of 100 trabeculectomies undertaken over a 7 month period, focal pressure was performed on 18 eyes. In 17 of these, filtering blebs developed immediately after focal pressure was applied in the first post-operative week. Although this technique is useful in the immediate post-operative period, the authors do not recommend it for long term IOP control.

Therapeutic ultrasound was investigated as a method for restoring failed trabeculectomies by Yablonski, Masonson, El Sayyad, et al., (1987). Twenty recently postoperative eyes with nonfunctioning trabeculectomies were treated at the site of the failed bleb with 2 to 4 ultrasound applications, each of five seconds duration at an intensity level of 10 kW/cm^2. The mean time between the therapeutic ultrasound treatment and the trabeculectomy was 91+/-71 days(+/- SEM). Fifteen of the 20 eyes demonstrated a long term pressure decrease to 21 mmHg or below with follow-up for 236+/-204 days (mean+/-SD). However complications included the immediate development of a cataract in one patient and an immediate post-ultrasound pressure rise over 10 mmHg in 13 eyes. Thus the mean pretreatment IOP was 32+/-8 mmHg (+/-SD) while the IOP within one hour of the procedure was 50+/-17 mmHg. All of these hypertensive eyes responded rapidly to acetazolamide and a hyperosmotic agent. The high post-operative pressure rise is likely to be detrimental to an already compromised optic nerve. Nevertheless the technique may prove to be useful in some cases of failed trabeculectomy.

Removable-suture closure of the lamellar scleral flap has been advocated by Shin, (1987) as a technique for coping with post-trabeculectomy elevated IOP. In a series of over 50 cases using this method of surgery he encountered no problems associated with using the suture, and particularly in the first week when the suture was easy to remove, was able to re-establish filtration in failing

trabeculectomies. Shin felt that the ability to remove a suture post-operatively enabled him to sew up the lamellar scleral flap tightly, thus diminishing the complications of post-operative flat anterior chambers and conjunctival wound leaks.

1.6.9. Other modifications of trabeculectomy

Numerous other technical modifications of trabeculectomy have been devised in an effort to reduce the complications of surgery and to improve its success rate. These have ranged from excising Tenon's capsule in the vicinity of the trabeculectomy in order to decrease the pool of potential fibroblasts that may form scar tissue, to incorporating aspects of older operations devised for glaucoma, eg. trabeculectomy with iridenclesis (Gess, Koeth and Grallee, 1985); subscleral Scheie procedures (Luntz, 1986)). None of the modifications of the original Cairns trabeculectomy (Cairns, 1968) has been shown consistently to provide improved surgical success or diminished complications. Accordingly, none of these modifications, other than the Watson trabeculectomy (Watson, 1970) has been adopted into general ophthalmic practice.

In the future, techniques of cutting the sclera less traumatically may replace surgery with the scalpel. Thus, the use of the Excimer laser to fashion sclerostomies is being explored in the hope that less tissue damage at the time of surgery may lead to less scarring at the operation site (Berlin, Rajacich, Duffy, et al., 1987). These techniques are yet to be applied outside of experimental studies, but they represent a potentially exciting field for future development.

In summary, it can be seen that none of the surgical modifications of the trabeculectomy have any advantage over the original Cairns procedure (Cairns, 1968) and that for all techniques of trabeculectomy there will be a significant failure rate and an incidence of surgical complications and side effects. The inescapable conclusion appears to be that mere surgical modifications are insufficient to improve the success rate of trabeculectomy. Other avenues need to be explored and there may be a role for adjuvant treatment with substances designed to retard wound healing. Any drug which is found to be successful will have to achieve a fine balance between wound healing which facilitates effective drainage and closure of the conjunctiva, and over vigorous wound healing which causes scarring and a failure of filtration.

1.7. THERAPEUTIC ADJUNCTS TO IMPROVE THE SUCCESS RATE OF TRABECULECTOMY

It is generally agreed that excessive wound healing at the site of a trabeculectomy accounts for the majority of surgical failures. In an effort to improve the success rate of surgery, a number of treatments designed to retard wound healing have been explored.

1.7.1. Steroids

The exact mode of action of glucocorticoids is not fully understood and some of the literature regarding their role as inhibitors of wound healing is rather conflicting. On theoretical grounds they would appear to be active in inhibiting wound healing. However, it is claimed by some authors, that while an inhibition of wound healing has been demonstrated in the experimental animal, in the dosages generally administered, glucocorticoids do not appear to influence wound healing in the human subject (Walter and Israel, 1979).

When given within the first 2 to 3 days following injury, glucocorticoids appear to act at many sites in the process of repair, in the experimental situation. They reduce inflammatory cell infiltration, particularly affecting macrophages (Leibovich and Ross, 1975). Capillary budding is inhibited and reduced numbers of fibroblasts are present in the wound with consequently less formation of fibrous tissue (Ehrlich, Tarver and Hunt, 1973). However, this is not corroborated by other studies. Some of the differences between the experimental findings and the clinical effects of glucocorticoids may be explained by the effect of triamcinolone on fibroblast proliferation in tissue culture. Triamcinolone acetonide and dexamethasone sodium phosphate demonstrated a bimodal effect on both conjunctival and dermal fibroblasts of pigmented rabbits, with stimulation of fibroblast proliferation at low doses and inhibition at higher dosages (Blumenkranz, Claflin and Hajek, 1984). When evaluated using different cell types, namely New Zealand White rabbit retinal pigment epithelial cells and corneal fibroblasts, dexamethasone had no significant effect on cell proliferation (Fiscella, Peyman, Elvart, et al., 1985). This makes it impossible to predict what the final outcome on wound healing will be when using glucocorticoids in vivo.

Steroids have been evaluated in rabbit models of glaucoma filtering surgery by a number of authors. McGuigan, Cook and Yablonski, (1986) found that dexamethasone phosphate in the form of 0.05% ointment applied twice daily, significantly prolonged the patency of a thermal sclerostomy in albino rabbits, with the time to failure extended from the control of about 23 days to just under 50 days. However, Miller, (1988) did not confirm the beneficial effects of steroids on filtration survival in New Zealand White rabbits. In Miller's study a suspension of dexamethasone 1% was administered twice daily to rabbits that had undergone a thermal sclerostomy.

Topical corticosteroids have been incorporated as an adjunct to trabeculectomy for many years, and their use has become almost universal. Excellent surgical results are obtainable without them and they were not advocated by Cairns, (1968 and 1981) or Watson, (1970). Even in high risk patients, namely Nigerian Blacks, success rates for trabeculectomy of 95.4% without the use of steroids, have been reported (Thommy and Bhar, 1979). Many of the studies in the literature are also difficult to compare because different corticosteroids at different doses have been investigated. Nevertheless, in a prospective randomised study, topical prednisolone acetate 1%, administered for 20 days post-operatively, has been shown to significantly improve the success rate of trabeculectomy. This was manifested by a greater average lowering of the IOP when compared to the controls, and a greater frequency of a clinically detectable filtration blebs. The oral administration of high dose prednisolone had no added benefit over topical use alone (Starita, Fellman, Spaeth, et al., 1985).

The best method of employing corticosteroids is yet to be determined. Many surgeons use topical drops post-operatively only and continue them for 4 to 6 weeks. However, Starita, et al., (1985) commenced them 1 day pre-operatively and only continued them for 20 days post-operatively. Giangiacomo, Dueker and Adelstein, (1986) administered subconjunctival triamcinolone 4 mg to 15 eyes at high risk of surgical failure due to episcleral scaring, one week pre-operatively. In this uncontrolled and anecdotal study very good results were achieved with only one eye deemed a surgical failure. This study opens up the possibility that corticosteroids may be more effective if delivered to

the target tissues well before the trauma of surgery. Clearly further controlled studies are needed.

The effect of steroids appears to be limited, however. Miller, Joseph, Wishart, et al., (1987) reported the surgical results in sixteen eyes that had previously failed trabeculectomy, undergoing a further trabeculectomy, that received hourly topical 1% prednisolone sodium phosphate for several months post-operatively. At the end of one year no improvement was seen in the surgical results when compared with a previously reported series of similiar patients treated with less steroid.

Thus, it can be concluded that while corticosteroids may be beneficial in improving the success rate of trabeculectomy, they have a relatively limited effect and are not the final answer in recalcitrant cases of excessive fibrosis.

1.7.2. Antiprostaglandins

Antiprostaglandins are known inhibitors of inflammation and have demonstrated activity in the eye in both experimental and in vitro situations (Unger, Cole and Hammond, 1975). Indomethacin has also been shown to reduce inflammation and cause less breakdown of the blood-aqueous humour barrier in patients' eyes after cataract extraction (Sanders and Kraff, 1984). The effect of antiprostaglandins on trabeculectomy was therefore investigated by Migdal and Hitchings, (1982 and 1983). In a double-masked placebo controlled trial incorporating 30 patients, indomethacin 0.5% drops were administered 60 and 30 minutes preoperatively, and continued three times daily for one month. There was persistent conjunctival and episcleral venous congestion in the eyes treated with indomethacin and a higher rate of surgical failure was recorded in this group. The authors postulated that this may be due to the fact that indomethacin is merely a cyclooxygenase inhibitor and thus blocks only one arm of the arachidonic acid cascade, leaving the unapposed lipoxygenase pathway to form leukotrienes which are powerful inflammatory agents. A drug which blocks both of these pathways may inhibit postoperative inflammation in glaucoma surgery. While such drugs are undergoing experimental evaluation, they are not yet widely available.

Indomethacin drops have also been employed as part of the post-operative regime in Japanese patients undergoing trabeculectomy although they appear not to have been evaluated as a single adjuvant treatment in the Japanese. Shirato, et al., (1982) prescribed 0.5% indomethacin drops 3 to 4 times daily in conjunction with topical 0.1% betamethasone, while Yamashita, et al., (1985), in addition to indomethacin, administered pre- and post-operative systemic prednisolone, as well as employing per-operative Helon (sodium hyaluronate). As mentioned in section 1.2.4. the success rate for trabeculectomy in the Japanese is generally less than in Caucasians. While the incidence of complications in the Yamashita series was significantly less than in the Shirato series, the length of follow-up in the Yamashita series did not allow the effect of this anti-inflammatory regime on the post-operative IOP to be evaluated.

Thus, on the basis of the current evidence, indomethacin drops are contra-indicated in Caucasian patients undergoing trabeculectomy. The use of indomethacin in association with glaucoma surgery remains unsubstantiated in other groups of patients.

1.7.3. Local ionising irradiation

The literature relating to the use of ionising radiation as an adjunct to glaucoma surgery is generally of extremely poor quality. Beta-irradiation has been used since the 1930's in the treatment of superficial conditions of the cornea and conjunctiva, eg. to inhibit the regrowth of pterygia after surgery. This use was based on the in vitro sensitivity of fibroblasts to irradiation. Beta-irradiation is particularly suitable for superficial conditions because of its relatively limited tissue penetration.

Iliff, (1944) employed beta-irradiation from radon, to reduce the failure of glaucoma filtering operations in Black patients, believed to be due to obliteration of the filtering bleb by scar-tissue formation. In this uncontrolled anecdotal study, 11 eyes were treated. Eight of the 11 cases were said to be successful. The author felt that these results warranted further trials of beta-irradiation. In 1959, Cohen, Graham, and Fry, reported on 10 cases of filtration surgery performed in Black patients, with the addition of three applications of beta-irradiation post-operatively. Success was claimed in 8 out of 10 cases, but again these observations were not controlled.

Similiarly, Cameron (1970) advocated the use of 2,500 rads of beta-irradiation when reoperating on patients who had previously sustained unsuccessful glaucoma surgery, or in cases of thrombotic glaucoma. However, his observations were anecdotal and he advanced no comparative data in support of his claim. Likewise, in the Japanese literature there are similiar uncontrolled observations claiming benefit from the use of beta-irradiation in filtering operations (Ogino, Masuda and Abe, 1966).

Other indirect evidence supporting the use of beta-irradiation to affect wound healing comes from a large study of 800 patients over 23 years who received beta-irradiation for a wide variety of conditions (Barron, McDonald and Hughes, 1970). Poor wound healing was found in 2 patients undergoing cataract extraction some time after receiving beta-irradiation for pterygia or severe vascularizing keratitis. In one patient a persistent flat anterior chamber with a wound leak developed, while the other developed a filtering bleb.

In animal studies, however, a clinically meaningful effect of beta-irradiation on enhancing the survival time of experimental drainage surgery has not been found. For example, in rabbit eyes undergoing surgery, Miller (1988) found that although beta-irradiation delayed the fibroblastic response in the wound, producing thinner scar tissue in the early stages, no long-term benefit was achieved. Similiar effects were found as long ago as 1961 by Grillo and Potsaid, (1961) who observed that although X-irradiation retarded the contraction of experimental skin wounds in guinea pigs, all wounds ultimately closed fully.

There have been few studies on the use of irradiation in conjunction with trabeculectomy. In a small study of patients undergoing repeat trabeculectomy, Miller et al., (1987) found no benefit from the preoperative application of 2,500 rads of beta-irradiation.

In conclusion, although the published studies of irradiation as an adjunct to filtration surgery are poor, there is no substantive evidence that it improves the success rate of surgery.

1.7.4. Anti-metabolites

A number of anti-metabolites have been evaluated as fibroblast

inhibitors. Much of the initial work was done at the Bascon Palmer Eye Institute, Miami, Florida, in relation to proliferative vitreoretinopathy. Blumenkranz, Ophir, Claflin, et al., (1982), having initially demonstrated that 5-Fluorouracil (5-FU) inhibited rabbit dermal fibroblast proliferation in tissue culture, showed that intra-vitreal 5-FU was effective in the treatment of an experimental model of massive peri-retinal proliferation in the rabbit. In a more formal evaluation of conjunctival and dermal fibroblasts in-vitro, Blumenkranz, et al., (1984) investigated a number of anti-proliferative agents. They found that while doxorubicin was more potent than fluorouracil, both drugs inhibited fibroblast proliferation in a dose dependent manner. However, the therapeutic range of fluorouracil appeared to be better than that of doxorubicin, indicating a potentially safer therapeutic index.

Based on these studies, intra-ocular and peri-ocular 5-Fluorouracil was used in patients with complicated retinal detachments and proliferative vitreoretinopathy (Blumenkranz, Hernandez, Ophir, et al., 1984). Although this initial study was not controlled, the retinal attachment rate of 60% at 6 months compared favourably with other published series. The relatively minimal side effects encountered led the authors to suggest the further use of 5-FU in future randomised studies.

At the same time as 5-FU was being evaluated as an inhibitor of proliferative vitreoretinopathy, the idea of using the drug extra-ocularly to inhibit wound healing in relation to glaucoma surgery was raised. Gressel, Parrish and Folberg, (1984), at the Bascon Palmer Eye Institute initially tested 5-FU in an animal model of glaucoma surgery. Posterior lip sclerectomies were performed in each eye of 10 normal owl monkeys. One eye of each animal received subconjunctival injections of 5-FU twice daily for the first post-operative week, once daily for the next week, and 4 further injections upto 25 days post-operatively. This dosage regime was empirical. In none of the control eyes was a drainage bleb present after the second postoperative week. Two animals died, from uncertain causes, on the eleventh and twenty-fourth postoperative days. In 5 of the 8 remaining 5-FU treated eyes, localised, thin-walled, polycystic blebs were present 14 weeks postoperatively. One additional animal showed a diffuse low bleb. Four of the eight 5-FU treated eyes demonstrated a patent sclerostomy at 14

weeks post surgery. The drug had relatively mild side effects in Gressel's study which encouraged other workers at the same institution to try the drug in human beings.

Heuer, Parrish, Gressel, et al., (1984) performed a pilot study with subconjunctivally injected 5-FU in complicated glaucoma patients with poor surgical prognoses for conventional drainage surgery. Patients suffering from medically uncontrollable glaucoma in aphakia, neovascular glaucoma, or glaucoma in phakic eyes following at least two unsuccessful filtering operations, were included in the study. A total of 58 eyes underwent either a trabeculectomy or a full-thickness drainage procedure. In the initial patients 3 mg of 5-FU was injected subconjunctivally on postoperative days 1 to 14. Thereafter, the dose was increased to 5 mg and administered twice on days 1 to 7 and then once for the following week. Thus the total dose of 5-FU ranged from 0 to 125 mg. Intraoperative and postoperative complications influenced the number of 5-FU injections.

Success in Heuer's study was defined as an IOP less than or equal to 21 mmHg with or without medication, or an IOP less than or equal to 25 mmHg but greater than 21 mmHg without medication. In patients with aphakia, surgery was successful in 79% of cases; in neovascular glaucoma a 69% success rate was achieved; while in patients undergoing reoperation, 89% of cases were successful. Forty five percent of the eyes developed corneal epithelial defects with occasional ones persisting for as long as 4 weeks following surgery. Conjunctival epithelial defects overlying the site of the 5-FU injections also frequently occurred. One eye developed a sterile conjunctival ulcer but no infections developed. Subepithelial corneal scarring developed in 3 eyes, but was only visually significant in 1. Forty one percent of the eyes developed conjunctival wound leaks or conjunctival needle track leaks. However, the latter complication was virtually eliminated by subsequently using taper point needles for suturing. The total subconjunctival dose used in this study was less than 3% of the total intravenous dose used in one course of systemic chemotherapy, and therefore systemic toxicity was neither expected nor observed. Since this was an uncontrolled study the results could not be compared with other published series. However, the authors believed that their results demonstrated an improvement in the surgical prognosis for eyes at high risk of surgical failure. The relatively limited side effects

of the treatment led the authors to plan a randomised clinical trial of 5-FU in filtering surgery.

These observations were further extended in an intermediate follow-up of the pilot study (Heuer, Parrish, Gressel, et al., 1986). Ninety-five patients (104 eyes) were enrolled in the study, on 84 of whom at least 6 months of follow-up was available. Four patients were excluded from the analysis of surgical outcome because of retinal detachment within six months of their glaucoma surgery. The dosage regime was the same as that described above. Using the same IOP criteria for success as in Heuer, et al., (1984) surgery was successful in 68% of the aphakic eyes with non-neovascular glaucoma, 81% of the phakic eyes with non-neovascular glaucoma after unsuccessful filtering surgery and 75% of the eyes with neovascular glaucoma. The initial 104 operations were complicated by corneal epithelial defects in 50% of cases; conjunctival wound and suture track leaks in 36%; suprachoroidal haemorrhages in 9%; retinal detachments in 3%; subepithelial scarring in 3%; endophthalmitis in 2% and malignant glaucoma in 1%. In spite of these side effects, the nine authors felt that postoperative subconjunctival 5-fluorouracil increases the likelihood of achieving IOP control in eyes with a high risk of failure from conventional surgery. These conclusions have led to the establishment of a multi-centre controlled clinical trial of 5-FU known as the Fluorouracil Filtering Surgery Study, in patients with non-neovascular glaucoma in aphakia and non-neovascular glaucoma after unsuccessful filtering surgery. Recruitment to the study ceased by the middle of 1988 and the trial will report one year thereafter.

A further follow-up of the same pilot study was reported in 1987 (Rockwood, Parrish, Heuer, et al., 1987). One hundred and fifty five patients were followed by life-table analysis for upto 3 years. The conclusions for this larger number of patients were essentially the same as those described above. However, statistical analysis failed to demonstrate a positive correlation between the total dose of 5-FU administered and surgical success. This suggested that either an undetermined lower dose of 5-FU might be as effective as the higher total dosages or that there is a critical period during postoperative wound healing such that 5-FU injections have their greatest effect in that period and the earlier or later injections have little or no added effect on filter survival. This is rather disturbing as the

Fluorouracil Filtering Surgery Study has been using a high dose of 5-FU, which may compromise its conclusions.

Adjunctive treatment with 5-fluorouracil has been adopted enthusiastically by a number of authors (Kitazawa, et al., 1987). However, there is increasing evidence of serious side effects from the use of the drug. Lee, Hersh, Kersten, et al., (1987), emphasised the importance of corneal epithelial toxicity and conjunctival wound leaks from 5-FU. Serious corneal complications were reported by Knapp, Heuer, Stern, et al., (1987), following the use of 5-FU. The patients concerned had pre-existing corneal epithelial defects including keratoconjunctivitis sicca, exposure keratopathy, and bullous keratopathy. The complications included bacterial corneal ulceration in 2 patients, sterile corneal ulceration and corneal perforation in one patient, and a keratinised corneal plaque with underlying stromal infiltration in one patient.

The toxic effect of 5-FU on the corneal epithelium was evaluated in a rabbit model by Capone, Lance, Friend, et al., (1987) who suggested that careful titration of the dose of 5-FU could minimize or eliminate corneal complications while still retaining anti-mitotic activity. The potential toxicity of 5-FU to the corneal endothelium has also been studied (Mannis, Sweet and Lewis, 1988), suggesting that the drug should be used with caution where access to the endothelium is a possibility eg. after glaucoma filtering surgery.

In an effort to minimize the side effects of 5-FU, Weinreb, (1987), has advocated titrating the dose of the drug according to the clinical response. Thus in his study, 5-FU was not administered if there was evidence of corneal toxicity as demonstrated by epithelial defects or filaments, flat anterior chamber, or a conjunctival wound leak. Using a lower total dose of 5-FU than that described in the Bascon Palmer series described earlier (range 17.5 mg to 62.5 mg), he demonstrated a similiar surgical success rate with a reduced incidence of complications. However, conjunctival wound leaks were still observed in 24% of the 63 eyes in the study and 29% demonstrated corneal epithelial toxicity.

In summary, 5-Fluorouracil is a potentially useful drug but with potentially serious side effects. Its mode of administration by

frequent subconjunctival injection makes it highly unacceptable to many patients. The results of a controlled trial using 5-FU are not yet available. As a result of these facts the drug has received very little use in the United Kingdom. Unequivocal demonstration of a beneficial effect from 5-FU may make its disadvantages easier to tolerate and lead to its more widespread use. In spite of a tremendous amount of investigative work having been performed on 5-FU, the drug is far from the ideal as an adjunct to glaucoma surgery. Nevertheless, this work has highlighted the need for further medications to modulate wound healing in relation to glaucoma surgery.

Concurrently with the investigation of 5-FU, a number of other anti-metabolites were undergoing evaluation in vitro and in animal models of proliferative vitreoretinopathy. Wiedemann, Kirmani, Santana, et al., (1983) demonstrated a dose-response relation using daunomycin to control experimental massive periretinal proliferation in pigmented rabbits, while after initial evaluation of Taxol in vitro, van Bockxmeer, Martin, Thompson, et al., (1985) showed that single intravitreal injections of the drug significantly reduced the incidence and extent of proliferative vitreoretinopathy in rabbits.

In an attempt to evaluate alternative antineoplastic drugs for use in glaucoma surgery, Kwong, Litin, Jones, et al., (1984) investigated the effect of bleomycin sulphate, cytosine arabinoside and 5-FU as inhibitors of fibroblast proliferation in rabbit aqueous humour. Previous work from Herschler's laboratory had shown that aqueous humour modulated fibroblast growth (Burke, Foster and Herschler, 1982/1983). Therefore in order to simulate conditions prevailing in the area of a trabeculectomy, the effect of the drugs was investigated in the presence of aqueous humour. Fibroblasts were derived from rabbit Tenon's capsule and skin and from human Tenon's capsule removed peroperatively. Different effects were observed depending on the cell type under evaluation. While all three drugs markedly inhibited fibroblast proliferation, the most potent effect on human fibroblasts was exhibited by bleomycin which caused a ten-fold reduction of fibroblast proliferation. Combination chemotherapy using various permutations of these three drugs was shown not to have added effectiveness in inhibiting human Tenon's capsule fibroblast proliferation (Litin, Kwong, Jones, et al., 1985). Although bleomycin has been demonstrated to have potent activity in inhibiting human

fibroblast proliferation in vitro, it appears to have only been evaluated in experimental animals to date.

In order to minimize unwanted toxic effects of anti-metabolites used as adjuncts to glaucoma surgery, and to improve the mode of delivery of the drugs, various ingenious techniques have been developed. These have sought by means of a single application, to lower the total dose of drug administered while still retaining effective drug concentrations at the operation site. Kay, Litin, Woolfenden, et al., (1986), investigated bleomycin containing collagen sponges and silastic disc implants with a microhole, implanted at the time of surgery. Collagen implants had the theoretical advantage that they were biodegradable at the operation site. Their initial studies confirmed that the implants markedly prolonged the half-life of the drug in the wound. This prompted the evaluation of 5-FU and bleomycin impregnated collagen implants in a small pilot study in rabbits undergoing filtration surgery. Both drugs improved bleb survival in the rabbit, with blebs treated with bleomycin surviving upto 50 days, which was 41 days longer than the untreated controls. No toxic effects were observed with bleomycin but the animal treated with a high dose of 5-FU showed complications, which were not stated in the paper, and poor bleb formation.

A similiar technique was employed by Lee, Flores, Anderson, et al., (1987) who incorporated 5-FU into a bioerodable polymer composed of bis (p-carboxyphenoxy) hexane and sebacic acid. Control animals received a drug-free seton. Favourable effects were seen in terms of bleb survival in rabbits treated with the drug containing implants. However, there were more complications such as extrusion of the seton, hyphaema and corneal haziness, seen in the group of animals receiving the active drug than in the control animals.

An alternative technique for improving the delivery of medications has involved incorporation of drugs in multivesicular liposomes which slowly leak. Skuta, Assil, Parrish, et al., (1987) entrapped 5-fluorouridine 5'-monophosphate (a potent metabolite of 5-FU) in liposomes, which were administered subconjunctivally to monkeys undergoing filtration surgery. Delayed wound healing was demonstrated at the site of the glaucoma surgery but at the expense of severe local and systemic toxicity. A similiar system for the delivery of 5-FU has

been devised (Simmons, Sherwood, Nichols, et al., 1988), but is yet to have its effectiveness demonstrated.

It is obvious that the optimal delivery system of antimetabolites to the site of glaucoma surgery remains to be determined. However, slow release biodegradable systems appear to have some theoretical advantages which still require to be realised in practice.

1.7.5. Collagen anti-cross linking agents (lathyrogens)

McGuigan, et al., (1986) investigated the effects of D-penicillamine and dexamethasone on glaucoma filtration surgery in rabbits, testing the drugs alone and in combination. Functioning of the filter was defined as an IOP in the operated eye 2 mmHg or lower than in the non-operated eye. No histology was performed in this study. Relative hypotony can merely be a response to surgery and in other studies histological failure of the filter has predated IOP failure (Lee, et al., 1987). Therefore, the results of this study have to be interpreted with caution. The authors found that intraperitoneal D-penicillamine, in spite of inducing systemic lathyrism was ineffective either alone or in combination with topical dexamethasone in extending the duration of function of the filter. Topical dexamethasone alone and subconjunctival D-penicillamine alone significantly prolonged the duration of function of the filter, however when combined, no additional effect was seen. The authors concluded that topical dexamethasone or subconjunctival D-penicillamine significantly prolonged the patency of a thermal sclerostomy in non-glaucomatous rabbit eyes, a conclusion that is hard to justify on the basis of the data presented.

McGuigan, Mason, Sanchez, et al., (1987), then investigated the use of D-penicillamine and beta-aminoproprionitrile (BAPN) in experimental glaucoma filtering surgery in 11 cynomolgus monkeys that had iatrogenic, laser trabeculoplasty induced glaucoma. Eyes treated with BAPN or D-penicillamine maintained successful filtration for at least 3 days longer than nondrug-treated controls. While this effect was statistically significant, it was clearly temporary and of limited clinical usefulness. In the trial D-penicillamine was administered subconjunctivally each day for seven days postoperatively, while BAPN was given both as an ointment and as an intramuscular injection, daily until filter failure, which was defined as an IOP exceeding 25 mmHg on

three consecutive days. The authors concluded that the moderate prolongation of the patency of the filter may have resulted from an inadequately effective drug concentration, or that the drugs may have limited potency because their action on inhibiting collagen cross-linking effects only a minor component of the healing process that causes filter failure.

The poor results seen in experimental filtering surgery did not prevent a pilot human trial from taking place. Moorhead, Smith, Stewart, et al., (1987) applied topical BAPN ointment to 23 eyes of 23 patients undergoing filtration surgery as a result of aphakic glaucoma, neovascular glaucoma or because they had previously sustained failed drainage surgery. The drug was applied as a 20% preparation in petrolatum-based ointment in order to improve contact time, four times a day for one month following surgery, and thereafter twice a day for two additional months. The study was uncontrolled, therefore no definitive conclusion is possible. The overall success rate, defined as an IOP less than 23 mmHg, was 74%. However, in the light of the minimal side effect encountered of stinging of the drug, the authors felt that further controlled studies using BAPN were justified.

In conclusion, collagen anti-cross linking agents or lathyrogens, have shown little promise in experimental situations of improving the success rate of filtration surgery. Use of the drugs in humans has been very limited and claims of efficacy remain unsubstantiated.

1.7.6. Combination chemotherapy

The use of drugs with different modes of action, in combination, has long been employed in cancer chemotherapy. The rational is to provide maximum efficacy, which can be either additive or synergistic, with minimal side effects. Similiarly, the combined use of a number of drugs with anti-wound healing activity, may be beneficial in attempting to reduce fibrosis in relation to trabeculectomies, with a minimal incidence of side effects. An alternative is to employ a single drug that is active at a number of different sites in the wound healing process.

A number of studies have been mentioned above in which two anti-wound healing agents have been employed simultaneously. For example,

prednisolone acetate 1% drops were administered in conjunction with 5-FU in the studies of Heuer, et al., (1984 and 1986) and dexamethasone 0.1% was used in association with 5-FU in the study of Kitazawa, et al., (1987). However, no additive or synergistic effect has been conclusively demonstrated.

Molteno, Straughan and Ancker, (1976b), attempted to control bleb fibrosis in the vicinity of a Molteno tube (Molteno, et al., 1976a), using a combination of anti-inflammatory drugs. In choosing appropriate drugs, Molteno, et al. used the fall in IOP produced by administration of the drug as evidence of anti-inflammatory efficacy. Control was provided by daily measurement of IOP in the fellow eye which was unoperated upon and was either normal or glaucomatous. The control eye received the same topical drugs as the surgically treated eye. Using this technique they found no anti-inflammatory activity exhibited by the antihistamine promethazine HCl, the protease inhibitor aprotinin, the serotonin inhibitor methysergide, and the antirheumatic agent diprocetyl. Moderately active agents which lowered the IOP by 5-10 mmHg were mefenamic acid and aspirin systemically, noradrenaline 5%, isopropyl-noradrenaline 2% and adrenaline 1% administered topically. Active agents lowered the IOP by 10-15 mmHg and included the prostaglandin synthetase inhibitor flufenamic acid, systemically, adrenaline 2% and dexamethasone and prednisolone applied topically. Since no single agent, even in very large doses, could completely overcome bleb inflammation, a combination of the three most active drugs, belonging to different classes of compound was used. The regime advocated was prednisolone 10 mg 3 times daily and flufenamic acid 200 mg 3 times daily for 4 days before the operation, as well as for 6 weeks after. In addition adrenaline 2% was applied topically 4 times a day.

The drug combination was used on 41 eyes with advanced glaucoma, undergoing drainage surgery by means of Molteno implants. Controls, which were not randomised, consisted of 125 eyes similiarly selected and drained by implants, 30 of which had been treated with topical steroids alone and 95 of which received high dose systemic prednisolone (40-60 mg per day). Post-operative IOP's were significantly lower in the combination chemotherapy group and in the group receiving systemic prednisolone, when compared to the controls who received topical steroids alone. Although the triple regime

afforded no extra advantage in terms of lower IOP, it did allow the dose of systemic prednisolone to be halved. In the same paper, Molteno, et al., (1976b) describe their experiences of using the triple regime in 42 eyes undergoing a conventional Cairns type trabeculectomy. A short follow-up precluded definite conclusions, however, the authors felt that use of the triple regimen extended the application of trabeculectomy to extremely advanced cases of glaucoma, which would have previously presumably undergone Molteno tube implantation.

In a subsequent paper, Molteno, (1980) advocated a modification of the combination regime, using systemic prednisolone 10 mg 3 times a day, beginning 2 days preoperatively and continued for 6 weeks thereafter, fluphenamic acid 200 mg 3 times a day for 7 weeks, systemic colchicine 0.3 mg 3 times a day for 8 weeks, topical betamethasone 0.1%, atropine 1% and adrenaline eye drops 2% all 3 times a day for 8 weeks. This paper is in the form of an anecdotal series of 4 case reports and accordingly no conclusion can be made as to the efficacy of the treatment.

Molteno's recommendations for an antifibrotic regime have not been widely adopted. Cairns, (1983) and Brown and Cairns, (1983) initially used Molteno's combination chemotherapy in association with implanting Molteno tubes but then abandoned it in all but fit young patients because of an unacceptably high incidence of side effects from the drugs. There does not appear to be any published experience of the use of the drugs in association with trabeculectomy, other than the brief report of Molteno, et al., (1976b).

As yet there is no well accepted regime of combination anti-fibrotic therapy. In vitro, attempts have been made to find new regimes of drugs to use in combination. McGuigan, Quigley, Lutty, et al., (1988) investigated the effects of D-penicillamine and daunorubicin on monkey conjunctival fibroblast and human Tenon's capsule fibroblast, proliferation and collagen synthesis. Both daunorubicin and surprisingly D-penicillamine inhibited fibroblast proliferation, although daunorubicin was 200 times as potent as D-penicillamine. As expected D-penicillamine significantly impaired collagen production by the cells. The authors hypothesized that a combination of these two drugs may be beneficial in inhibiting fibrosis at the site of a

trabeculectomy, although in tissue culture they did not attempt to demonstrate the effect of the two drugs when applied simultaneously to their test system.

In conclusion, no truly acceptable regime of antifibrotic medication is yet available. There is clearly an urgent need to find new and useful drugs to modify wound healing in order to reduce fibrosis in relation to trabeculectomies.

1.8. WOUND HEALING

1.8.1 General features

In order to be able to explore new therapeutic options to modify wound healing at the site of a trabeculectomy, it is necessary to understand the fundamental mechanisms underlying the healing of tissues. This is critical in order to appreciate the possible stages in the process where inhibition can be targeted. As yet our knowledge of wound healing is still relatively rudimentary but the processes involved are beginning to be understood. They consist of both simultaneous and consecutive inter-relating sequences or cascades, involving numerous inflammatory mediators, growth factors, local hormones and chemoattractants. These affect a variety of cell types some of which are already present at the site of injury, and others which are recruited from the vascular system or the surrounding connective tissues.

Animal studies have demonstrated that wound healing at the site of glaucoma filtration surgery is essentially the same as that occurring at other sites in the body (Miller, 1988). However, the situation is complicated by the presence of aqueous humour at the operation site which may influence the healing response (Hitchings and Grierson, 1983; Spaeth and Craven, 1987).

The trauma of surgery to the conjunctiva, Tenon's capsule, sclera and iris produces acute inflammation at the operation site. This is manifested by a microvascular response with a brief vasoconstriction of the arteriolar vessels, followed by vasodilation (Dawson and Willoughby, 1985). The endothelial cells of vessel walls constrict, particularly in post-capillary venules, leading to the extravasation of plasma proteins (including fibrinogen and fibronectin) and blood cells. The clotting system is activated; platelet aggregation occurs; mast cells degranulate and damaged cell membranes initiate the arachidonic acid cascade. The clotting system activates Hageman factor, resulting in the formation of kinins. Platelets degranulate, releasing ATP and 5-Hydroxytryptamine, while mast cells release histamine. The arachidonic acid cascade leads to the formation of prostaglandins, leukotrienes and platelet activating factor. The initial movement of solute and proteins out of vessels is followed by a wave of migration of polymorphonuclear leukocytes. Monocytes, which

form macrophages, and lymphocytes follow the emigration of polymorphs. These inflammatory cells are induced to migrate by gradients of chemical mediators in a process which is known as chemotaxis. A great number of substances which are chemotactic for inflammatory cells have been identified (Schiffmann and Gallin, 1979) and include fibroblast derived factors (Sobel and Gallin, 1979), lymphocyte derived chemotactic factor (Snyderman and Pike, 1978), and macrophage derived factors (Wahl, 1985). The macrophages, together with the polymorphs, phagocytose damaged tissue debris. In contrast to the polymorph, the macrophage is capable of replication at the site of inflammation.

When exposed to tissue factors, plasma and blood clot, forming a fibrin-fibronectin matrix. Inflammatory cells migrate into this clot. Macrophages are fundamental in initiating wound angiogenesis (Thakral, Goodson and Hunt, 1979), and new vessels also grow into the clot. Fibrocytes in the edges of the wound, Tenon's capsule and the episclera become activated, proliferate and migrate to the operation site. In rabbit (Seetner and Morin, 1979) and in monkey eyes (Desjardins, et al., 1986) after glaucoma surgery, fibrocytes are activated around the fifth post-operative day.

The fibrin-fibronectin matrix is eventually removed by a combination of fibrinolysis and phagocytosis. Fibroblasts in turn synthesise a matrix consisting of collagen, fibronectin and glycosaminoglycans, and form young fibrovascular connective tissue or granulation tissue. In common with granulation tissue at other sites in the body, this tissue has the capacity to contract, which is seen clinically as anterior migration of the conjunctival scar in a trabeculectomy with a limbus based conjunctival flap. The fibrous tissue becomes organised and remodelled, eventually forming a mature fibrous scar which will vary in extent and in density in different eyes. The reasons for the variability of scar formation are at present unknown.

The wound healing processes culminating in the final common pathway of the activation of fibroblasts are exceedingly complicated. Each one of these processes is theoretically amenable to pharmacological inhibition. However, clinically significant interruption of wound healing has not been convincingly demonstrated by interfering with the wound healing mechanisms at these early stages. Very little is known of the factors which trigger the activation of a "quiescent" fibrocyte

to become an active fibroblast (Silver, 1973; Skuta and Parrish, 1987). As yet, fibroblast behaviour has not been clearly deliniated. However, there are a number of relatively distinct features which may be suitable for pharmacological inhibition. In reality, these processes are not separate entities but are in fact occurring both simultaneously and consecutively. For the sake of simplicity these can be considered as:

1.) Fibroblast activation,
2.) Fibroblast migration,
3.) Fibroblast proliferation,
4.) Synthesis and secretion of the intercellular connective tissue matrix,
5.) Fibroblast contraction, and,
6.) Remodelling to form a mature scar.

A great deal of work has been performed on studying cellular and particularly fibroblast proliferation. Numerous substances which occur in healing wounds and which appear to have a central role in controlling cell proliferation have been identified. These include fibroblast growth factor, epidermal growth factor, platelet derived growth factor, and transforming growth factor beta (Reviewed by Gospodarowicz, 1983; and Pessa, Bland and Copeland, 1987). In addition to their action as fibroblast mitogens, some of these substances are also known to be powerful chemotactic agents for fibroblasts. Thus platelet derived growth factor (Seppä, Grotendorst, Seppä, et al., 1982), brain-derived growth factor which resembles acidic fibroblast growth factor (Senior, Huang, Griffin, et al., 1986), and transforming growth factor beta (Postlethwaite, Keski-Oja, Moses, et al., 1987) are all known to be chemotactic for fibroblasts.

Investigative work on fibroblast anti-proliferative agents has been considerable and has resulted in the use of 5-Fluorouracil in potentially "complicated" trabeculectomies (see section 1.7.4.). Anti-proliferative agents that have been investigated are not specific to the cells involved in wound healing and in particular fibroblasts, but instead affect all rapidly proliferating cells, including corneal epithelial cells. Thus corneal epithelial defects account for one of the major side effects of the treatment with 5-Fluorouracil. Clearly, other angles of attack to inhibit fibroblasts should be explored.

Various substances have been evaluated in an attempt to influence both the secretion of collagen at wound sites, and to influence its subsequent remodelling to form a mature scar. The lathyrogens, D-penicillamine and BAPN, which affect the cross-linking of collagen, have been investigated in both experimental models and in patients undergoing glaucoma surgery (see section 1.7.5.), without convincing evidence of their success. A potentially exciting development of the inhibition of collagen accumulation using amino acids such as cis-hydroxyproline and other analogues, has been demonstrated experimentally, (Lane, Bora, Prockop, et al., 1972; Uitto, Tan and Ryhanen, 1982) however, no clinically successful use of these substances has yet been shown. New compounds such as Malotilate reduce collagen synthesis of fibroblasts in vitro (Poeschl, Rehn, Dumont, et al., 1987) and may show therapeutic efficacy in future.

Fibroblast migration and fibroblast contraction are two aspects of fibroblast behaviour that have received little attention in relation to glaucoma surgery. It is one of the purposes of this thesis to investigate these relatively unexplored fibroblast activities with a view to controlling them. If fewer fibroblasts can be induced to migrate to the wound site, and if those that are there can be prevented from contracting, then wound healing may be inhibited, thereby improving the success rate of glaucoma surgery.

1.8.2. Fibroblast migration

The migration of fibroblasts from the surrounding connective tissues to the operation site has been shown to be fundamental to the healing of surgical wounds in general (Grillo, 1963; Ross, 1968) and to the healing of glaucoma filtration sites in particular (Miller, 1988). An improved understanding of the factors influencing the migration of these cells may allow their behaviour to be manipulated, thus either enhancing or inhibiting wound healing.

Fibroblasts exhibit different types of migratory behaviour (Lackie, 1986). Chemotaxis is defined as the directed migration of cells along a concentration gradient of a chemical substance. Such chemicals are generally diffusable. Most types of chemotaxis are positive, but negative chemotaxis may also occur, ie. migration away from the source of the chemical. Chemotaxis differs from chemokinesis, which is merely the accelerated random migration of cells in response to a chemical

substance. Both processes can however, result in the accumulation of cells in a particular site. Surface-bound gradients of a chemical substance also affect the migration of fibroblasts, and this type of cell movement has been termed haptotaxis (Carter, 1967). It is unclear what the relative importance of these three types of migration is in vivo, but it is likely that they are all interrelated. Fibroblasts are substrate dependant cells and cannot swim. Clearly their ability to move will depend on their attachment to the substate. Substances such as fibronectin which enhance the adherence of fibroblasts to the connective tissue matrix (Hynes and Yamada, 1982) would therefore be expected to influence the movement of these cells.

The exact mechanisms involved in fibroblast migration are not completely understood. Fibroblasts project lamellaepodia which appear to be produced by the rapid assembly of cell membrane at the leading margin of the cell (Harris, 1973). The lamellaepodia adhere to the substrate, and the body of the fibroblast is drawn up to the new adhesion sites by the contraction of the cytoplasmic actomyosin system of the cell (Harris, 1982; Postlethwaite, 1983).

The study of cell migration in the intact animal is extremely difficult due to the complicated nature of wound healing. Therefore, various in vitro experimental techniques for studying fibroblast migration have been devised and some of these are listed in table 1.3:

Table 1.3. Cell motility assays employed in fibroblast migration studies (modified from Albini, Adelmann-Grill and Müller, 1985)

Assay	Authors
Tissue fragment assay	Abercrombie and Heaysman (1953)
Agar overlay technique	Carpenter (1963)
Time lapse cinematography and image analysis	Parkinson (1963)
Wound culture technique	Vasiliev et al. (1969)
Boyden chamber assay	Postlethwaite et al. (1976)
Phagokinetic tracks technique	Albrecht-Buehler (1977)
Agarose droplet assay	Varani et al. (1978)
Three dimensional collagen gels	Bell et al. (1979)

Many of these techniques have subsequently been modified by other authors. The Boyden chamber assay (Boyden, 1962) was originally developed for the study of neutrophil migration, and was modified by Postlethwaite, Snyderman and Kang, (1976) for the investigation of fibroblast chemotaxis. This technique has the advantage of simplicity and the potential for providing quantitative data. Development of the Boyden chamber has produced the 48-well micro-chemotaxis assembly (Falk, Goodwin and Leonard, 1980). Since most in vitro investigations of fibroblast chemotaxis have been undertaken using Boyden chamber-like devices, a similiar technique was adopted in this thesis.

1.8.3. Fibroblast contraction

Healing wounds undergo contraction (Van Winkle, 1967). This is fundamental to the healing process, particularly where excision of tissue has occurred. Contractional forces arise from cells within the granulation tissue of the wound (Ross, 1968). The matrix components of the wound have no direct part to play in the generation of the contractile force as shown by the fact that wound contraction proceeds normally in scorbutic animals which have normal cells numbers but grossly deficient collagen at wound sites (Abercrombie, Flint and James, 1956). It is generally agreed that the cells responsible for wound contraction are fibroblasts in the wound, but the mechanism by which this contraction is brought about is still not resolved.

A fibroblast specialised for contraction was identified in granulation tissue by Gabbiani, Hirschel, Ryan, et al., (1972). This cell was called a "myofibroblast" because of its resemblance to smooth muscle cells. By electron microscopy myofibroblasts demonstrate bundles of parallel fibrils in the cytoplasm, resembling those of smooth muscle cells. These fibrils have subsequently been shown to contain actin, myosin, alpha-actinin and tropomyosin (Eddy, Petro and Tomasek, 1988) and have been called "stress fibres". Myofibroblast nuclei showed multiple indentations or folds, quite unlike that of normal fibroblasts. There were numerous intercellular connections, identified as maculae adhaerentes and gap junctions, between these modified fibroblasts. The connections between the cells were thought to allow the transmission of contractile forces to other cells or to the stroma (Gabbiani, Majno and Ryan, 1973). It was thought that the cells functioned like small muscles, pulling the wound edges together, until newly formed collagen was laid down to maintain the contracted

position. This type of tonic contraction which can occur for protracted periods of time without fatigue has been likened to that occurring in the Catch muscle of molluscs which allows prolonged closure of their shells (Rüegg, 1971).

Isenberg, Rathke, Hulsmann, et al., (1976) demonstrated that stress fibres microdissected by laser from living fibroblasts contract on the application of ATP, and Kreis and Birchmeier, (1980) by using video-intensified fluorescence microscopy showed that stress fibres of fibroblasts are contractile. However, these views have been challenged (Burridge, 1981) and it has been proposed that the presence of stress fibres in fibroblasts indicates cells that have previously contracted and remain in a state of isometric tension (Ehrlich, 1988). A similiar appearance is seen in tissue culture where fibroblasts stop dividing and remain firmly tethered to the unyielding plastic substrate of the culture flask.

A different mechanism for the generation of fibroblast traction has been proposed by Harris, Wild and Stopak, (1980) and by Harris, (1982). By studying fibroblast movement on very thin sheets of cross-linked silicone fluid they observed that cells exert tractional forces which are manifested by elastic distortion and wrinkling of the substratum. Thus compression wrinkles running transverse to the long axis of the cell and tension wrinkles radiating from its ends are seen. Contractile forces generated by fibroblasts would thus be caused by the shear stress produced at the cell-substrate interface, but the generation of this force is still ultimately dependant on the actin-myosin contractile system. This results in the constant rearward movement of the cell membrane in a manner analagous to a tank track. However, unlike a tank track, both the dorsal and ventral surfaces of the cell undergo rearward movement, in a process which requires continual replacement of the cell membrane. The shearing force produced by a fibroblast has been calculated as 2 to 3 orders of magnitude greater than that needed to propel a cell of this size at normal speeds (Harris, Stopak and Wild, 1981). In vivo transmission of the force to the surrounding collagen fibres occurs, resulting in wound contraction and wound remodelling. This traction exerted by the cells is distinct from simple contraction like that of a muscle, as the cells elongate instead of shorten as they compress and stretch the

collagen around them. Lewis, (1984) compared this process to the hand-over-hand hauling in of a rope by a sailor.

Whatever the mechanism of fibroblast contraction, it is clearly a powerful force involved in wound healing. A clear understanding of the process may allow it to be inhibited pharmacologically which may reduce fibrosis at a wound site.

Many different approaches to studying wound contraction have been undertaken. In vivo wound contraction was investigated in guinea pigs by Abercrombie, et al., (1956) and by Grillo and Potsaid, (1961), who concluded that the process was cell mediated. Early in vitro studies were performed by Higton and James, (1964) who studied the contraction of granulation tissue strips from excised rabbit skin wounds, and found that contraction could be inhibited by potassium cyanide which stops aerobic respiration. James and Taylor, (1969) grew chick fibroblasts in vitro and measured the tension generated by sheets of cells using a micro-spring balance. The contraction of granulation tissue was further investigated by Majno, Gabbiani, Hirschel, et al., (1971) who demonstrated pharmacological inhibition of contraction using various drugs. In studying the mechanism of tractional retinal detachment, Kirmani and Ryan, (1985) excised intravitreal fibrocellular membranes produced by penetrating injury in rabbit eyes, and demonstrated both contraction and relaxation of these membranes when they were placed in a tissue-bath attached to a micro-strain gauge.

These experimental systems of wound contraction are complicated. In an intact animal it is impossible to divorce wound contraction from the other factors intrinsic to wound healing, while in strips of granulation tissue the activity of a number of cell types inter-relating with the fibrous matrix, makes interpretation difficult. These techniques would also be very difficult if not impossible to apply to wound contraction at the limbus of the eye.

In an effort to understand fibroblast contraction more clearly, single cell contractile assays have been developed using cells grown in tissue culture. Masuda, Owaribe and Hatano, (1983) developed a contractile model using a variety of mammalian and avian cells, which were permeabilised to allow the ingress of ATP, by exposure to the

non-ionic detergent Triton X-100, thus forming "cytoskeletons". The cells were then placed in a contraction buffer, the constituents of which approximated to the internal environment of the cells. Contraction, which is now well recognised to be actin-myosin based, was initiated by the addition of 3 mM ATP to the cytoskeletons. The contraction elicited by ATP is achieved by the ATP acting as an energy source (Izzard and Izzard, 1975; Isenberg, et al., 1976; Kreis and Birchmeier, 1980). This is the same regardless of whether the cytoskeletons are produced by detergent extraction with Triton X-100 or by glycerination.

The technique of cell permeabilisation using Triton X-100 was developed as an improvement of an alternative method using glycerol. Although glycerination of cells to form cytoskeletons has been used with some success by various authors (Ehrlich, Rajaratnam and Griswold, 1986), Masuda, et al., (1983) found that many cells became detached from the substrate when using this technique, thus making it extremely difficult to apply in a predictable fashion. They also found that the cytoskeletal organisation was better preserved in cells treated with Triton X-100 than with glycerol.

Other models of whole cell contraction have been described. One of these involves introducing cells into semisolid gels of collagen (Bell, Ivarsson and Merrill, 1979). Fibroblasts can condense a hydrated collagen lattice to a tissue-like structure 1/28th of the area of the starting gel in 24 hours. In an effort to provide a suitable environment for the study of proliferative vitreo-retinopathy, various cell types were introduced into vitreous explants (Forrester, Docherty, Kerr, et al., 1986). These techniques of gel contraction do not however distinguish among cell proliferation, migration and contraction. The nature of the collagen in the gel has also been shown to affect the rate and extent of gel contraction since gels rich in type III collagen contract faster and to a greater degree than ones made from type II collagen (Ehrlich, 1988). Likewise, heparin which modulates the organisation of hydrated collagen gels but has no direct effect on the fibroblasts, will inhibit gel contraction (Guidry and Grinnell, 1987). Thus, while cell-containing gels may be useful for studying wound healing, they do not provide a specific assay for cell contraction as they are sometimes purported to do.

In order to try to overcome some of the problems associated with the techniques described above, a novel single cell contractile assay was developed in this thesis. Various drugs were selected to attempt to elucidate the mechanism of fibroblast contraction produced in the assay, as well as to inhibit this contraction. Drugs with anti-contractile activity may ultimately be useful as inhibitors of wound healing at the site of trabeculectomies.

1.8.4. The role of aqueous humour

The role of aqueous humour in the success or failure of glaucoma drainage surgery has long been disputed.

Kornblueth and Tenenbaum, (1956) presented experimental evidence demonstrating an inhibitory effect of aqueous humour on the growth of chick and rabbit fibroblasts and rabbit iris in tissue culture. By contrast, based on observations of patients in whom glaucoma drainage tubes had been implanted, Epstein, (1959) concluded that aqueous excited a proliferation of fibroblasts which was responsible for drainage failure. Exactly the opposite effect was reported by Teng, Chi and Katzin, (1960) who felt that aqueous humour had a degenerative effect on collagen. This effect was not seen if the collagen was protected by intact endothelium.

The literature contains little further information about the effect of aqueous humour on fibrosis until a series of papers from Herschler's laboratory starting in 1980. Herschler, Claflin and Fiorentino, (1980) investigated the effect of human aqueous humour on the growth of autologous subconjunctival fibroblasts. Aqueous humour was taken from 19 patients undergoing cataract extraction who were free of other ocular diseases, and from 45 patients with a variety of glaucomas undergoing filtering surgery. Of the 45 glaucoma patients, 22 had primary open angle glaucoma, 9 neovascular glaucoma, 9 aphakic glaucoma, 1 uveitic glaucoma, and 4 angle closure glaucoma. It is unclear from the paper whether any of these patients exhibited preoperative inflammation. A biopsy of Tenon's capsule was performed at the time of surgery, and plated out in tissue culture with the aqueous humour from that particular patient constituting 100% of the growth medium. Monolayer cultures of fibroblasts derived from unrelated human cadaver skin and sub-Tenon's tissue were also exposed to various concentrations of aqueous humour derived from different

donors. The aqueous humour failed to inhibit further growth of monolayer cultures. However, aqueous humour obtained at the time of routine cataract extraction inhibited outgrowth of fibroblasts from the patient's own Tenon's capsule biopsies in all 19 specimens studied. After 14 days of incubation, the explants were transferred into a tissue culture growth medium, where outgrowth of fibroblasts occurred from 6 of the 19 samples. Eight of the 19 aqueous samples so tested were then used as the growth medium for unrelated Tenon's capsule biopsies. Three of the 8 samples supported outgrowth from the unrelated tissue explant. With 16 of the 45 samples of glaucomatous aqueous humour, fibroblast outgrowth occurred from the autologous Tenon's capsule. This differed significantly ($p < 0.01$) from the results seen with the cataractous specimens. The response to glaucoma surgery was evaluated in the 45 patients from whom samples were taken. A positive relationship between surgical success and fibroblast inhibitory activity was seen ($p < 0.01$), ie. surgery was more likely to be successful where fibroblast outgrowth from the Tenon's capsule biopsy was inhibited by the patient's own aqueous humour. Weaknesses of this study, however, are that no correlation was made between the type of glaucoma, pre-operative inflammation, the fibroblast inhibitory effect of the patient's aqueous humour, and the final surgical result. Thus, most of the surgical failures are likely in the patients with secondary glaucomas, many of whom may have had damaged blood-aqueous humour barriers, with a consequent change in the fibroblast growth promoting activity of their aqueous humour.

These results led Herschler, et al., (1980) to conclude that a primary defect in aqueous humour biology may be an important factor in the pathogenesis of glaucoma and that fibroblast inhibitory activity of aqueous humour may be correlated with success of filtration surgery. However they emphasised that secondary aqueous humour, formed after surgery, was likely to be more important than the primary aqueous humour as sampled in this study.

The hypothesis that changes in aqueous humour may effect the results of filtering surgery was tested by performing experimental surgery in monkeys (Radius, Herschler, Claflin, et al., 1980). Aqueous humour was sampled at the time of surgery and again at various time periods postoperatively. The outgrowth from subconjunctival biopsy specimens was significantly reduced ($p < 0.001$) when preoperative aqueous humour

was used as the growth medium when compared to controls where normal tissue culture medium was used. However, when aqueous humour taken two days postoperatively was used as the growth medium, this trend was reversed and considerably more explants showed outgrowth of fibroblasts. Aqueous humour sampled at 1,2, and 4 weeks post-operatively also supported the growth of subconjunctival fibroblasts in tissue culture. Filtration surgery was routinely unsuccessful. The authors concluded that either material added to the aqueous humour postoperatively as a result of break down of the blood-aqueous humour barrier, inactivated an inhibitor normally present in primary aqueous humour, or that primary aqueous, in contrast to secondary aqueous, lacks sufficient nutrient material to support fibroblast growth in tissue culture.

Herschler's work led him to propose that there was a fibroblast inhibitory factor in aqueous humour which appeared to be diminished or lacking in many patients with glaucoma. He felt that the lack of inhibitory activity in aqueous was related to surgical failure and possibly that this may be a causative factor in the elevation of intra-ocular pressure in primary open angle glaucoma (Herschler, 1981a).

The situation was further complicated by the demonstration of Burke, et al., (1982/1983) that primary aqueous humour was mitogenic for fibroblast cultures but that at high concentrations, cell survival was impaired. The authors concluded that aqueous contains a number of bioactive agents that act in conjunction to both affect cell proliferation and cell survival. They postulated that the ultimate effect on tissues exposed to aqueous humour is therefore dependant on the relative concentration of these substances. A candidate for one of the mitogens in aqueous humour is the 17,000 Dalton polypeptide identified by gel electrophoresis, which appears to resemble fibroblast growth factor (Tripathi, Millard, Tripathi, et al., 1988; Tripathi, Millard and Tripathi, 1989).

Litin, Jones and Herschler, (1985) studied the effect of aqueous humour on protein biosynthesis of rabbit and human Tenon's capsule fibroblasts. They found that 20% aqueous humour stimulated the synthesis of DNA with a concommitant increase of protein synthesis. However, when compared with the generalised stimulation of protein

synthesis elicited by 10% foetal bovine serum, it was found that aqueous humour selectively increased the synthesis of specific proteins. The authors hypothesized that these specific proteins may be involved in cell proliferation, and that targeting drugs to inhibit their activity may be of benefit in reducing fibrosis in relation to glaucoma surgery.

You-qin, Nagy and Spaeth, (1985) investigated the effect of aqueous humour factors on phytohaemagglutinin stimulated lymphocyte transformation. Fresh or frozen aqueous samples derived from cataract patients inhibited lymphocyte transformation. However, aqueous samples derived from patients with primary open angle glaucoma showed a different response. Approximately one third of the samples of fresh glaucomatous aqueous enhanced lymphoproliferation, while two thirds of specimens that had previously been frozen demonstrated such enhancement. Although this paper lacks a great deal of experimental detail, the authors suggested that an inhibitory factor in aqueous humour may be absent in some patients with glaucoma, and that this inhibitory factor could be destroyed by freezing.

Herschler and Litin, (1987) have demonstrated by gel electrophoresis a difference in the relative concentrations of proteins present in the 130,000 to 135,000 Dalton range in the aqueous humour of patients with cataracts when compared to patients with chronic open angle glaucoma. They postulated that the outflow obstruction in primary open angle glaucoma may be related to abnormalities in the aqueous humour protein distribution of susceptible individuals.

The effect of aqueous humour on the migration of inflammatory cells has been investigated by a number of authors. Rosenbaum, Wong, Perez, et al., (1984) found that normal rabbit aqueous humour had no influence over the migration of rabbit polymorphonuclear leukocytes. By contrast, rabbit monocyte migration was enhanced by normal rabbit aqueous humour (Rosenbaum and Raymond, 1985), and a similiar response was seen with human monocytes and aqueous humour derived from patients undergoing cataract extraction. However, the enhanced migration was found to be due to chemokinesis or accelerated random migration (Rosenbaum, Raymond, Fujikawa, et al., 1987).

No enhancement of bovine capillary endothelial cell migration by normal rabbit aqueous humour was demonstrated by Tapper, Scheiner, Frissora, et al., (1981). However, when aqueous humour from patients undergoing cataract extraction or from normal post-mortem eyes was applied to similiar cells, a marginal increase in cell migration was demonstrated (Tapper, Albert, Robinson, et al., 1983).

The influence of aqueous humour on the migration of fibroblasts does not appear to have been studied previously. Since the migration of fibroblasts to the site of a trabeculectomy is fundamental to the healing response, and because the peri-ocular connective tissues in the vicinity of a functioning trabeculectomy are bathed in aqueous humour, it is important to determine the role, if any, of aqueous humour on the migration of these cells. Consequently, the effect of aqueous humour on the migration of ocular fibroblasts has been investigated in this thesis.

1.9. SELECTION OF DRUGS

A number of drugs were investigated as potential inhibitors of migration and contraction, which are the main fibroblast activities studied in this thesis. The drugs were selected on the basis of their mode of action on various cells or systems in the body.

1.9.1. Drugs with activity against the cytoskeleton

Taxol, cytochalasin B and colchicine affect the cytoskeleton of cells in different ways. Taxol is derived from the bark of the Yew tree, Taxus brevifolia (Wani, Taylor, Wall, et al., 1971) and has a unique chemical structure comprising a taxane derivative with an oxetan ring. This ring system is rare in natural products. Taxol has been shown to promote microtubule assembly and blocks cells in mitosis (Horwitz, Parness, Schiff, et al., 1981). Cytochalasin B disrupts microfilaments, leading to a condensation of the actin-containing contractile network, with blebbing of the cell membrane (Britch and Allen, 1981). Colchicine leads to the depolymerization of microtubules, and the consequent "blocking" of cells in mitosis (Borisy and Taylor, 1967).

Since both the contraction and the migration of fibroblasts are dependant on the functioning of the actin-myosin cytoskeleton of the cells, drugs that interfere with the cytoskeleton may be useful inhibitors of these processes. Consequently taxol, cytochalasin B and colchicine were investigated as fibroblast contraction and migration inhibitors.

1.9.2. Drugs which increase intracellular cyclic-AMP levels

Cyclic nucleotides, including cyclic-AMP (cAMP), have a complicated role in cell growth and differentiation (Friedman, 1976). Cyclic-AMP regulates the shape, the motility, the adhesiveness and the growth rate of cultured fibroblasts (Willingham and Pastan, 1975). The shape changes are partially produced by the accumulation of microfilaments and microtubules in bundles near the plasma membrane of the cells. In addition, elevated levels of cAMP have been found to lead to a selective decrease in collagen production by cultured human skin fibroblasts (Baum, Moss, Breuel, et al., 1978). When fibroblasts were exposed to 1.2 mM dibutyryl cAMP they showed greatly reduced migration and a reduction of activity in their ruffled membranes. Cell division

was also diminished, but these effects were reversible on removing the excess cAMP (Johnson, Morgan and Pastan, 1972).

Intracellular cAMP levels can be elevated by a variety of methods. The administration of dibutyryl cyclic-AMP allows passage across cell membranes into the cytoplasm, where the butyryl moieties are cleaved, resulting in elevated cAMP levels. One effect of prostaglandin E_2 is the stimulation of cell membrane adenyl cyclase activity, resulting in increased intracellular levels of cAMP. Theophylline inhibits phosphodiesterase, the enzyme which breaks down cAMP, and therefore leads to the intracellular accumulation of cAMP (Laurence and Bennett, 1980).

In view of the known actions of these drugs, theophylline, dibutyryl cyclic-AMP and prostaglandin E_2 were therefore investigated as inhibitors of fibroblast migration and contraction using the models which have been developed in this thesis.

1.9.3. Calmodulin antagonist

Calmodulin is a peptide which plays a central role in cellular regulation. Among its diverse functions, it activates myosin light chain kinase, which catalyses the phosphorylation of myosin, which in turn brings about the contraction of smooth muscle. It is also concerned with microtubule disassembly and cell membrane phosphorylation (Ganong, 1983). Trifluoperazine and the other phenothiazines, chlorpromazine and promethazine, are calmodulin antagonists.

In view of the concept of fibroblasts behaving like small smooth muscles (myofibroblasts) it seems reasonable to inhibit this activity using calmodulin antagonists. Trifluoperazine was therefore investigated as a representative calmodulin antagonist.

1.9.4. Calcium antagonist

It is widely accepted that calcium is critical for excitation-contraction coupling of all types of muscle cells (Ganong, 1983). Nifedipine is a calcium-channel blocker which interferes with the inward displacement of calcium ions through the slow channels of active cell membranes (British National Formulary, 1988). Its main use clinically is as an anti-anginal agent and as an anti-hypertensive

agent because of its principal mode of action which is to relax arterial smooth muscle in both the coronary and peripheral circulations.

Since fibroblast locomotion and contraction is dependant on an actin-myosin system very similiar to that occurring in smooth muscle cells, it is reasonable to expect that inhibiting the movement of calcium across cell membranes by employing a calcium channel blocker may affect these activities of fibroblasts.

1.9.5. Cholinergic antagonist

Ipratropium bromide is a cholinergic antagonist used for treating asthma. Its mode of action is as a bronchial smooth muscle relaxant, thus producing bronchodilation (British National Formulary, 1988). It seemed worth trying to inhibit the smooth muscle-like activity of fibroblasts using this drug.

1.9.6. Beta$_2$-adrenoreceptor stimulants

Ritodrine hydrochloride is a beta$_2$-adrenoreceptor stimulant used to relax uterine muscle in premature labour and to inhibit hypertonic uterine action (British National Formulary, 1988). Salbutamol is a similiar compound, also used in premature labour, but encountered more commonly as a bronchodilator by virtue of its action in relaxing the smooth muscle of the bronchi. These drugs have powerful actions in inhibiting smooth muscles and in an effort to inhibit the muscle-like action of fibroblasts they were applied in the assays described in this thesis.

1.9.7. Smooth muscle antagonist

Trocinate or beta-diethylaminoethyldiphenylthio acetate is a powerful directly acting smooth muscle antagonist. It has been used clinically to inhibit bladder and ureteric spasm. Madden, Morton and Peacock, (1974), applied trocinate directly to skin wounds of Californian white rabbits. They found that the drug inhibited wound contraction in a dose dependant manner. Local toxicity was not a problem and on removal of the drug, the wounds contracted normally.

Trocinate was therefore investigated as a fibroblast anti-contractile agent in this thesis.

1.9.8. Retinoids

Retinoids have been shown to have pronounced effects on a wide variety of normal and neoplastic cells (Lotan, 1980). The responses seen are diverse and are poorly understood but it is clear that different types of the same cell eg. fibroblasts, do not always respond in the same way to the same stimulus. Retinoids have been shown to inhibit the migration of embryonic human skin fibroblasts. There is also evidence that they inhibit collagen production and proliferation of embryonic and adult human skin fibroblasts and of chick tendon fibroblasts (Hein, Mensing, Müller, et al., 1984). Retinoic acid has, in addition, been demonstrated to have potent effects in reversing squamous metaplasia in keratinising conditions of the conjunctiva (Wright, 1985; Tseng, 1985).

This group of drugs seemed to have properties that would inhibit wound healing in relation to trabeculectomies. The effect of two representative retinoids, retinol (vitamin A) and retinoic acid, was therefore investigated on fibroblast migration.

1.9.9. Drugs used to characterise the contractile response of fibroblasts

A number of drugs were investigated in order to characterise the contractile response of intact fibroblasts in the assay developed in this thesis. These included adenosine, arylazido aminopropionyl adenosine triphosphate ($ANAPP_3$), and Reactive Blue 2.

1.10. IN VITRO STUDIES

Animal experimentation is clearly essential before new therapeutic substances or new surgical techniques are applied to human beings. However, in vivo studies have a number of serious limitations. If large numbers of potential drugs are to be evaluated, then vast numbers of experimental animals will be required. A wide range of drug concentrations will need to be assessed in order to ensure that a failure of therapeutic effect is not merely due to an inappropriate dosage regime. Likewise an unsuitable mode of administration which does not allow access of the drug to its intended site of action may produce a false negative result. Maintaining large numbers of experimental animals is extremely expensive and long follow-up may be time consuming.

Many of these problems can be overcome by the use of in vitro techniques employing cells grown in tissue culture. These techniques allow many of the complicated pathways of wound healing to be dissected, providing the means to study a particular small aspect. A specific cell type can be grown in tissue culture and studied in isolation without the interactions of the numerous cell types involved with wound healing in vivo. The cellular environment can easily be manipulated in vitro, and the effects studied, in a manner which cannot be done in vivo. Direct application of drugs at their intended site of action is easily accomplished in vitro. Tissue culture systems also allow the testing of numerous potential therapeutic compounds at a number of dose levels, in a relatively easy manner. This is facilitated by the fact that in vitro experiments on drug effects are generally of short duration when compared with their in vivo counterparts. Thus many more potential compounds can be assessed in a relatively cheaper environment in vitro than in vivo.

While in vitro systems have many advantages and the reduction in use of experimental animals is desirable, it must be emphasised that in vivo testing of new treatment modalities is still critical. The biggest single problem with in vitro systems is that they may not be clinically relevant. Therefore once potential drugs have been identified in vitro, it is essential to demonstrate their potential

in animal experiments, followed if the drug shows promise by controlled trials in human beings.

1.11. AIMS OF THIS THESIS

The aims of this thesis can conveniently be considered in four categories:

1.) To develop an in vitro system for the study of fibroblast chemoattraction, employing cells appropriate to the investigation of wound healing at the limbus of the eye. These types of fibroblasts viz. Tenon's capsule, scleral and conjunctival fibroblasts will therefore been used throughout this thesis.

2.) To investigate the effects of aqueous humour as a potential chemoattractant for these fibroblasts. Differences between normal aqueous humour and glaucomatous aqueous humour will be evaluated.

3.) To establish a model of fibroblast contraction. This model should be specific for cell contraction, allow the direct observation of the cells undergoing the contraction, involve minimal manipulation of the cells, and be pathophysiologically relevant to wound healing at the limbus of the eye.

4.) To evaluate in vitro a range of drugs as potential inhibitors of fibroblast contraction and fibroblast migration, thereby narrowing down the range of suggested substances to test in experimental animal models of failing glaucoma surgery. Some of the more promising drugs could then be administered to patients undergoing glaucoma surgery in order to improve the success rate of these operations.

2.1. CELL CULTURE

2.1.1. Rabbit cells

Six New Zealand White rabbits of approximately 2.5 kg in weight were used to provide fibroblasts. The animals, which were free of ocular and systemic disease, were sacrificed with an overdose of phenobarbitone. Immediately after death the eyes from 5 animals were enucleated under sterile conditions and transported to the tissue culture laboratory in sterile humidified plastic containers. The dorsal skin of two of the rabbits was shaved and biopsies of approximately 4 cm^2 were taken superficial to the panniculus carnosis. These samples were similiarly transported to the laboratory. The ten eyes from the 5 animals were washed with phosphate buffered saline containing penicillin and streptomycin (Gibco, Paisley, Scotland). Biopsies of conjunctiva, subconjunctival connective tissue (the equivalent of Tenon's capsule in human eyes), and sclera, measuring approximately 2 by 2 mm, were taken and placed in 25 cm^2 plastic tissue culture flasks (Sterilin, Feltham, England). Between 1 and 4 pieces of tissue were placed in each flask. The specimens were allowed to adhere to the base of the flask by drying slightly or were maintained in position under rectangular glass coverslips adhered to the flask with wax (50% petroleum jelly and 50% paraffin). One millilitre of new born calf serum and 3 ml of Ham's F10 medium with 10% new born calf serum containing 100 units/ml of penicillin, 100 ug/ml of streptomycin, and 0.25 units per ml of amphotericin B (Gibco) was added to each flask (complete F10). The flasks were placed at 37°C in an incubator with an atmosphere of 5%CO_2/95%air and left undisturbed for 3 days. The skin samples were similiarly treated.

After 3 days the cultures were observed by inverted phase contrast microscopy (Olympus and Nikon, Tokyo, Japan) and cells growing from the explants were photographed. By the end of one week virtually all primary explants had shown growth. Cultures were fed twice weekly with 5 ml of complete F10 medium. After approximately two weeks the primary tissue explants were removed from the original flasks and transferred to fresh flasks where they were cultured as described above.

By the end of four weeks, cells were removed from their flasks by washing with calcium and magnesium free phosphate buffered saline

(PBS) followed by the addition of 2 ml of 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (BDH Chemicals, Poole, England). Exposure to trypsin was limited to 3 minutes and the reaction was terminated by the addition of complete F10 to each flask. Cultures of ocular fibroblasts from the 5 rabbits were bulked together during passaging and split at a ratio of about 1 to 4 into new flasks. Similiarly, skin fibroblasts from the two rabbits were bulked at first passage.

First passage fibroblasts were grown to confluence within about 2 weeks and then the cultures were prepared for frozen storage. Cells were removed from their flasks as described above and suspended in F10 medium with 20% new born calf serum and 10% ANALAR grade dimethyl-sulphoxide (BDH Chemicals). The cell suspension was centrifuged at 300g for 10 minutes. The supernatant was poured off and the cell pellet resuspended in 1 ml of the above medium. The cell suspension was placed in screw capped vials (Nunc, Denmark) and slowly reduced in temperature to the final storage conditions of -196^{o}C under liquid nitrogen (Taylor-Wharton, Indianapolis, USA). When required for use in the experiments, the vials of cells were rapidly thawed, the cells were suspended in 10 ml of complete F10 and centrifuged at 300g for 10 minutes to wash off the dimethyl-sulphoxide. The cell pellet was resuspended in complete F10 and, at a split ratio of 1 - 2 to 1 - 4, was distributed to plastic flasks. Cells were passaged by similiar methods to those described above at split ratios varying from 1 - 4 to 1 - 10 and were employed between passages 2 and 5 in the experiments.

Fibroblasts were grown initially in complete F10 medium. This was chosen because most culturing of rabbit fibroblasts in the laboratory had previously been done with this medium. In order to check that growth of the cells was optimal in F10, three other media were investigated viz. RPMI (Flow Labs., Irving, Scotland), Eagle's minimum essential medium (Gibco), and 199 (Gibco). Four confluent flasks of 1st passage rabbit conjunctival fibroblasts were bulked and passaged into sixteen flasks. The flasks were divided into four groups and fed with one of the four complete media. Cells were counted in each flask at 1, 2, 3 and 7 days, using a premeasured grid which was placed within the phase contrast microscope. Cells in randomly selected areas were counted within the grid and the total cell count within each flask derived by extrapolation (grid or direct count). At 8 days, one

flask in each group was trypsinised and the cells indirectly counted with a Coulter counter (model ZF, Coulter Electronics Ltd., Luton, England).

Growth curves were obtained by passaging a known number of cells into four flasks. Cell counts were made at 1, 2, 3, 7 and 10 days by a grid count under phase contrast microscopy. Counts beyond 10 days were difficult and probably inaccurate by this technique due to cells piling on top of each other. Comparisons were made between the growth curves of the various cell types that were being cultured.

2.1.2. Human Cells

Human fibroblasts were grown from two sources: peroperative biopsies and from eye bank eyes. Peroperative biopsies were taken in the course of glaucoma surgery from five patients. Pieces of tissue up to about 4 mm^2 in area were taken from the edge of conjunctival flaps and from exuberant Tenon's capsule and placed in transport medium 199 with additional glucose. The patients ranged in age from 37 to 64 years with a mean of 52.2 years. Eye bank eyes reached the laboratory 48 hours post mortem when they were no longer suitable for corneal donation for transplantation. Three donor eyes were obtained that had been removed from cadavers with no previous eye disease. The donors were aged 55 and 75 years in the case of two eyes; the age of the third donor could not be established but was likely to be relatively young as the cause of death was a sub-arachnoid haemorrhage. Specimens of sclera, Tenon's capsule and conjunctiva were obtained from each donor eye.

Primary explants of upto 4 mm^2 were placed in flasks as described in Section 2.1.1. Between 1 and 4 specimens were placed in each flask. Initially the explants were fed with 1 ml of new born calf serum and 3 ml of complete F10 with an additional 10% foetal calf serum (Gibco). The flasks were incubated in identical conditions to the rabbit cells described above. Cultures were fed weekly with 3 ml of complete F10 with an additional 10% foetal calf serum. Growth from the primary explants was slower than with rabbit tissues. All explants were photographed once outgrowth of cells commenced. Explants that had not grown by three weeks were discarded. Proliferation of cells to the point where passage could be contemplated was very variable from culture to culture, and did not appear to be related to the age of the

donor. In some profusely growing cultures removal of the primary explant to a new flask was possible.

Within 4 to 8 weeks cultures that were growing well were passaged as described in section 2.1.1. with split ratios varying from 1 - 2 to 1 - 4. Unlike the rabbit cells, human cells from different donors were not bulked into a single culture. After first passage, growth was again variable, and not all cultures continued to proliferate. Once flasks had confluent cultures, within 4 to 8 weeks, cells were prepared for frozen storage. When required, cells were recovered as described in section 2.1.1.

Previous experience in the laboratory had indicated that F10 was the most appropriate medium for the growth of human fibroblasts. Because of slow growth of these cells, and the evidence from the rabbit cells (see section 2.1.1), experimentation with alternative media was not undertaken.

2.2. CHEMOTAXIS

Chemotaxis was undertaken in 48-well micro-chemotaxis chambers (Neuro Probe, Cabin John, Maryland), based on the original single well Boyden chamber (Boyden, 1962). Both the 48-well chamber and the original Boyden chamber are shown in figure 2.1.

Each of the 48-wells of the micro-chemotaxis apparatus is divided into two. Chemoattractants were placed in the lower wells, which have a volume of 25 ul. The upper wells have a volume of 50 ul, and the cell suspension was placed in these wells, separated from the lower wells by a polycarbonate membrane with pores of 8 um in diameter bored through it (Nucleopore, Pleasanton, California). The membranes are 10 um thick, 25 mm wide and 80 mm long. The pores comprise about 5% of the surface area of the membrane. Cells on the proximal surface of the membrane which sensed the chemoattractant on the distal side, migrated through the pores and remained adherent to the distal side of the membrane. After incubation of the chamber for appropriate time intervals (see section 2.2.1.) at 37°C in an atmosphere of 5%CO_2/95%air, the membrane was removed, fixed in 100% ethanol for 30 seconds, air dried, and finally stained with haematoxylin. The membrane was mounted on a glass slide with aqueous mounting medium and covered with a glass coverslip. Cells that had migrated through the pores in the membrane were counted at 1000X magnification (Olympus, Tokyo, Japan). At this magnification, with its shallow depth of field, it was possible to focus easily on the distal surface of the membrane alone, which enabled migrated cells to be differentiated from non-migrated cells on the proximal surface of the membrane 10 um away. Twenty fields were examined for each of the 48 wells of the chamber; this represented 0.54 mm^2 or 1/15 of the area of each well. A count of 20 fields was chosen as this was consistent with much of the literature on this subject (Postlethwaite, et al., 1976). Quantification of the number of cells migrating through the pores gives an assessment both of the strength of the chemoattractant as well as the capability of the particular cell type to migrate.

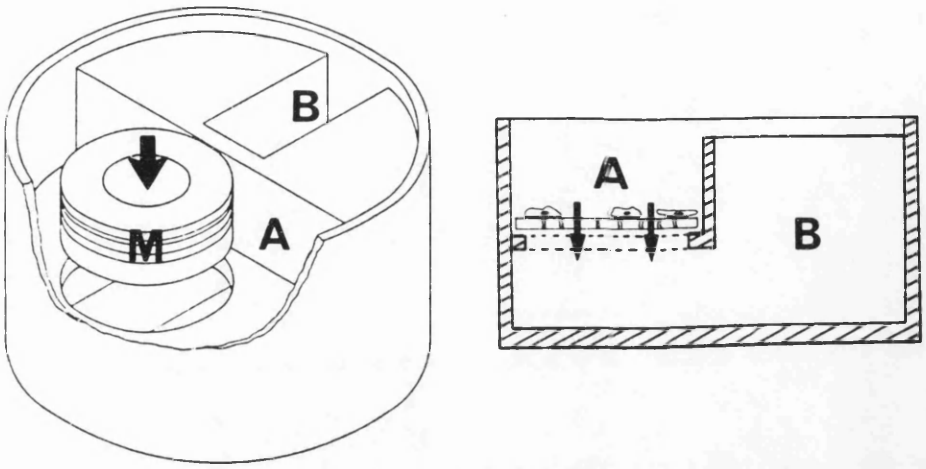
Cells were also counted on the proximal surface of the membrane, ie. non-migrated cells. This enabled the "plating efficiency" or the efficiency of fibroblast adherence to the membrane to be determined. These counts were made in the presence and absence of chemo-

attractants, as well as after subjecting cells to various drugs (see Section 2.2.6.).

A number of different devices are available for the investigation of cell migration, based on the original Boyden chamber (Boyden, 1962). The original Boyden chamber required 3.6 ml of cell suspension and 3 ml of test solution, which obviously makes it unsuitable for the investigation of small volumes of fluid such as aqueous humour (fig.2.1.a.). Smaller modified Boyden chambers or "Blind Well" chambers which are commercially available, employ volumes of test solution as small as 25 ul (Neuro Probe) (fig.2.1.b.). Similiar "in house" devices can also be constructed (Postlethwaite, et al., 1976). A number of these individual "Blind Well" chambers can be run in an array to evaluate a number of test substances simultaneously. However, individual polycarbonate membranes are required for each chamber. Since there is variation between membranes, potential errors are introduced into the experiment. The assembly and disassembly of numerous chambers is also extremely time consuming. The 48-well micro-chemotaxis chamber (fig.2.1.c. and d.) suffers from none of these problems. The 25 ul lower wells allow small volume fluids such as aqueous humour to be evaluated. A single membrane covers all 48 wells, thus reducing variation between wells, and allowing rapid and easy assembly of the apparatus. The 48-wells also allow the simultaneous assessment of numerous test substances or drug dilutions, thus reducing intra-experimental error. Once the incubation period for cell migration has elapsed, subsequent manipulation, orientation and staining of a large single membrane from a 48-well chamber is far simpler and consistent than for 48 individual membranes from "Blind Well" chambers.

Fibroblasts were utilised just as they were becoming confluent, 4 to 7 days after passaging. Rabbit cells were employed between passages 2 and 5 in order to provide sufficient cells for experimentation. Human cells were used at passages 2 and 3 in the hope of more closely mimicing the in vivo situation by employing low passage number cells (Albini, Pontz, Pulz, et al., 1988). Removal of fibroblasts from their flasks was carried out as described in section 2.1.1. Exposure to trypsin was limited to three minutes, since exposure for longer than this is considered to reduce chemotactic activity (Postlethwaite, et al., 1976). The reaction was terminated by the addition of 8 ml of

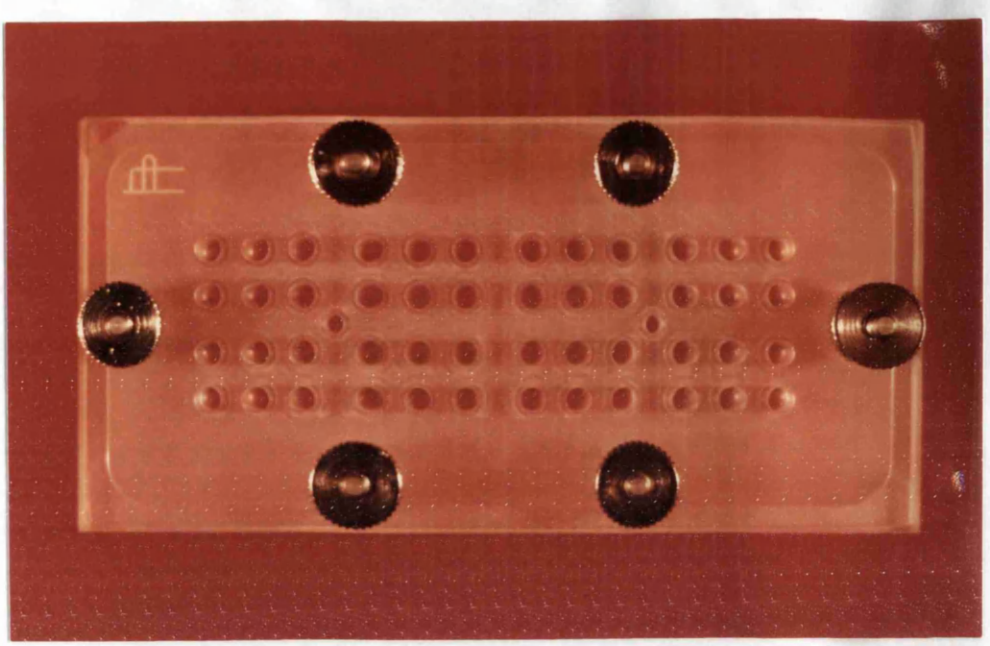
a



b

Fig.2.1. Apparatus employed for studying chemotaxis. a.)Diagram of the original Boyden chamber. The cell suspension of volume 3.6 ml is placed in compartment A. Compartment B holds 3 ml of the test solution. The porous membrane (M) is placed between the two chambers.; b.)Photograph of a single blind-well chemotaxis chamber. The lower well within the clear plastic cylinder has a volume of 25 ul. The upper well within the white screw-on cap has a volume of 50 ul.

c



d

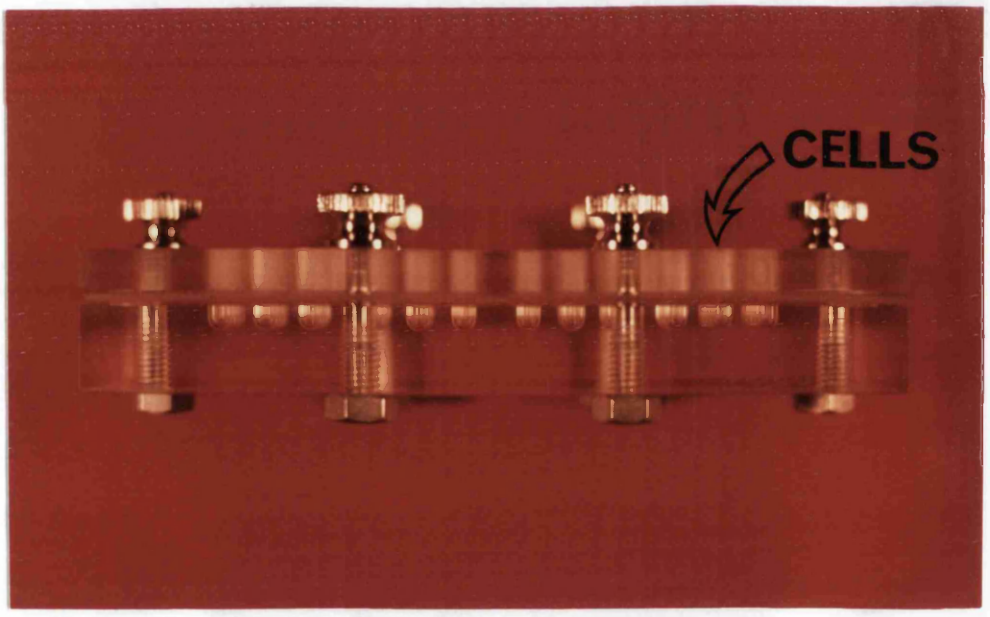


Fig.2.1. (Cont'd) Apparatus employed for studying chemotaxis. c. and d.)Photographs of the 48-well micro-chemotaxis chamber, c.)Plan d.)Elevation. Cells are placed in the upper wells (50 ul) as indicated. The lower wells (25 ul) contain the chemoattractant and are separated from the upper wells by the porous polycarbonate membrane.

complete F10 medium to each flask. The cell suspension was centrifuged at 300g for 10 minutes, and washed once with serum free F10. Cell counts were made in a Coulter counter (model ZF) and the cells were finally suspended at a concentration of $7x10^5$ cells/ml. Thus 35,000 cells were added to each of the upper wells of the chemotaxis chamber.

2.2.1. Base line evaluations

A pore size of 8 um in the polycarbonate membranes was chosen as this had been shown by most other investigators to be an effective diameter for fibroblast migration (Albini, et al., 1985). Polycarbonate membranes are available with or without the wetting agent polyvinyl-pyrrolidine (PVP). Untreated PVP free membranes have previously been shown not to allow the adherence of fibroblasts and required coating with gelatin (Postlethwaite, et al., 1976), while PVP free membranes are said to promote the adherence of these cells (Neuro Probe catalogue). In order to determine the optimal type of membrane to use and whether any treatment of the membranes with substances known to facilitate the adherence of fibroblasts was required, the following types of membranes were prepared:

a.)Untreated polyvinyl-pyrrolidine free membranes (-PVP)

b.)Untreated membranes with PVP.

c.)Gelatin. PVP membranes were placed in a glass beaker containing 0.5% acetic acid at 50°C for 20 minutes. They were then washed twice in glass distilled water, following which they were placed in a solution containing 5 mg/l Porcine gelatin type I, 300 bloom (Sigma, Poole, Dorset) in distilled water at 100°C for 1 hour. The membranes were then air dried, and subsequently placed in an oven at 100°C for 1 hour (Modified from Postlethwaite, et al., 1976).

d.)Fibronectin. Membranes were placed in a solution of 100 ug/ml of bovine fibronectin (Sigma) in distilled water for 30 minutes and then air dried (Ungari, Katari, Alessandri, et al., 1985).

e.)Poly-L-Lysine. Membranes were soaked in 1% Poly-L-Lysine (MW > 300,000 Sigma) in distilled water for 2 hours and then washed in distilled water for 2 hours. They were finally air dried (Senior, Griffin and Mecham, 1982).

In order to determine the optimal time for incubation of the chemotaxis apparatus, a gelatinised PVP membrane was cut into quarters and each piece was used in 4 separate 48-well micro-chemotaxis chambers. An optimal concentration of bovine fibronectin was used as

the chemoattractant (see section 2.2.2.); rabbit Tenon's capsule fibroblasts and human scleral fibroblasts were used as the indicator cells. One chamber was sampled after incubation for 2, 3, 4, and 6 hours.

2.2.2. Fibronectin

Fibronectin has previously been found to be a chemoattractant for various types of fibroblasts (Postlethwaite, Keski-Oja, Balian, et al., 1981; Seppä, Yamada, Seppä, et al., 1981; Mensing, Pontz, Müller, et al., 1983). A dose-response curve was established for fibronectin employing both human and rabbit ocular fibroblasts. The optimal chemoattractant dose of fibronectin was then applied in all assays as a positive control for migration.

Bovine fibronectin derived from plasma (Sigma) was utilised throughout. It was dissolved in distilled water to a concentration of 1000 ug/ml, aliquotted, and stored at -20°C. When required, samples were rapidly defrosted and great care was taken to minimize storage at room temperature in order to reduce the avid binding of fibronectin with the walls of its storage vessel. Further dilutions were made with serum free F10 medium and concentrations of fibronectin varying from 5 to 1000 ug/ml were assessed.

Once the dose-response curves for fibronectin had been established, fibronectin was submitted to a Zigmond-Hirsch chequer-board analysis to determine whether the chemoattractant effect observed was due to chemotaxis or chemokinesis (Zigmond and Hirsch, 1973). In this procedure the cells are subjected to three types of situation, which are clarified in fig.2.2.:

i.)By adding the chemoattractant (fibronectin) to the lower wells of the chamber alone, the cells are exposed to a progressively increasing positive gradient of chemoattractant. An increase in the number of migrating cells indicates a chemotactic effect (red bar), ie. enhanced directed migration of the cells along a concentration gradient of a chemical substance.

ii.)By adding fibronectin to the upper wells alone, in conjunction with the fibroblasts, the cells are exposed to a progressively increasing negative gradient of chemoattractant. An increase in cell migration indicates a chemokinetic effect (green bar), ie. enhanced random migration of the cells in response to a chemical substance.

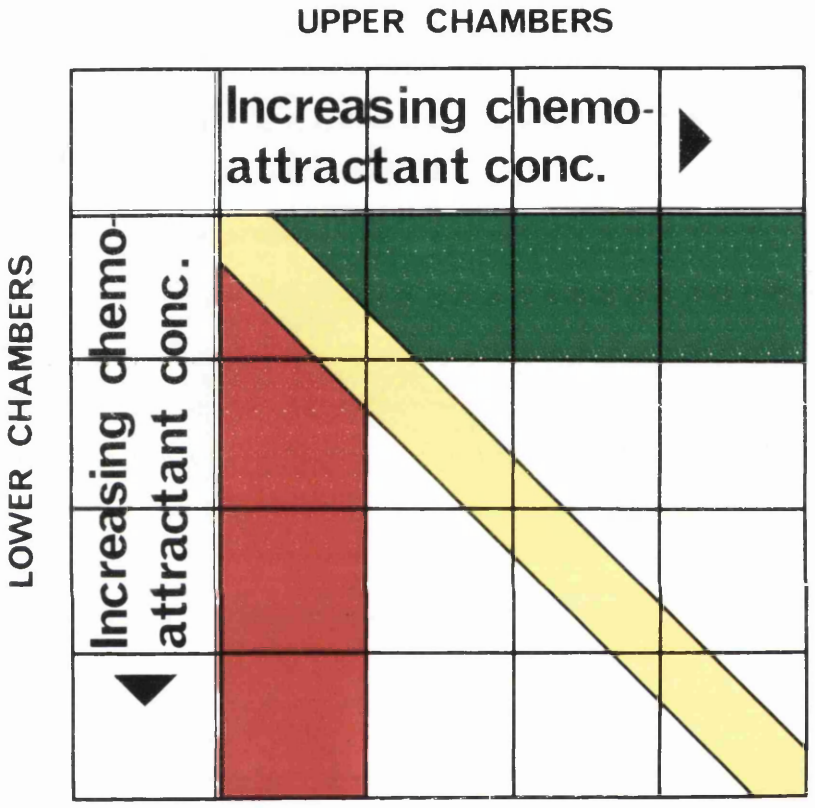


fig.2.2. The organisation of a Zigmond-Hirsch chequer-board

iii.)By adding fibronectin to both upper and lower wells of the chamber, the cells are exposed not to a gradient but merely to an increasing concentration of chemoattractant, equal in both upper and lower wells. An increase in cell migration also indicates a chemokinetic effect (yellow bar).

2.2.3. Fibroblast conditioned medium

In this procedure the potential chemoattractants synthesised by cultured cells and secreted into the growth medium were collected. This provided a cheap, readily available source of chemoattractants. A confluent culture of rabbit scleral fibroblasts at second passage was washed three times with phosphate buffered saline. It was then washed twice with 10 ml of serum free F10 and a further 5 ml of this medium was incubated in the flask for 24 hours. This fibroblast conditioned medium was centrifuged at 300g to remove floating cells. The supernatant was aliquotted and frozen at -20^{o}C until used in the chemotaxis assay (Mensing, et al., 1983).

2.2.4. Rabbit aqueous humour

A total of 20 young adult New Zealand White rabbits (40 eyes) weighing approximately 2 kg were used in the study. All rabbits were free of ocular and systemic disease. Seventeen of the rabbits were sacrificed by an overdose of barbiturates. Immediately post mortem, a paralimbal transcorneal paracentesis was made using a 25G needle, which was introduced into the mid anterior chamber, with great care taken not to touch any intra-ocular structures. Aqueous humour was allowed to drip from the end of the needle under the influence of the intra-ocular pressure, until approximately 100 ul had been collected in plastic vials, from each eye. The specimens were either used immediately in experiments or were frozen to -20^{o}C for storage. Under these conditions chemoattractant activity was maintained for at least 1 year.

The aqueous humour was used as a chemoattractant for rabbit ocular fibroblasts, and concentrations from 4 to 100% were evaluated. Having established that aqueous had a chemoattractant effect, chequer-board analyses (Zigmond and Hirsch, 1973) was then performed to establish whether the migration was due to chemotaxis or chemokinesis. In order to define the chemoattractants, aliquots of rabbit aqueous humour were boiled for five minutes and were then tested for chemoattractant

activity. Boiling was performed in sealed containers, thus maintaining the volume of the sample. Boiled samples were also submitted to chequer-board analysis (Zigmond and Hirsch, 1973).

Paracentesis of rabbit aqueous humour has been shown to disrupt the blood-aqueous barrier in the secondarily accumulated aqueous humour (Unger, et al., 1975). In order to determine whether the described technique of sampling rabbit aqueous humour was causing a breakdown of the blood-aqueous barrier in the primarily sampled aqueous, three rabbits were pretreated before paracentesis with drugs known to stabilize the blood-aqueous barrier.

Animal 1 Platelet activating factor inhibitor (PAF inhibitor).
The rabbit was treated with an oral dose of 40 mg of the PAF inhibitor, BN52021 in water (Ipsen, Paris, France, kindly supplied by Prof.DA Willoughby) one hour before sacrifice (van Haeringen, Verbeij and van Delft, 1987). Aqueous humour was sampled from both eyes as described above.

Animal 2 Aspirin.
The rabbit was anaethetised with intravenous phenobarbitone and 600 mg soluble aspirin was administered per rectum (Miller, Eakins and Atwal, 1973). After 1 hour the animal was sacrificed and aqueous was sampled as above.

Animal 3 Indomethacin.
The rabbit was anaethetised, 20 mg of indomethacin (Sigma) was administered intravenously and one drop of 10 mg/ml indomethacin to each eye every 10 minutes. (Unger, et al., 1975). After 30 minutes the rabbit was sacrificed and aqueous humour sampled as above.

Aqueous from both eyes of the three pre-treated animals was compared for chemoattractant activity with aqueous taken from both eyes of a randomly selected control animal, at concentrations varying from 4 to 40%.

2.2.5. Human Aqueous Humour

Samples of aqueous humour were taken from patients undergoing intraocular surgery. All specimens were taken as the first intraocular manoeuvre as close to the start of surgery as possible. A 25 or 27G

needle was passed into the anterior chamber either through a paracentesis made as part of the normal surgical procedure or through a partial thickness groove prior to completion of a cataract section. The needle was observed at all times under the operating microscope and great care was taken not to make contact with any intra-ocular structures and to aspirate the aqueous as gently as possible. Aqueous was aspirated into a 1 ml syringe. Approximately 100 ul was obtained from each patient, without totally flattening the anterior chamber. Due to the small volume of the specimens, not all of the experiments could be performed on all of the specimens. Samples were therefore chosen at random for a particular experiment.

A total of 58 specimens of aqueous humour, from 58 eyes, were obtained as outlined in table 2.1. Nine of these specimens of aqueous were obtained from patients undergoing reoperation, and are documented in table 2.2. All previous surgery on these patients had been undertaken at least 10 months before the present study. Of the nine patients in this group, seven had previously undergone glaucoma surgery that had failed. The other two patients were prospectively included in this category. One patient had not had previous glaucoma surgery, but was included as she was 20 years old, aphakic, with a previous encirclement for a retinal detachment, and with previous strabismus surgery. The other patient was 27 years old with Rieger's syndrome and high myopia. She had not previously undergone intraocular surgery but underwent implantation of a silicone tube and gutter, based on the expectation of an unsuccessful trabeculectomy.

Of the 23 patients undergoing a first trabeculectomy, 17 patients had primary open angle glaucoma, while 6 had primary open angle glaucoma with narrow angles. Of the 6 patients with a narrow angle component to their glaucoma, 3 had undergone YAG-laser iridotomy 9, 24 and 32 months, respectively, pre-operatively while a fourth patient had undergone a surgical transcorneal peripheral iridectomy 12 months preoperatively. Thus, 22 of the 23 patients had undergone no previous ocular surgery. For each of the 23 patients the following data was recorded from the notes: Intra-ocular pressures (IOP) at diagnosis of glaucoma, immediately before surgery, and at each follow-up visit; pre- and post-operative visual acuities; pre- and post-operative disc appearance; and pre- and post-operative visual fields, although the latter were not available for all patients. These parameters are

Table 2.1. Showing the derivation of all samples of human aqueous humour

Source	Number of specimens	Mean age (+/-SD) [years]
Extracapsular cataract extractions	26	70.8+/-12.8
Trabeculectomy as primary antiglaucoma therapy (Primary trabeculectomy)	8	67.2+/-12.0
Trabeculectomy after failed medical therapy (Secondary trabeculectomy)	15	71.9+/-11.6
Reoperations:		
Second trabeculectomy	2	41.8+/-21.7
Implantation of silicone tube	7	

Table 2.2 Clinical histories of patients undergoing reoperation

Number	Sex	Age	Diagnosis	Operation	No. of previous glaucoma operations	Lens status	Other operations
1	F	49	Congenital cataracts	Tube	4	Aphakic	2
2	M	69	Penetrating injury 3lyrs ago	Tube	2	Aphakic	3
3	F	59	Chronic narrow angle glaucoma	Tube	2	Aphakic	1
4	M	37	Chronic anterior uveitis	Tube	3	Aphakic	1
5	F	51	Heterochromic cyclitis	Tube	1	Aphakic	1
6	F	20	Congenital cataracts	Tube	0	Aphakic	4
7	F	61	Primary open angle glaucoma	Trab.	3	Phakic	0
8	M	3	Buphthalmos	Trab.	1	Phakic	0
9	F	27	Rieger's syndrome	Tube	0	Phakic	0

Tube = Inplantation of silicone tube and gutter (Schocket, et al., 1982)

Trab. = Trabeculectomy

detailed in table 3.1. (page 201). Observations on the optic discs of the patients were made by a number of different observers, which precluded obtaining accurate comparative data. Discs were therefore categorised as normal, mildly cupped with a cup/disc ratio of less than 0.5, moderately cupped with a cup disc ratio between 0.5 and 0.8, and severely cupped with a cup disc ratio greater than 0.8. Likewise visual fields were performed on a number of different perimeters with different operators. Consequently, fields were categorised as normal, mildly abnormal, moderately abnormal with a field defect in one hemifield only, or severely abnormal with field defects in both upper and lower hemifields. While these estimates of the optic disc and the visual field are crude, they allow some quantification of the severity of glaucoma in an individual patient.

All 26 patients undergoing cataract extraction had no other ocular disease and had not previously undergone ocular surgery.

The aqueous specimens were derived from 2 sources: Moorfields Eye Hospital, High Holborn, London, and from Addenbrookes' Hospital, Cambridge (Mr.Peter Watson). However, at each hospital, a number of different surgeons contributed to providing the samples. All the patients undergoing primary trabeculectomies were under the care of Mr.Watson and he also provided 8 of the secondary trabeculectomy specimens. Cataract extractions were performed by two surgical techniques: via a corneal section or via an ab externo limbal approach. Accordingly, aqueous specimens from patients with cataracts were either taken via a corneal groove or via a limbal groove after first elevating a conjunctival flap.

Specimens of aqueous humour were assessed shortly after they had been removed from the patients or were stored at -20^{o}C. There was no difference in chemoattractant activity after freezing, and specimens maintained their activity for at least 12 months of storage at this temperature. Dilutions of samples were made with serum free F10 medium. Chemoattractant activity was assessed at concentrations varying from 4 to 100%. Initially rabbit Tenon's capsule fibroblasts were used as the indicator cells and results were then confirmed using human cells.

In order to determine whether pH gradients might exist across the polycarbonate membrane in the chemotaxis chamber, partially accounting for the cell migration, the pH was measured in the top and bottom wells. Cataractous aqueous humour from one patient, with concentrations varying from 4 to 40%, provided the chemoattractants. The pH of the wells was measured three hours after the start of the assay using a dipcast plastic pH electrode (Modified from Band, Fry and Treasure, 1977) which allowed accurate measurements in volumes as small as 25 ul (the volume of the lower wells of the chemotaxis chamber). The chamber was allowed to equilibrate in air before the measurements were made.

Chequer-board analyses were performed on aqueous to determine whether the chemoattractant effect was due to chemotaxis or chemokinesis (Zigmond and Hirsch, 1973). This was done both on cataractous and glaucomatous aqueous humour, using both rabbit and human ocular fibroblasts as the indicator cells.

Ultrafiltration experiments were undertaken initially using rabbit aqueous humour. Then aqueous from one patient undergoing extracapsular cataract extraction was passed through an ultrafilter with a 30,000 Dalton cut-off (Amicon Centrifree with YMT membrane, Danvers, USA). The ultrafiltrate was run in a 48-well chemotaxis chamber with unfiltered aqueous from the same patient and fibronectin for comparison. A filter of this type was chosen as protein adsorbs to it very poorly.

In order to determine the stability of the aqueous humour chemoattractants to pH changes, 0.1 M NaOH or HCl was added to aliquots of cataractous aqueous humour with an initial pH 7.2 to increase the pH to 11 or reduce it to 3. The altered pH was maintained for 45 minutes and then returned to the baseline by the addition of acid or alkali as appropriate. A chemo-attraction assay was run with untreated aqueous for comparison, at concentrations varying from 4 to 40%.

The thermal stability of the aqueous humour chemoattractants was evaluated by boiling aliquots of aqueous humour for 5 minutes in sealed containers, thus maintaining the volume of the samples. Chemoattractant activity was then compared with that of aliquots of

the same unboiled specimens. Boiled aqueous humour was also assessed in chequer-board arrays.

In order to assess the integrity of the blood-aqueous barrier, the aqueous humour from patients undergoing cataract extraction was subdivided according to whether the patients were placed on Guttae Indomethacin 1% q.d.s. to the operated eye pre-operatively. This was used by some of the surgeons to reduce peroperative miosis. The decision to use indomethacin was made entirely independently of the current study. Cataractous aqueous humour was also subdivided for analysis according to the surgical approach to cataract extraction and the surgeon operating.

Protein estimation was performed on nine randomly selected specimens of cataractous aqueous humour using the method of Lowry, Rosebrough, Farr, et al., (1951). Human quality control serum (NEQAS, Birmingham, England) was used as standards and the results were read using a Bichromatic Analyzer 100 (Abbott Laboratories, Chicago, USA) between 550 and 650 nm.

2.2.6. Drugs

Drugs were used to attempt to inhibit the migration of fibroblasts to chemoattractants. Initially an optimal dose of fibronectin was used as the chemoattractant. Drugs that showed activity as inhibitors of chemo-attraction were then assessed with an optimal dose of rabbit aqueous humour used as the chemoattractant.

In the experimental work described in this thesis, rabbit ocular fibroblasts responded in a qualitatively similiar manner to human ocular fibroblasts, when exposed to chemoattractive stimuli, including aqueous humour. However, in general fewer human cells migrated in a similiar time period, than rabbit cells, although this was not invariable. Human cells proved more difficult to grow than rabbit cells. They multiplied at a slower rate and passaging was inconsistent with all but a few cultures. Since human cells were in relatively short supply and were more difficult to manipulate than rabbit cells because they are less robust, it was decided to perform drug testing using rabbit cells as a first line.

The following drugs were assessed after addition to the upper wells of the 48-well micro-chemotaxis apparatus, together with the cells:

Colchicine 10^{-9} to 10^{-3}M (Sigma)
Cytochalasin B 10^{-10} to 10^{-5}M (Sigma)
Taxol 10^{-10} to 10^{-5}M (National Cancer Institute, Bethesda, USA)
Theophylline 10^{-12} to 10^{-3}M (Sigma)
Dibutyryl cyclic-AMP 10^{-12} to 10^{-3}M (Sigma)
Prostaglandin E_2 10^{-11} to 10^{-3}M (Sigma)
Trifluoperazine 10^{-12} to 10^{-3}M (Sigma)
Nifedipine 10^{-11} to 10^{-3}M (Bayer, Newbury, England)
Ritodrine HCl 10^{-8} to 6.18×10^{-3}M (Duphar Laboratories, Southampton, England)
Ipratropium Bromide 10^{-9} to 10^{-4}M (Boehringer Ingelheim, Bracknell, England)
Salbutamol 10^{-7} to 10^{-3}M (Allen and Hanburys Ltd, Greenford, England)

The following drugs were incubated with the cells for 3 days prior to the addition of the pretreated cells, without further drugs, to the upper wells of the apparatus (Hein, et al., 1984):

Retinol 10^{-12} to 10^{-4}M (Sigma)
Retinoic acid 10^{-13} to 10^{-5}M (Sigma)

All drugs besides taxol, cytochalasin B, prostaglandin E_2, nifedipine, retinol and retinoic acid were readily water soluble and were diluted with serum free F10 medium. Taxol and cytochalasin B were dissolved in ANALAR grade DMSO (BDH Chemicals), aliquotted and stored at -20°C. Further dilutions were then made with serum free F10. The other water insoluble drugs were solubilised in a small amount of ANALAR grade absolute ethanol, and aliquotted. The retinoids were stored in vials in liquid nitrogen. Further dilutions were made with serum free F10 medium. Appropriate controls using diluent and no drugs were incorporated into the experiments.

Ritodrine HCl was obtained both as pure substance and dissolved in buffered sodium chloride for intravenous injection. Ipratropium bromide was obtained in the form of unit dose vials for administration via nebuliser. This contained the drug dissolved in sodium chloride with the pH maintained at 3.4 with HCl. Pure ipratropium bromide

powder was also obtained. Salbutamol was obtained only in the form of the intravenous preparation which consisted of salbutamol sulphate in sodium chloride with the pH maintained at 3.4-3.6 with small amounts of sulphuric acid or NaOH (Personal communications from the manufacturers).

2.2.7. Error check

Inter-observer error for counting cells through the membrane was checked by getting an additional experienced observer to count the same membrane. Intra-observer error was assessed by recounting, by the same observer, of cells migrated through a membrane. The percentage error of the counts is given by the equation:

$$\text{Percentage error} = \frac{\frac{X_1+X_2}{2} - X_1}{\frac{X_1+X_2}{2}} \times \frac{100}{1} \%$$

Where X_1 is the first count and X_2 is the second count.

2.3. CELL CONTRACTION

2.3.1. Triton X-100 cytoskeletons

Cytoskeletons were prepared according to the modified technique of Masuda, et al., (1983) and Grierson, Millar, De Jong, et al., (1986). A culture of rabbit ocular fibroblasts was passaged at a split ratio of 1 - 10. Approximately 3 days later when the cells were still sparsely distributed with about 10 cells per field of the phase contrast microscope (X20 objective), the cells were washed twice with phosphate buffered saline and exposed to a 0.2% solution of the non-ionic detergent Triton X-100 (BDH Chemicals) in a stabilisation buffer at room temperature. The stabilisation buffer consisted of 0.01 M Tris-HCl, pH 7.6, 0.14 M NaCl, 0.005 M $MgCl_2$, and 4% polyethylene glycol 6000. Thereafter, the cells were washed in phosphate buffered saline and the flask mounted on the stage of an inverted phase contrast microscope (Olympus). The stage of the microscope was encased by an incubation chamber maintained at 37°C. Cells were observed with the X20 objective lens and photographed on 35 mm film (fig.2.3.). The phosphate buffered saline was aspirated via a fine polythene tube secured at the neck of the flask, and replaced via another tube, with either contraction buffer alone or contraction buffer containing disodium Adenosine Triphosphate (ATP) (Sigma) varying from 0.01 mM to 1 mM. Photographs were taken every minute for the first ten minutes and then every 10 minutes up to one hour. The contraction buffer after Kreis and Birchmeier, (1980) consisted of 30 mM KCl, 10 uM $CaCl_2$, 10 mM Tris-Cl (pH 7). The cytoskeletons were measured according to the method of Grierson, et al., (1986): the 35 mm negatives were examined with a film reader (Zeiss, Jena, East Germany) and the cell outline drawn. The area of the drawing was measured with a semi-automated image analyser (MOP-Videoplan, Kontron, West Germany). A dose-response curve and a time-function curve were generated for fibroblast cytoskeleton contraction.

2.3.2. Whole cells

Rabbit ocular fibroblasts growing in sparse culture approximately three days after passaging, were washed three times in Dulbecco's phosphate buffered saline with calcium (0.9 mM) and magnesium (0.5 mM). The flask was then mounted on the stage of the microscope as described above. The Dulbecco's PBS was aspirated and replaced with serum free F10 medium containing 25 mM Hepes buffer (Gibco) or with a similiar medium containing concentrations of ATP varying from 0.1 mM

to 20 mM. The additional buffering capacity of the Hepes was required since the cells were maintained in air and not their usual atmosphere containing 5%CO_2.

Cells were photographed as described above in 2.3.1. (fig.2.3.). In addition they were filmed using time-lapse photography on 16mm cine film (Bolex, Switzerland).

Dose-response curves were generated using all four types of rabbit fibroblasts that had been cultured viz. conjunctival, Tenon's capsule, scleral, and skin fibroblasts. From the dose-response curves a dose of ATP that produced half the maximal response was selected for use in experiments with various drugs. In practice this varied somewhat from day to day. Therefore, a number of doses of ATP were tested at the start of each experiment in order to establish the half maximal response.

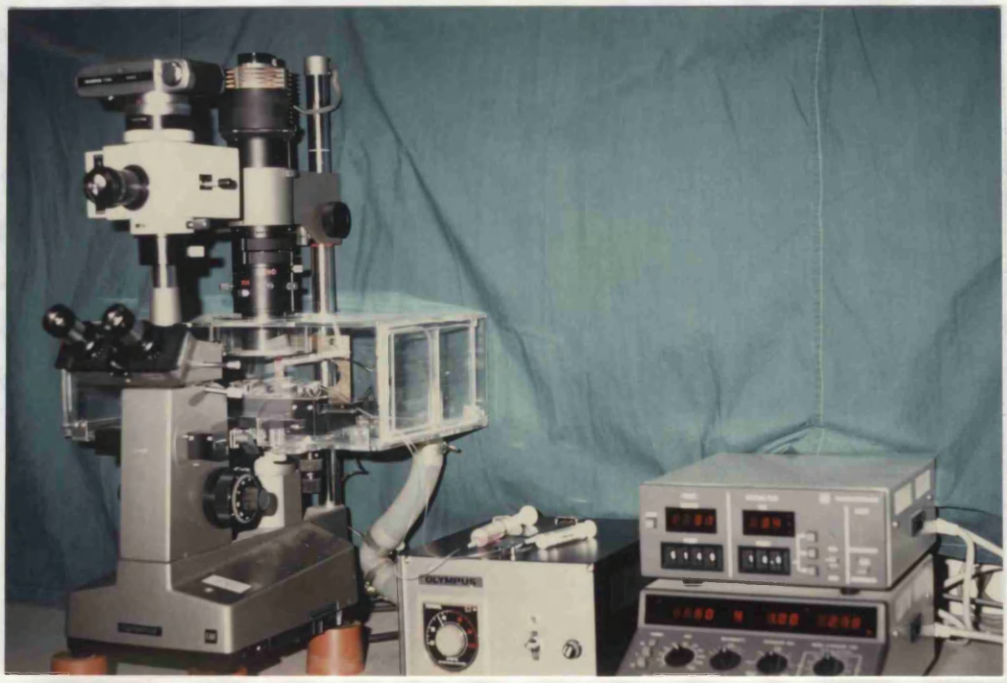
A time-function curve for the response was generated by measuring the area of the contracting cells at each time interval that the cells had been photographed.

Relaxation of the whole cells was achieved by aspirating the ATP and washing the cells three times with serum free F10 medium with 25 mM Hepes. The cells were then left in this medium for one hour during which they were photographed at ten minute intervals. ATP was then added to the cells and the process repeated. This was filmed using time-lapse cine photography upto seven cycles of contraction and relaxation.

The osmolality of F10 medium containing various concentrations of ATP was measured by the technique of freezing point depression using an Osmometer 3L (Advanced Instruments, Massachusetts, USA). This was compared with the osmolality of Dulbecco's phosphate buffered saline and F10 medium without ATP.

Whole cells were placed in Dulbecco's PBS without calcium and magnesium. Calcium and magnesium were then separately replenished, in steps, to the levels found in F10 medium and Dulbecco's PBS with calcium and magnesium. The cells were monitored as described above.

a



b

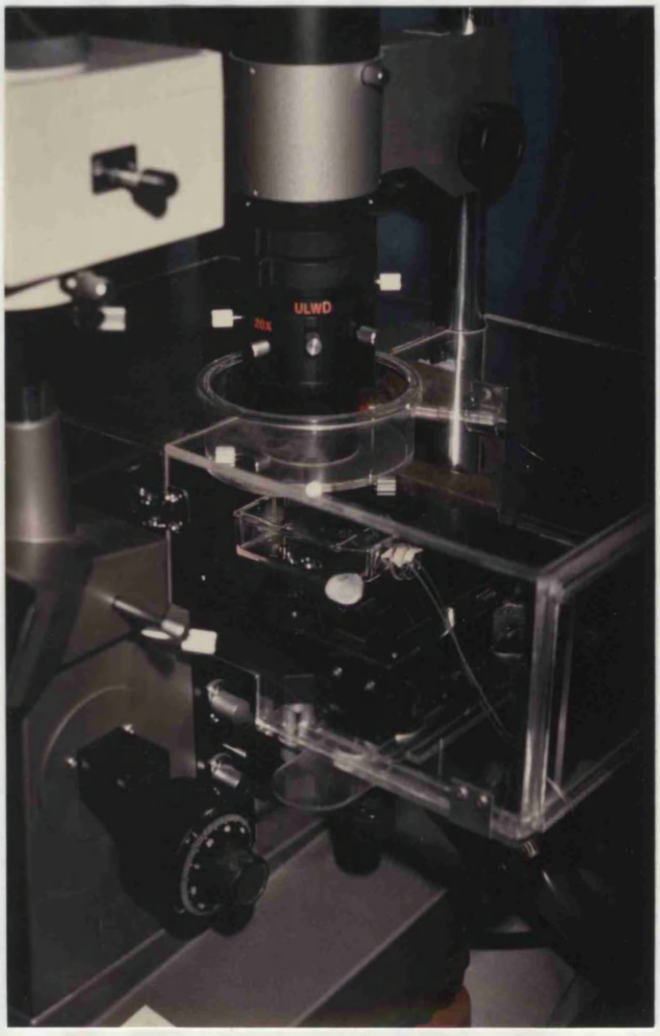


Fig.2.3. Photographs of the contraction apparatus a.)On the left of the photograph is the inverted phase-contrast microscope. The stage of

the microscope is encased by a perspex incubation chamber. A plastic flask of cells is seen on the stage. Polythene tubes attached to syringes are used to add and remove fluids from the flask. The black box in the middle is a heating unit which maintains the incubation chamber at 37°C. The camera control unit is on the right. This controls either a 35 mm camera (shown in the picture on top of the microscope) or a 16 mm cine camera. b.)Close-up view of a flask on the microscope stage.

2.3.3. Drugs

Various drugs were used in order to clarify and characterise the contractile response of the intact cells to ATP. Drugs with potentially antagonistic effects were also tested on the contractile assay.

Drugs used to characterise the response of the cells to ATP.

a.)Adenosine. In order to establish whether ATP was directly active or was first being broken down to adenosine, adenosine (Sigma) was applied to the intact cells in an identical manner to that described for ATP.

b.)Arylazido aminopropionyl adenosine triphosphate ($ANAPP_3$). This drug was kindly donated as a gift by Jeffrey S. Fedan. The compound has been shown to be a specific antagonist of ATP in some tissues (Hogaboom, O'Donnell and Fedan, 1980) via an effect on P_{2X}-Purinoceptors (Burnstock and Kennedy, 1985). All manipulations of the drug were carried out in the dark because of the change in $ANAPP_3$ receptor binding produced by exposure to light. Dilutions were made with Hepes buffered serum free F10 medium. Flasks of rabbit Tenon's fibroblasts were treated with concentrations of $ANAPP_3$ from 10^{-6} to 10^{-4}M, and maintained in the dark for 40 minutes, except for 1 photograph every 10 minutes. A dose of ATP was then added to the flask so that after dilution with the $ANAPP_3$ containing solution, the cells were exposed to a concentration of ATP which elicited half a maximal contraction. In practice this varied slightly from day to day, depending on the response of the cells. Control cells were therefore tested at the start of each experiment, and 5 or 7 mM ATP was selected as approximately half the maximally effective dose. The cells were then photographed at one minute intervals for the first 10 minutes and at ten minute intervals up to 1 hour, but were otherwise maintained in the dark.

c.)Reactive Blue 2 (Sigma). This drug has recently been renamed Cibacron Blue 3GA and has previously been shown to antagonise ATP mediated responses in some tissues via a P_{2Y}-Purinoceptor (Burnstock and Warland, 1987). The drug was dissolved in Hepes buffered serum free F10 and the cells were pretreated with doses of reactive blue 2 ranging from 10^{-5} to 10^{-2}M for 10 minutes. ATP was then added to the cells as in b.) and the cells were photographed up to one hour.

Drugs with potential anti-contractile activity.

a.)Taxol. As described in section 2.2.6., taxol was dissolved in DMSO. Further dilutions were made with Hepes buffered serum free F10. Cells were exposed to concentrations of taxol varying from 10^{-9} to 10^{-4}M. Exposure was from 30 minutes upto 2 hours, and 24 hours, whereupon half the maximally effective dose of ATP was added to the cells and the response monitored upto 1 hour.

b.)Cytochalsin B. Cytochalasin B was also dissolved in DMSO as described in section 2.2.6. with further dilutions made in Hepes buffered serum free F10. Cells were exposed to the drug at concentrations varying from 10^{-12} to 10^{-5}M for 30 minutes, ATP was added and the response monitored for 1 hour.

c.)Colchicine. Cells were exposed to colchicine at concentrations varying from 10^{-9} to 10^{-3}M for periods ranging from 30 minutes to 3 hours, and for 24 hours. ATP was added as above.

d.)Trocinate. Trocinate was applied to the cells for 30 minutes at concentrations varying from 10^{-7} to 10^{-4}M, followed by ATP as above.

e.)Trifluoperazine. This drug, at concentrations varying from 10^{-6} to 10^{-3}M was applied to the cells for 30 minutes, followed by ATP.

f.)Prostaglandin E_2. PGE_2 at concentrations varying from 10^{-10} to 10^{-4}M was applied to the cells for 24 hours. All drug manipulations were carried out in darkness because of the light sensitivity of prostaglandin E_2. ATP was then added to the cells as described above.

g.)Ritodrine HCl. Ritodrine HCl at concentrations varying from 10^{-6} to 6.18×10^{-3}M was applied to the cells for 30 minutes, followed by ATP.

2.3.4. Error check

The procedures of drawing around the outline of cells on the film viewer and the semi-automated image analyser are subject to errors of interpretation. Inter- and intra-observer errors were therefore assessed for these procedures, using an additional experienced observer. The percentage error was calculated using the same equation as in section 2.2.7.

2.4. CELL VIABILITY AFTER DRUG EXPOSURES.

Cell viability after 4 hours of exposure to many of the drugs described above was assessed by the cell's ability to exclude the vital dye Trypan Blue. One drop of Trypan Blue 0.25% was added to 1 ml of cell suspension. The cells were observed in an haemocytometer under phase contrast microscopy. Viable cells did not take up the dye, while dead cells appeared blue and non-refractile. Cells not exposed to drugs were used as controls.

2.5. CELL REPLICATION AFTER 24 HOURS EXPOSURE TO DRUGS.

Recently passaged cultures of rabbit Tenon's fibroblasts were exposed to many of the drugs described above for 24 hours. The cultures were then washed three times with F10 medium and were fed with further fresh complete F10. Cell counts were performed by a grid count under phase contrast microscopy. Cell proliferation was monitored upto two weeks after the drug exposure, and was compared to untreated control cells.

2.6. CELL MORPHOLOGY

The methods described above of chemotaxis and cell contraction allow for the observation of individual fibroblasts. The following techniques were employed to determine the morphological effects of migration and contraction on the cells. Drug effects, including potential toxicity, were also studied by these means:

2.6.1. Light microscopy

Cells migrating through the pores in the polycarbonate membrane of the chemotaxis apparatus were observed, after fixing in ethanol and staining with haematoxylin, using a light microscope (Olympus) and counted at 1000X magnification. The cells that had not migrated and were adherent to the proximal side of the membrane were also studied. Morphological effects of drugs on cells were observed using this technique as were effects of drugs on cell adherence to the membrane.

2.6.2. Phase contrast microscopy

Cells growing in tissue culture were routinely observed with inverted phase contrast microscopes (Olympus and Nikon). This technique allows living, unstained cells to be seen. Cell counts can also be made using this type of microscope without destroying or otherwise interfering with the cells. The contraction assay described in section 2.3.2. was monitored with phase contrast microscopy, and cells were photographed using 35 mm still film or 16 mm cine film.

2.6.3. Differential interference contrast microscopy

Normally growing cells and cells after exposure to ATP and various drugs were observed using differential interference contrast microscopy (Reichert Jung, Vienna, Austria), which allowed a "three dimensional" visualization of living cells to be made.

2.6.4. Scanning electron microscopy

Fibroblasts in their normal growth medium and after exposure to ATP and various drugs were prepared for scanning electron microscopy. The flasks of cells were washed initially with Dulbecco's phosphate buffered saline with calcium and magnesium. They were then fixed in 2% glutaraldehyde in Dulbecco's PBS for 1 hour. The specimens were postfixed in 1% buffered osmium tetroxide and dehydrated through graded alcohols. A section of the floor of the plastic flasks was then removed with a hot knife, and the specimens were critical-point dried

(Polaron Equipment Ltd., Watford, England), sputter coated with gold (Polaron Equipment Ltd.), and observed in a scanning electron microscope, type S520 (Hitachi, Tokyo, Japan). Membranes were removed from the chemotaxis apparatus and were similiarly prepared for scanning electron microscopy. This technique was used to provide high power views of cell shapes and cell surfaces, as well as information about the interaction between the polycarbonate membranes, the pores, and the fibroblasts.

2.6.5. Transmission electron microscopy

In order to observe cells in the process of migrating through the pores in the polycarbonate membrane, membranes bearing fibroblasts were removed from the chemotaxis apparatus and prepared for transmission electron microscopy. The initial preparation was as described in section 2.6.4. After dehydration in graded alcohols sections of the membrane were placed in 50% Araldite/50% propylene oxide. After one change of resin, the specimens were finally embedded in Araldite which was polymerized and allowed to harden. Ultrathin sections were cut using an ultramicrotome (Reichert-Jung) and placed on copper grids. They were ultimately stained with uranyl acetate and lead citrate. Membranes were observed and photographed in a JEOL 100C (Tokyo, Japan) or an Hitachi H600 (Tokyo, Japan) electron microscope.

2.6.6. Indirect immunofluorescence

The technique of indirect immunofluorescence was employed with two objectives:

i.)To check the fibroblast cultures for epithelial cell contamination. Monoclonal anti-vimentin (Euro-Diagnostics, Holland) and polyclonal anti-keratin (Dako, California, USA) antibodies were chosen.

ii.)To explore drug effects.

Monoclonal anti-actin (Euro-Diagnostics), anti-beta tubulin (Chemical Credential, Illinois, USA), and anti-vinculin (Chemical Credential) antibodies were utilised.

Cultures for immunostaining were grown on 8-well slides (Lab-Tek, Miles Scientific, Illinois), washed twice with phosphate buffered saline and fixed in cold methanol and acetone ($-20^{o}C$) for four minutes and two minutes respectively. The specimens were incubated in 1% normal goat serum for ten minutes to block non-specific binding. Primary antibodies were made up in 1% normal goat serum in phosphate

buffered saline at dilutions recommended by the manufacturers. After 60 minutes exposure to the primary antibody and subsequent washing in phosphate buffered saline, the samples were exposed to a 1 in 40 dilution of FITC-labelled goat anti-mouse IgG (Sigma) in the case of the monoclonal primary antibodies, or a similiar anti-rabbit IgG (Sigma) in the case of the polyclonal antibody. Exposure to the secondary antibody was for 60 minutes, whereupon the slides were washed and mounted with Fluorostab (Euro-Diagnostics) prior to examination in an epifluorescent microscope (Zeiss, West Germany, or Reichert Jung). Appropriate controls were conducted to determine the specificity of the fluorescent staining (Hiscott, Grierson, Trombetta, et al., 1984). This involved omission of the primary antibody, and the use of an inappropriate primary monoclonal antibody (in the case of the monoclonal antibodies) or the substitution of non-immune rabbit serum (for the polyclonal anti-keratin anti-serum).

Normal subconfluent rabbit Tenon's fibroblasts and fibroblasts that had been exposed to cytochalasin B, taxol, colchicine and ATP were viewed and photographed.

2.7. STATISTICAL METHODS USED IN THE ANALYSIS OF RESULTS

Consultation was made with a statistician at the start of the experimental work (Dr.Peter Clark). On-going statistical advice was frequently obtained from my supervisor, Prof.Ian Grierson during the course of this thesis.

Results were analysed using parametric statistics, where the data was normally distributed. Data that was not normally distributed was analysed using non-parametric statistics. In some situations where it was unclear whether parametric or non-parametric tests where most appropriate, the statistics were computed using both techniques. In practise, very similiar results were obtained using either parametric or non-parametric tests, and the situation never arose where a result was considered statistically significant using one type of test and non-significant using another.

The following statistical tests were employed:

Parametric tests

- Student t test.
- Paired t test.
- One way analysis of variance.
- Correlation analyses.

Non-parametric tests

- Wilcoxon two-sample test.
- Correlation analyses.

3.1. CELL CULTURE

3.1.1. Rabbit cells

Rabbit primary explants virtually all showed outgrowth of fibroblasts within one week of setting up. However there was variation in cellular growth rates between different primary cultures. After bulking together the fibroblasts from the 5 different animals, used to start the primaries, the resulting cultured cells became homogeneous (fig.3.1.). Indirect immunofluorescence staining indicated that all cells were keratin negative, showing an absence of epithelial contamination in the fibroblast cultures (fig.3.2.).

Rabbit cells that had been grown initially in F10 medium and then passaged, continued to proliferate at the same rate as cells in F10 when fed with RPMI or Eagle's minimum essential medium. However, cells that were fed with medium 199 immediately after passaging failed to settle well on the culture flasks, and those cells that settled did not proliferate over the ensuing seven days. Therefore, since F10 medium was the initial choice of culture medium, it was decided to continue with this regime (fig.3.3.).

After passaging fibroblasts there was a lag period of 24 to 72 hours before cell multiplication resumed. Thereafter, cell numbers increased logarithmically. Within 7 to 10 days, flasks contained approximately 4 million cells in a confluent monolayer. Further increases in cell numbers continued and the cultures became multilayered (fig.3.4.).

Cells were used in the chemotaxis assays 4 to 7 days after passaging when the cultures were undergoing logarithmic increases in cell numbers. For the cell contraction studies, fibroblasts were used 2 to 3 days after passaging when the cells were just emerging from their lag phase.

3.1.2. Human cells

Out growth from the primary explants was much slower than with rabbit specimens, and a number of explants that did not show growth by three weeks were discarded. The initial growth from the explants was not maintained in all cases. Some cells adopted a widely spread morphology

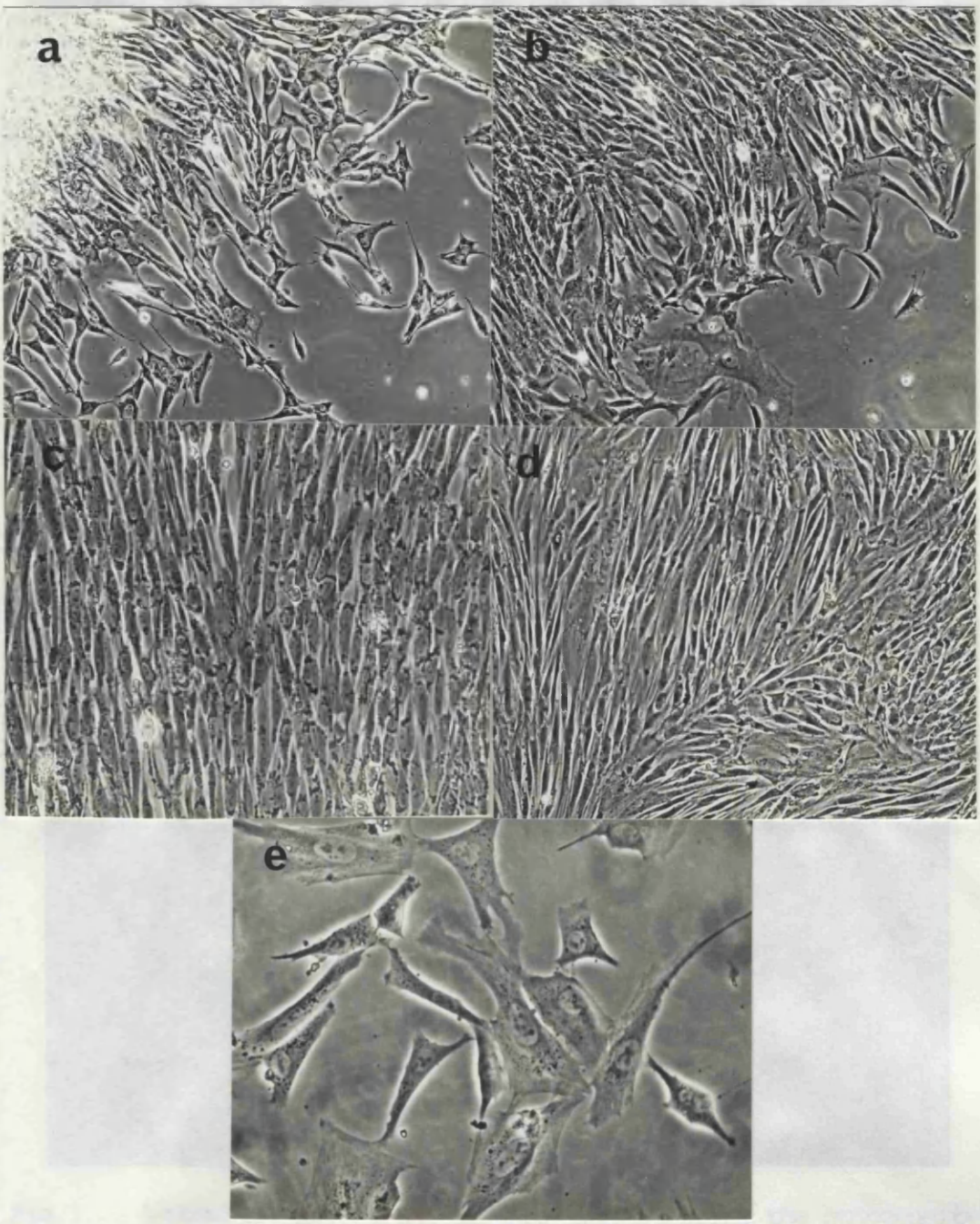


Fig.3.1. Photographs of rabbit fibroblasts in tissue culture. a.)Outgrowth of fibroblasts from the primary explant. b.)Cells at the leading front of the outgrowth. c.)Confluent culture of cells at first passage. d.)Confluent culture of cells at fifth passage. e.)Culture of cells at fifth passage in early log phase. (Magnification X110 a. to d.; e. X220)

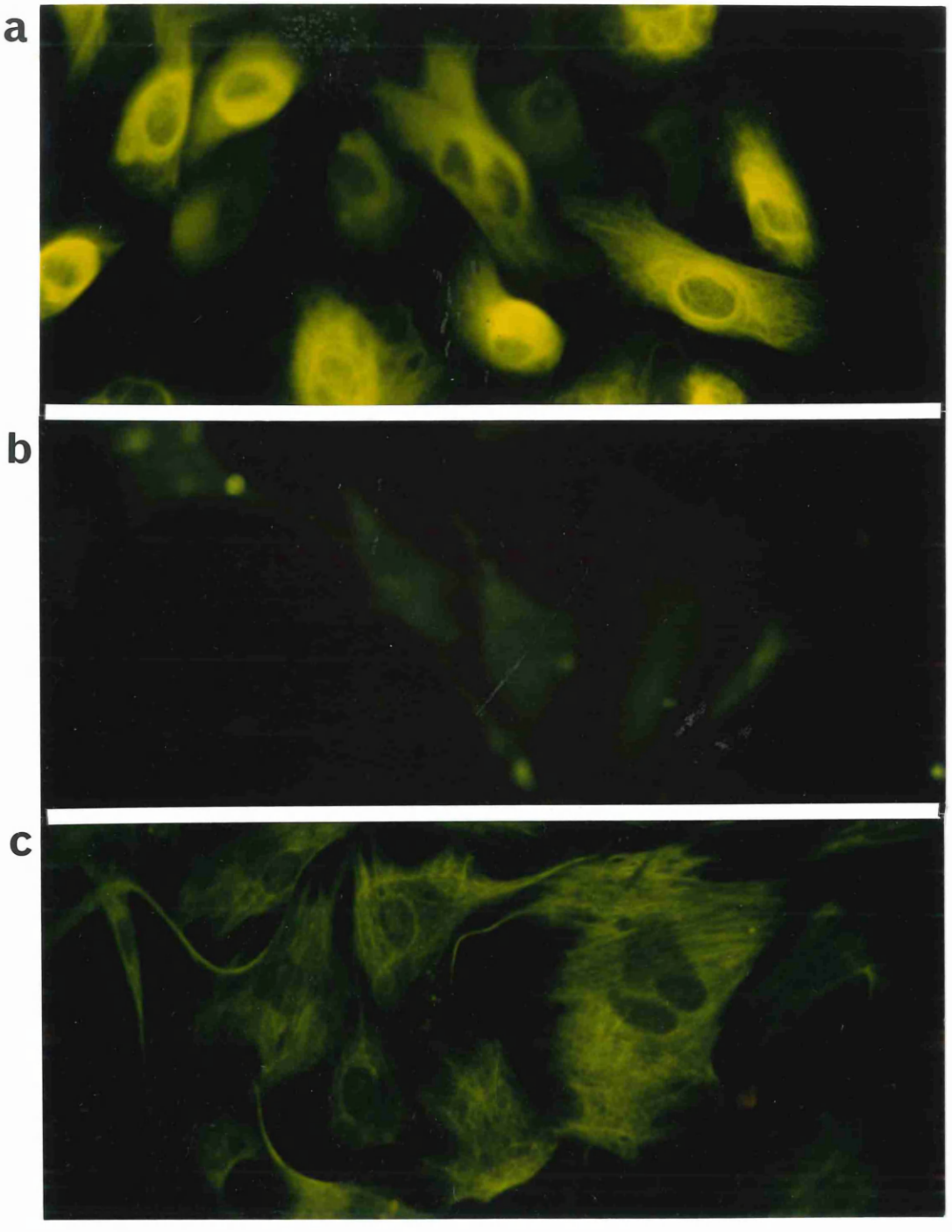


Fig.3.2. Immunofluorescent photographs demonstrating the homogeneity of fibroblast cultures. a.)Culture of bovine retinal pigment epithelial cells labelled with a polyclonal anti-keratin anti-serum. Since these are epithelial cells they are strongly keratin positive. b.)Culture of rabbit Tenon's capsule fibroblasts labelled with a similiar anti-serum. There is no definite fluorescence indicating that none of the cells contain keratin and that there is therefore no epithelial cell contamination of the fibroblast culture. c.)Similiar rabbit fibroblasts labelled with a monoclonal anti-vimentin antibody. All cells are strongly labelled. Vimentin is one type of filament occurring in fibroblasts. (Magnification X450).

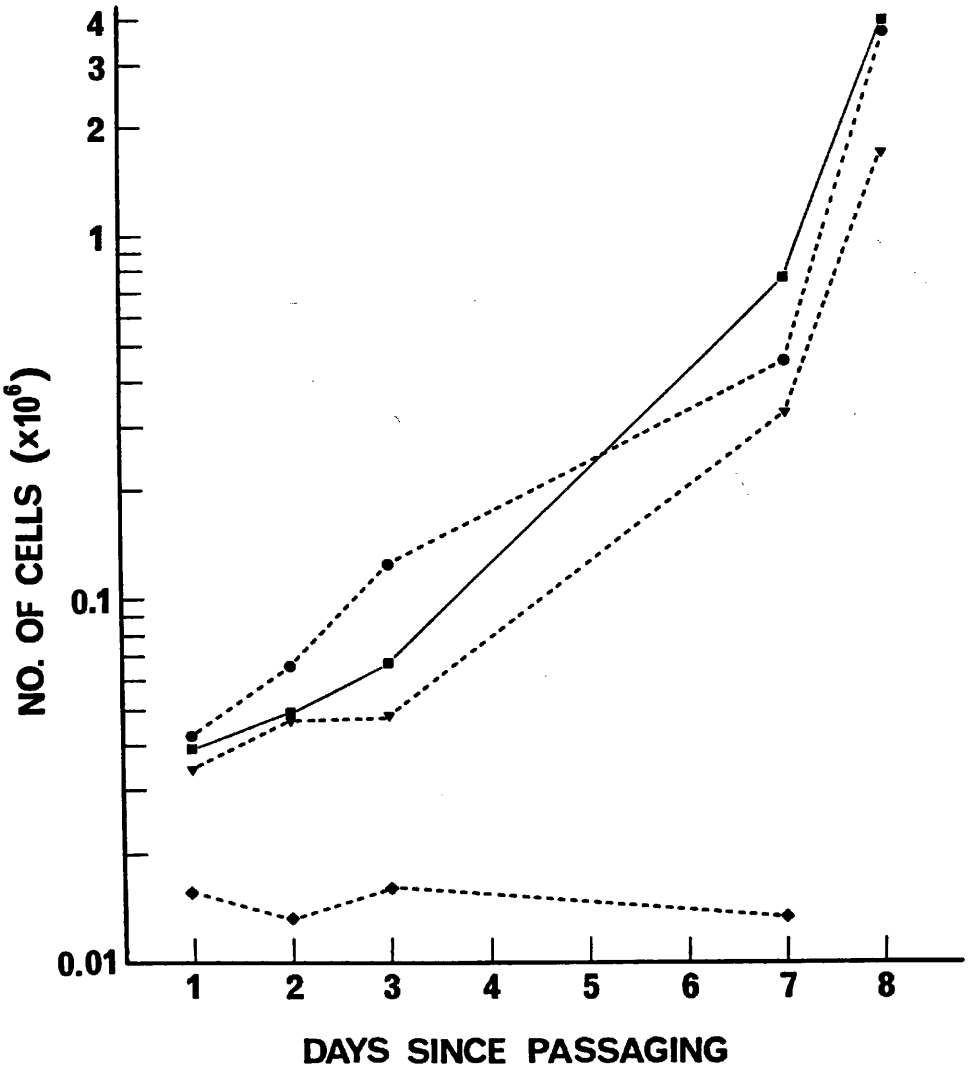


Fig.3.3. Confluent cultures of rabbit conjunctival fibroblasts growing in complete F10 medium at first passage were bulked together and distributed into 16 flasks. At day 0 there were equal numbers of cells in each flask, whereupon the cells were fed with the four different media as shown: --●--F10;—■—RPMI;--▼--Eagle's;--◆--199. Cells fed with medium 199 settled poorly. The few cells that did settle barely proliferated. There was little difference in the growth rates in the other three media. Points show the mean of four counts. Between days 1 and 7 direct cell counts were made with a grid count under phase contrast microscopy. At day 8 cells were indirectly counted with a Coulter counter.

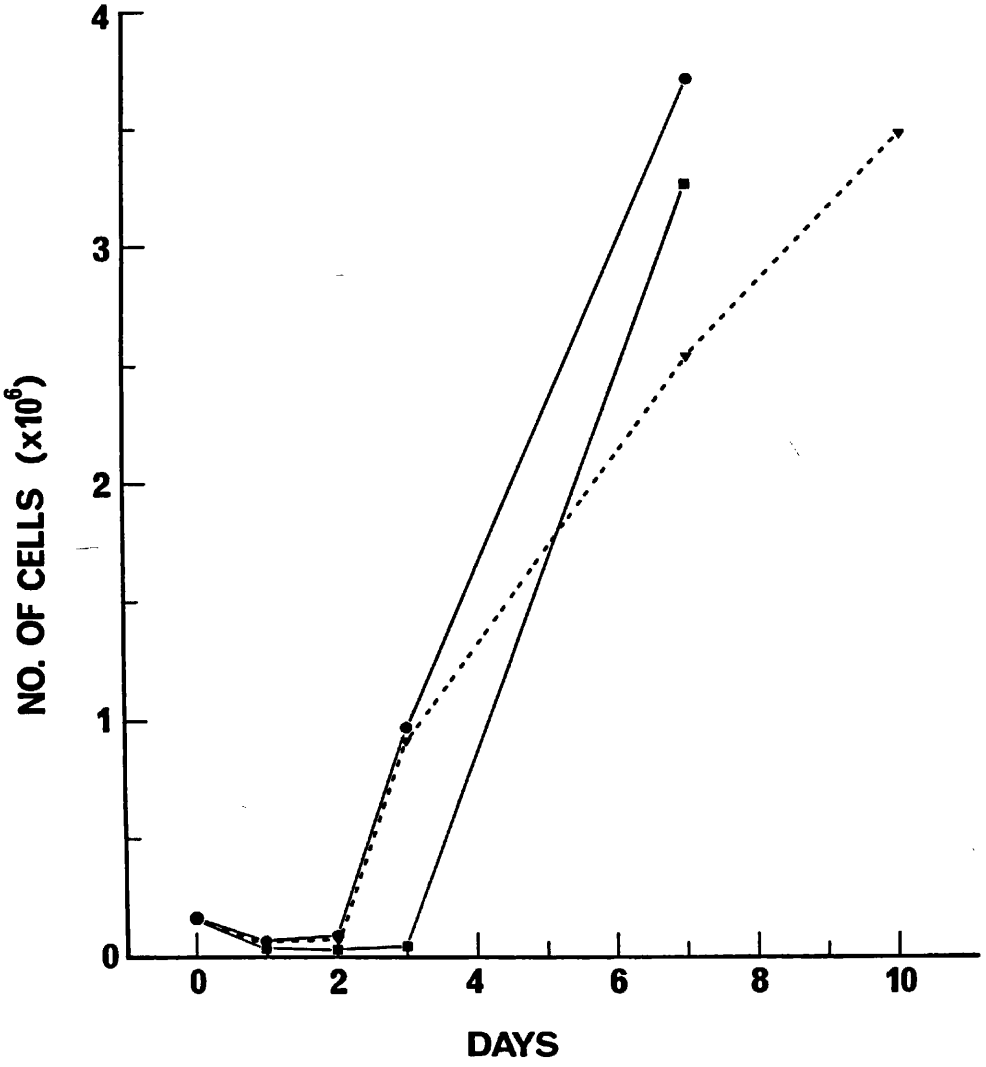


Fig.3.4. Growth curves of rabbit skin, conjunctival and Tenon's capsule fibroblasts at 3rd passage. 1.6×10^5 cells were placed in each flask at day 0. The experiment was conducted in quadruplicate. Cells counts were made by a grid count under phase contrast microscopy. Cells were used in the chemotaxis assays 4 to 7 days after passaging during the log phase of growth, while cells were employed in the contraction experiments 2 to 3 days after passaging when they were emerging from the lag phase. —●— Skin; —■— Conjunctival; --▼-- Tenon's capsule fibroblasts.

with the appearance of prominent stress fibres, and stopped proliferating (fig.3.5c.).

Cultures that appeared sufficiently vigorous were passaged, but after first passage, further cell multiplication was not always continued. Ultimately the following cultures showed reasonable growth such that cells at first passage could be frozen down and stockpiled for later use:

The most profuse growth was shown by the scleral fibroblasts from the eye bank eye of unknown age. These cells were used in most of the experiments where human cells were employed. The cells were further passaged at split ratios varying from 1 - 2 to 1 - 6 and maintained good growth up to third passage, which was the latest stage at which they were utilised (fig.3.5.). Conjunctival fibroblasts from this eye also grew well.

Conjunctival and Tenon's fibroblasts from the per-operative specimen taken from the 59 year old man undergoing retinal detachment surgery grew well and were also employed in the chemotaxis experiments.

Sparse growth was shown by the fibroblasts from the four other sources that continued to grow up to first passage. These cells were frozen down but were not required for any experiments.

All cells were maintained in complete F10 medium with a further 10% foetal calf serum. Since there was no difference in growth in rabbit cultures between F10, RPMI and Eagle's media, it was decided to maintain the human cells in this medium. Further experimentation with alternative media was not thought advisable because of a.) the relatively limited numbers of human cells, b.) their relatively slow growth rate and c.) so that comparisons between human and rabbit cells could be made without having the additional variable of different growth media.

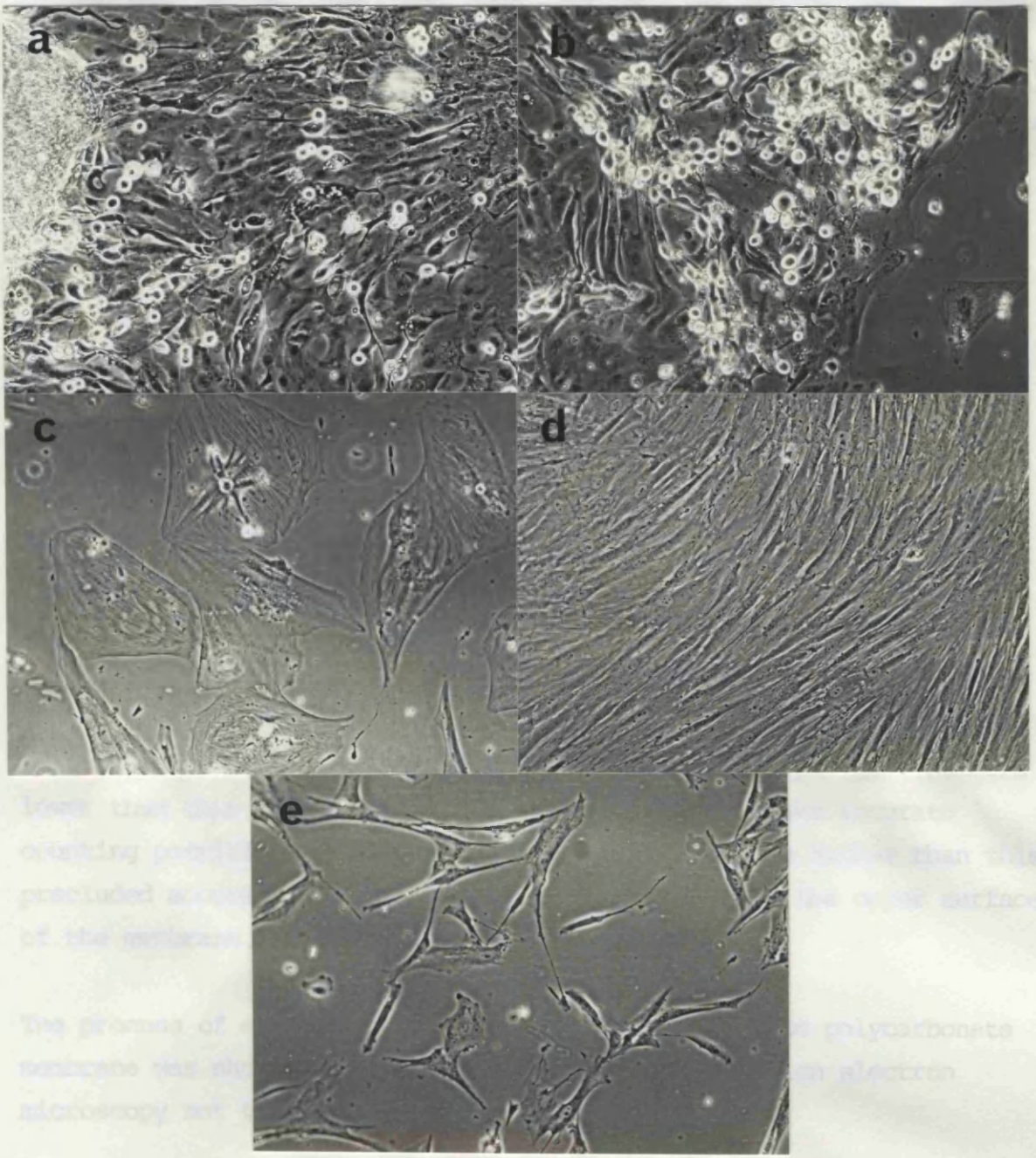


Fig.3.5. Photographs of human ocular fibroblasts in tissue culture. a.)Out growth from the primary explant after 7 days in tissue culture. b.)The leading front of cells from a similiar out growth of a primary explant. Numerous mitotic figures are evident. c.)Cells with prominent stress fibres from a culture that had stopped proliferating. d.)Scleral fibroblasts in a confluent culture after 2nd passage. e.)Similiar cells to those in d.) in a sparse culture in early log phase. (Magnification X110)

3.2. CHEMOTAXIS

3.2.1. Base line evaluations

When evaluated in a comparative experiment, fibroblasts attached best to polycarbonate membranes containing the wetting agent polyvinyl-pyrrolidine (PVP), and treated with 5 mg/l gelatin. The cell morphology also appeared normal on this type of membrane. Accordingly, gelatinised PVP membranes were adopted for all the chemotaxis experiments (fig.3.6.).

The number of rabbit fibroblasts that migrated through the pores in the polycarbonate membrane continued to increase up to 4 hours of incubation of the chemotaxis apparatus. Beyond four hours, no further increase in cell migration occurred.

Human fibroblasts, by contrast, continued to migrate in increased numbers for up to 6 hours of incubation. Accordingly, chemoattraction assays using human cells were incubated for 6 hours, and those using rabbit cells for 4 hours (fig.3.7).

7×10^5 cells per ml or 35,000 cells proved to be the most suitable number to add to the upper wells of the chemotaxis apparatus. Numbers lower than this provided too few migrated cells to make accurate counting possible (see section 3.2.7.), while numbers higher than this precluded accurate counting of the cells adherent to the upper surface of the membrane.

The process of migration through the 8 um pores in the polycarbonate membrane was shown by light, scanning, and transmission electron microscopy not to be harmful to the cells (fig.3.8.).

3.2.2. Fibronectin

Fibronectin was chemoattractive for both rabbit and human ocular fibroblasts. The optimally effective dose for rabbit cells was 20 ug/ml while for human cells this was 30 ug/ml. These doses were used as positive controls in all subsequent experiments. Concentrations of fibronectin upto 1000 ug/ml were tested. Beyond 100 ug/ml there was a slow reduction in the chemoattractant response. Qualitatively the responses of the rabbit and human cells were very

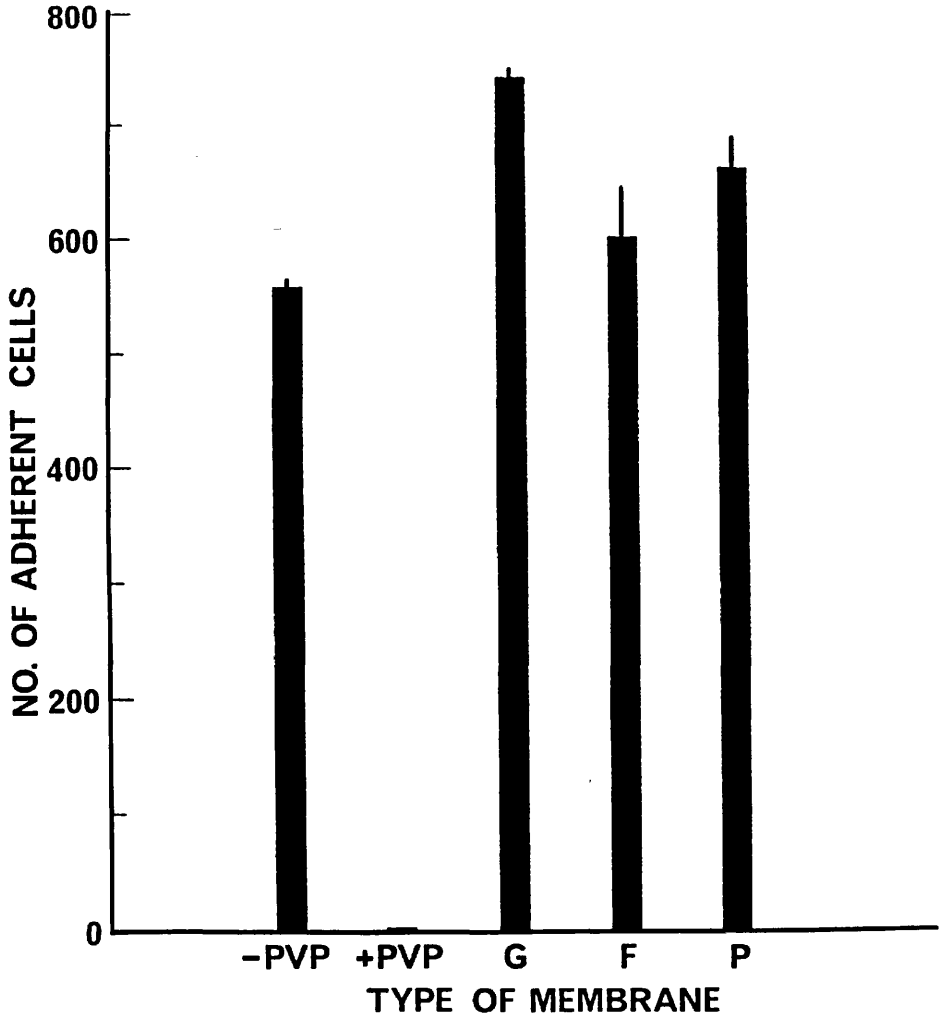


Fig.3.6. Histogram showing the plating efficiency of various types of polycarbonate membrane. 35,000 rabbit Tenon's fibroblasts were placed in the upper wells of the micro-chemotaxis chambers containing the 5 different types of membrane. After four hours of incubation the number of cells adherent to the membrane in each well was counted in 20 fields at 1000X magnification. Four wells were counted per membrane. Bars show the mean; lines the SEM. -PVP=Polyvinylpyrrolidine free; +PVP=PVP containing membrane; G=PVP membrane treated with 5 mg/l gelatin; F=PVP membrane treated with 100 ug/ml fibronectin; P=PVP membrane treated with 1% poly-L-lysine.

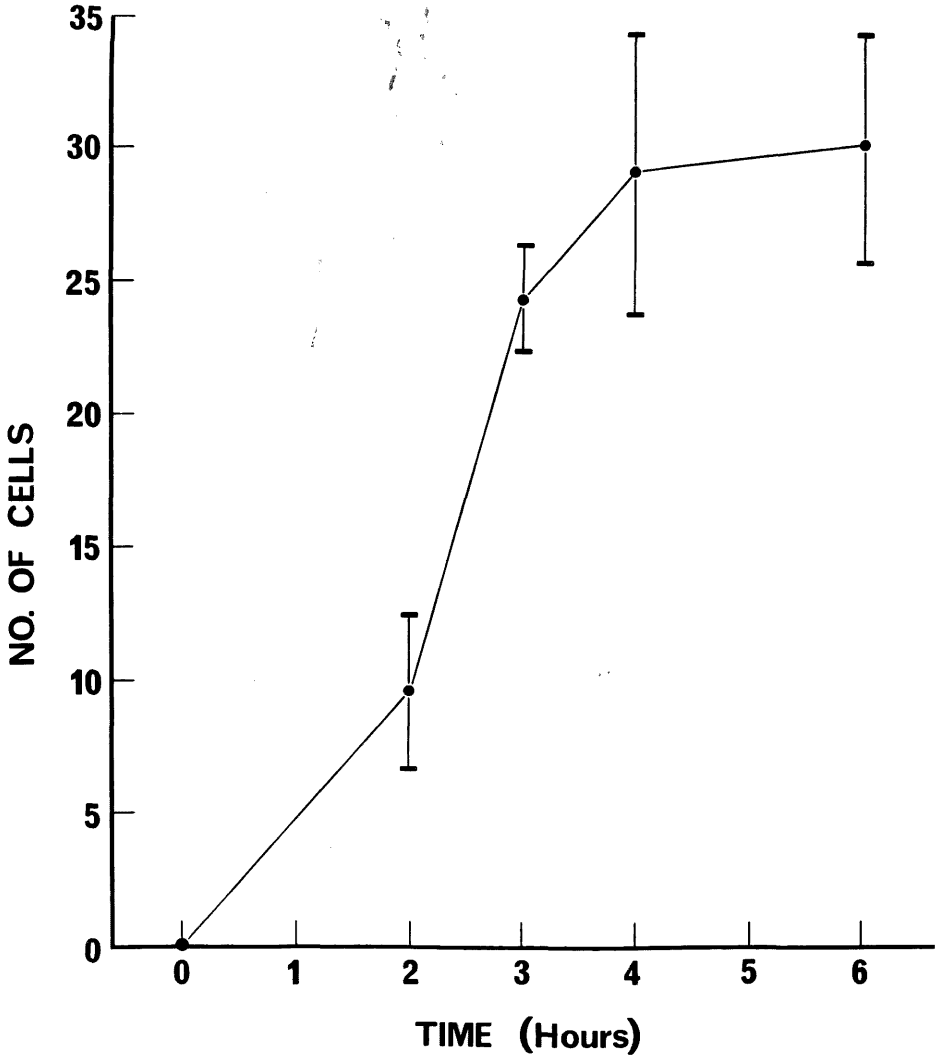


Fig.3.7a. Time function curve for the migration of rabbit Tenon's capsule fibroblasts. A gelatinised polycarbonate membrane was divided into quarters, each of which was placed in a separate 48-well micro-chemotaxis chamber. 20ug/ml fibronectin was used as the chemo-attractant and 35,000 cells were placed in each upper well. The chambers were sampled at the time intervals shown. Points show the mean counts in 4 wells; bars are SEM. Human scleral fibroblasts were run simultaneously in the same experiment for comparison (see fig.3.7b.).

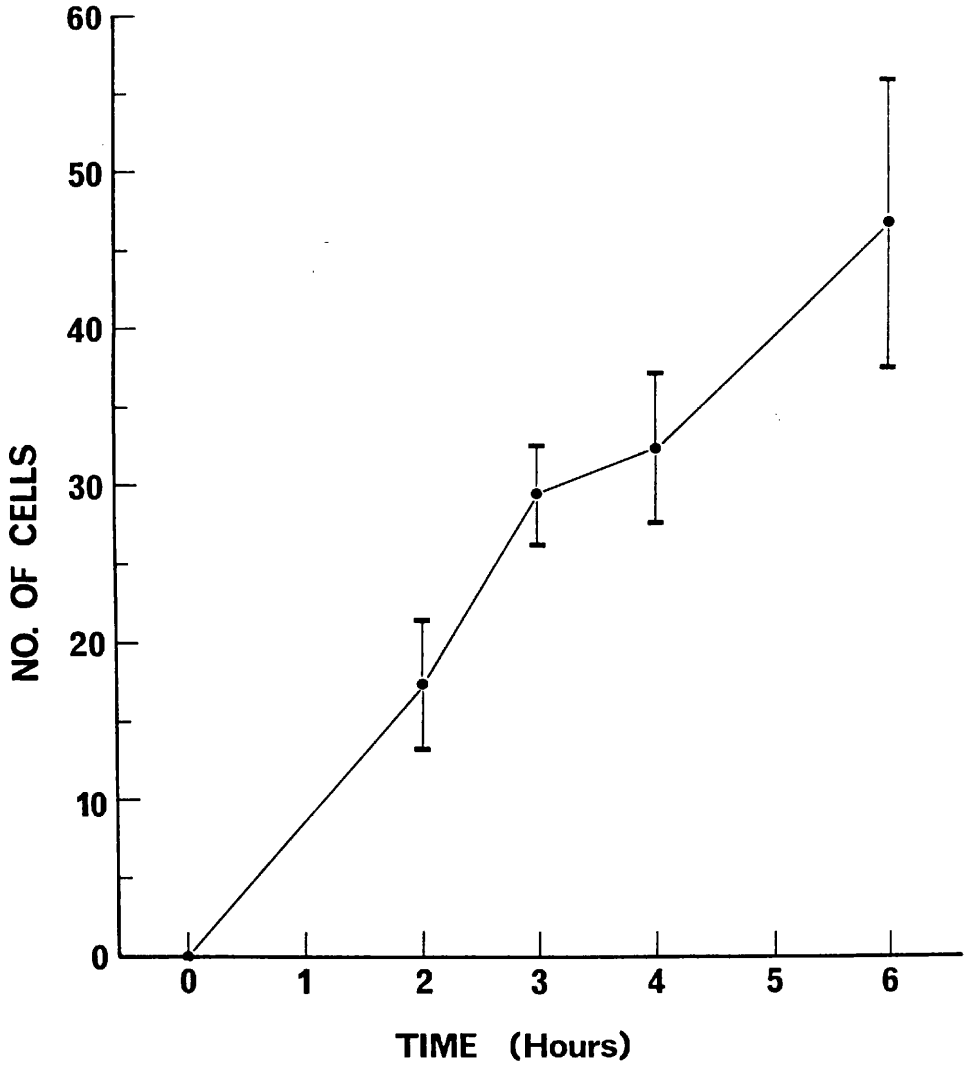
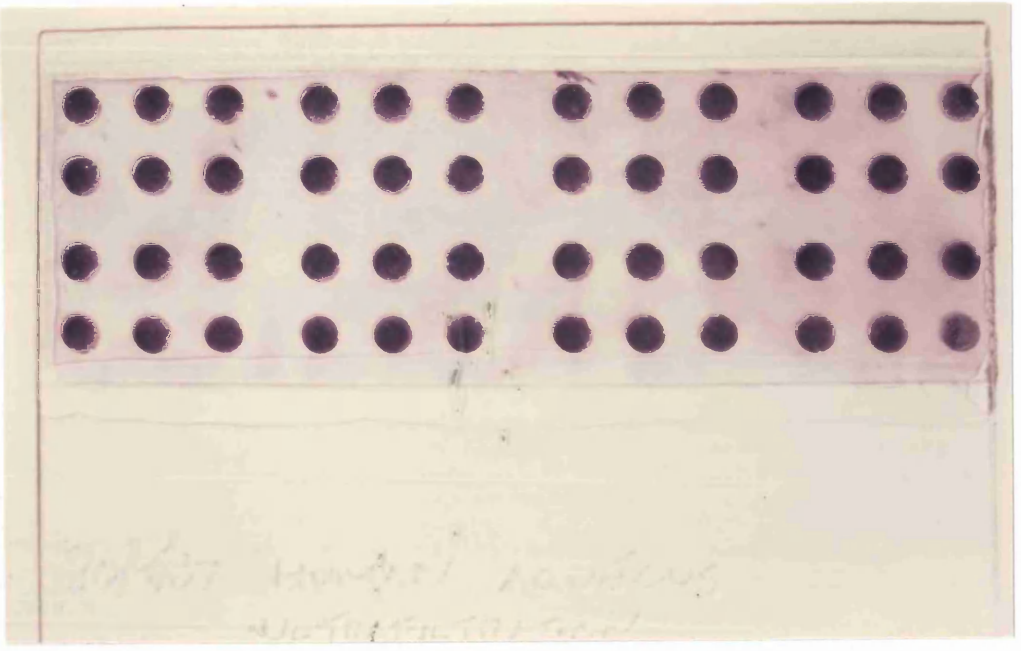


Fig.3.7b. Time function curve for the migration of human scleral fibroblasts. A gelatinised polycarbonate membrane was divided into quarters, each of which was placed in a separate 48-well micro-chemotaxis chamber. 30 ug/ml fibronectin was used as the chemo-attractant and 35,000 cells were placed in each upper well. The chambers were sampled at the time intervals shown. Points shown the mean counts in four wells; bars are SEM. Rabbit Tenon's capsule fibroblasts were run simultaneously in the same experiment for comparison (see fig.3.7a.).

a



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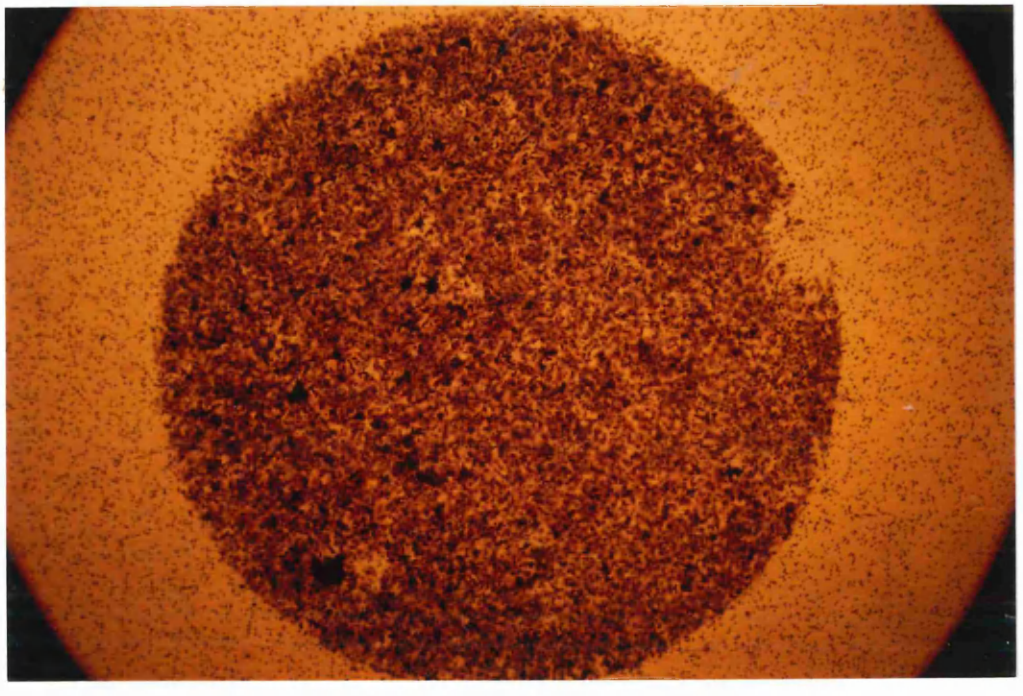


Fig.3.8. Photographs of fibroblasts attached to a gelatinised polycarbonate membrane. a)Discs of cells on a haematoxylin stained membrane, produced by each of the 48-wells of the micro-chemotaxis chamber. A disc of cells is 8 um in diameter. b.)Enlarged view of a disc of cells corresponding to one of the micro-chambers. Most of these cells are on the upper surface of the membrane and are yet to migrate.

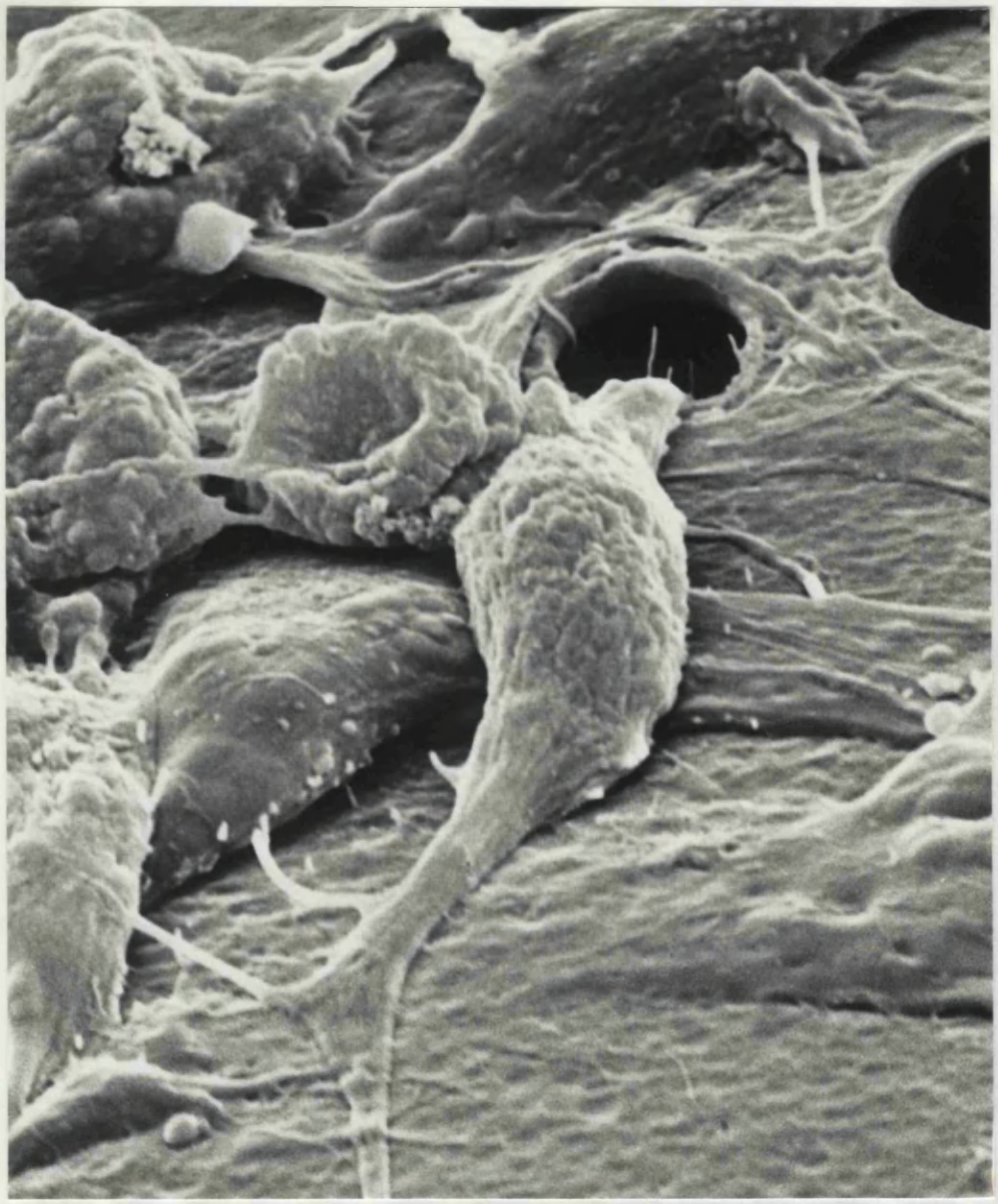


Fig.3.8. (Continued) Photographs of fibroblasts attached to a gelatinised polycarbonate membrane. c.)Scanning electron micrograph (SEM) of cells settled on the upper surface of a membrane, one of which is passing a process down an 8 um diameter pore (X4 800).

d

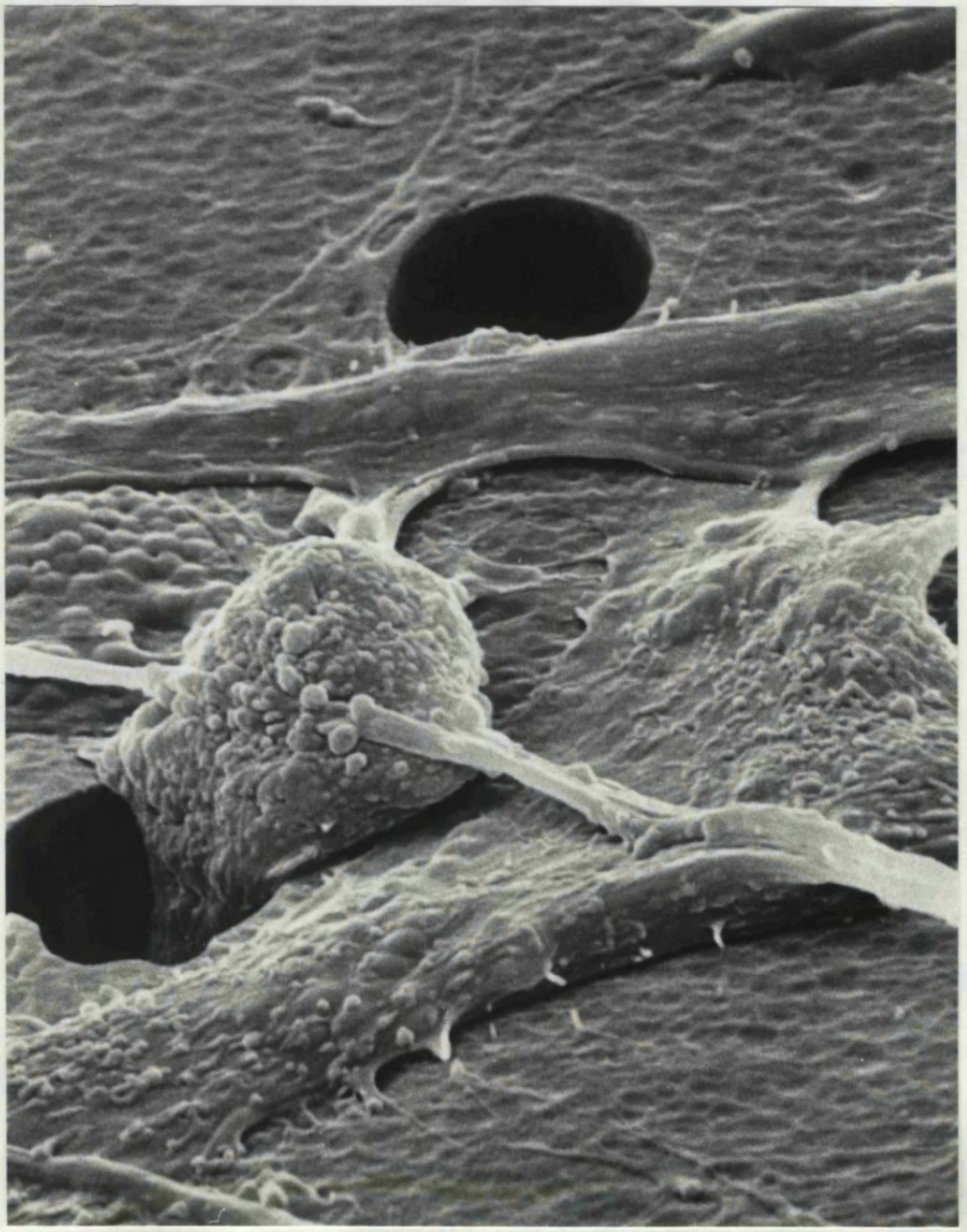


Fig.3.8. (Continued) Photographs of fibroblasts attached to a gelatinised polycarbonate membrane. d.)A fibroblast which has passed a little further into a pore (X5 500).

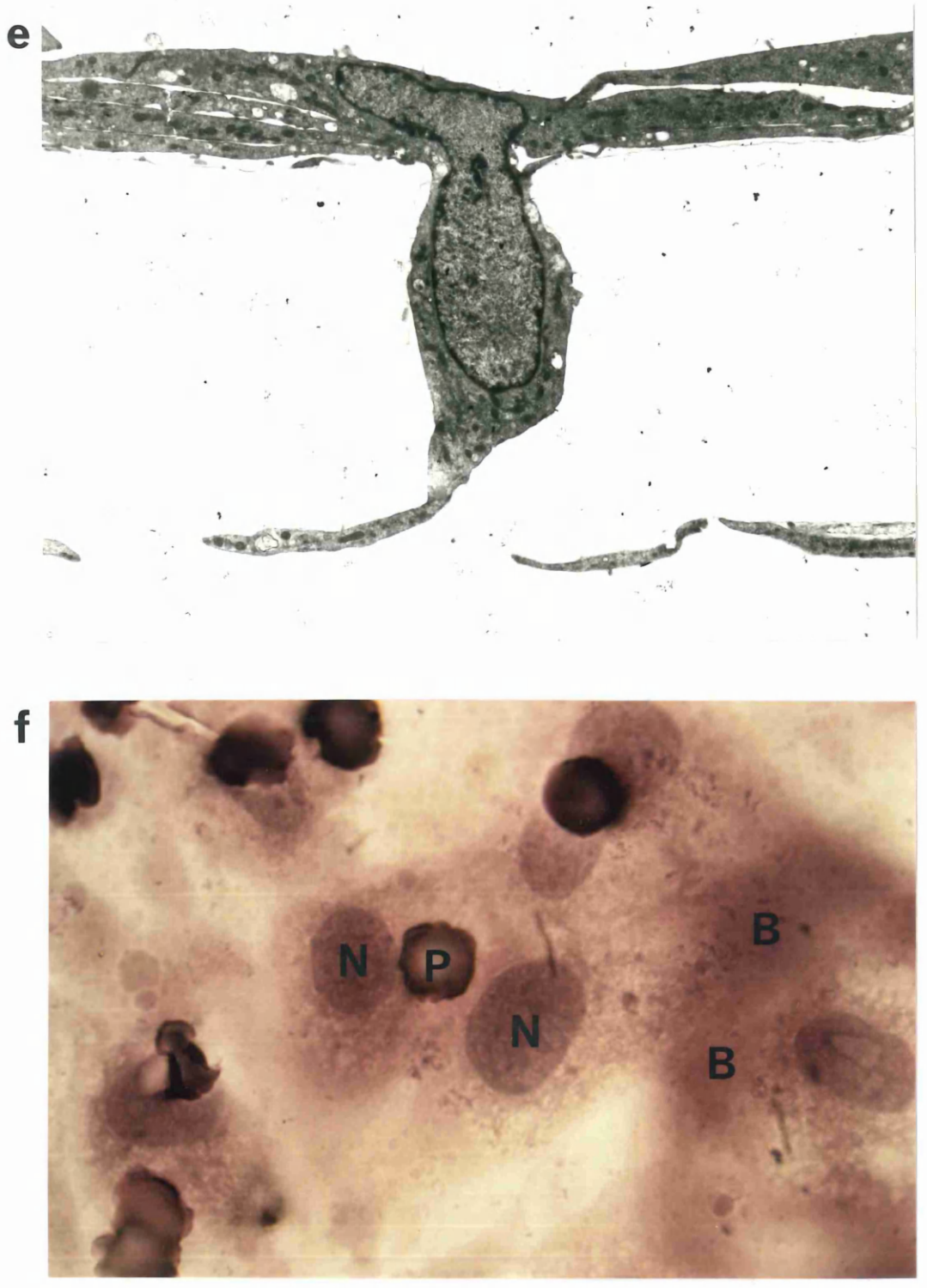


Fig.3.8.(Continued) Photographs of fibroblasts attached to a gelatinised polycarbonate membrane. e.)Transmission EM of a fibroblast caught in the act of migrating through a pore (X5 000). f.)Light micrograph of cells on the distal surface of the membrane (Xl 500). The nuclei (N) of the cells have been stained with haematoxylin. These cells have successfully migrated through the 8um pores (P). The blurs in the background (B) are cells on the other surface of the membrane that are yet to migrate. Attachment to the membrane and passage through its pores is not harmful to the cells.

similiar. However, quantitatively fewer human cells generally migrated than rabbit cells although this was not invariable (fig.3.9.).

Human Tenon's fibroblasts responded in a similiar manner to human scleral fibroblasts, although the magnitude of the migration was less. Human scleral fibroblasts were therefore used in most chemoattraction assays in which human cells were evaluated.

The four types of rabbit fibroblasts showed similiar dose-response curves to the Tenon's fibroblasts. Since fibroblasts that are primarily responsible for the failure of a trabeculectomy appear to originate in Tenon's capsule (see section 1.4.), these cells were used in most of the experiments where rabbit cells were employed.

There was considerable day to day variability in the response of both rabbit and human cells to fibronectin and to the negative control of serum free F10. In order to establish the cause of this variation, data from 31 experiments was evaluated where rabbit Tenon's fibroblasts migrated to both 20 ug/ml fibronectin and the negative control of serum free F10 medium. The variability could not be accounted for by the number of cells used in each experiment, since 7×10^5 cells per ml were routinely used. In spite of using the same number of cells per well, there was considerable day to day variation in the number of cells adherent to the membrane. However, there was no statistically significant correlation between the number of cells attached to the membrane and the number of cells migrating through the pores in the membrane to the standard control of 20 ug/ml fibronectin ($r = 0.117$ Pearson correlation, $p > 0.5$), (fig.3.10.).

In order to determine whether the day to day variability in the migration of the cells could be accounted for by the time elapsed since their last passage, the number of cells migrating to 20 ug/ml fibronectin was correlated with the time elapsed since the last passage. Between 3 and 10 days after passaging the results were widely scattered and there was no significant correlation. In a few experiments cells were used between 13 and 24 days after passaging; these cells migrated poorly (fig.3.11.). Since the vast majority of experiments were performed with cells 4 to 7 days after passaging, it is apparent that within these limits, the time elapsed since the passaging of the cells does not influence their ability to migrate.

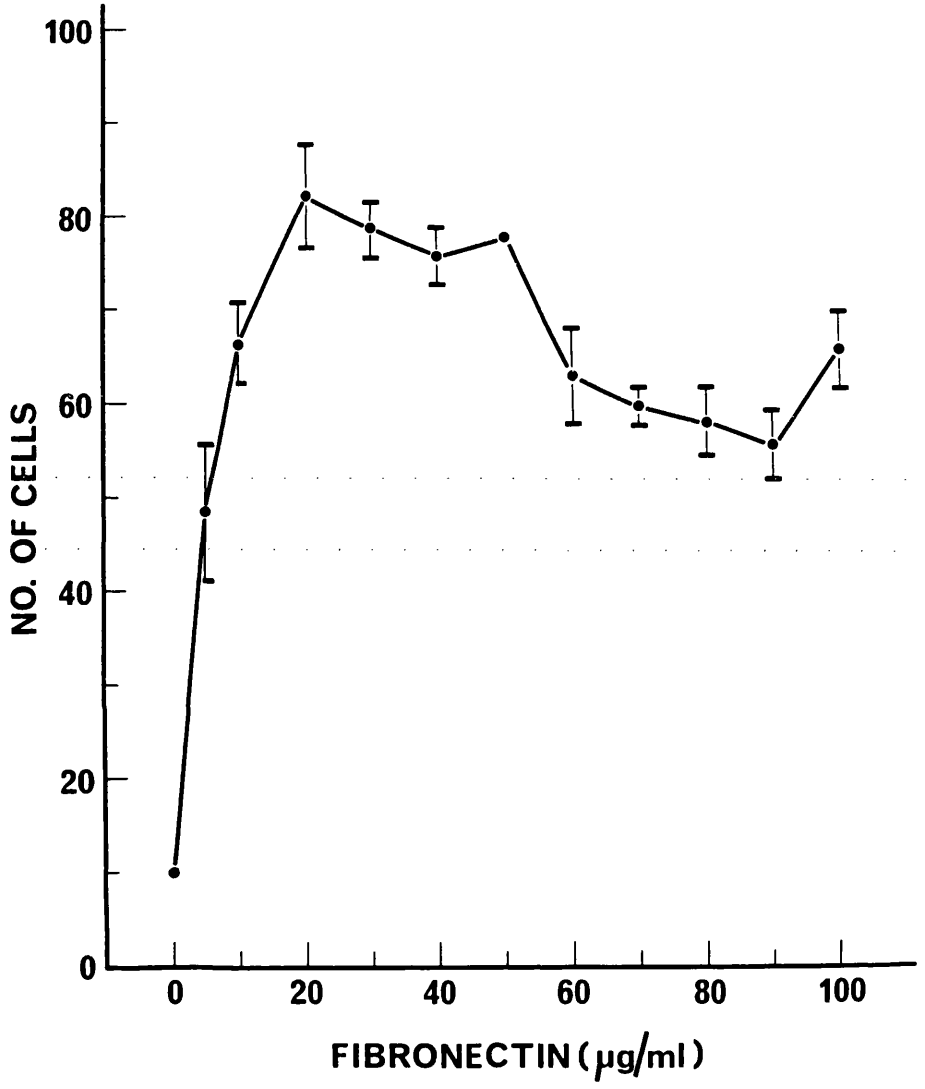


Fig.3.9. Dose-response curves for fibronectin. a.)Rabbit Tenon's capsule fibroblasts were employed as the indicator cells. Each point is the mean of 3 wells; bars are SEM. 20 ug/ml was used as the positive control for rabbit cells since optimal migration occurred in response to this dose.

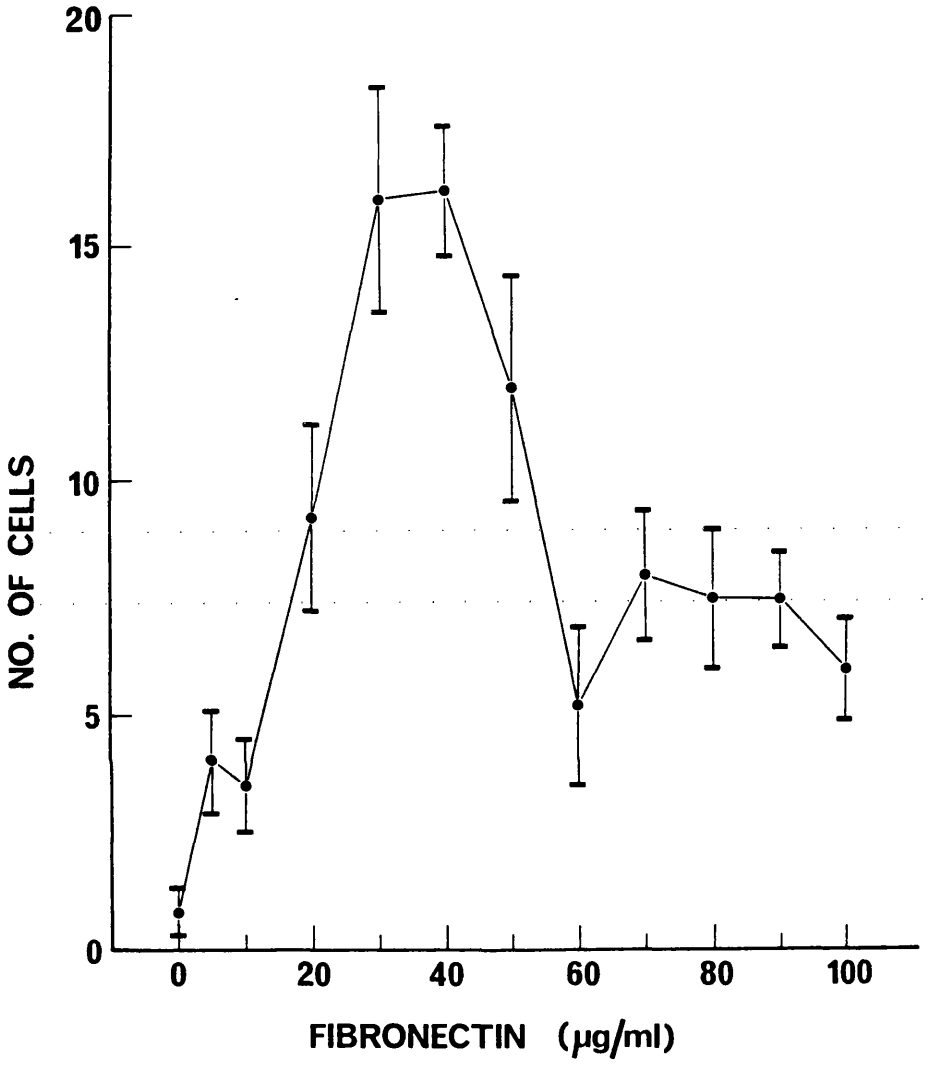


Fig.3.9. Dose-response curves for fibronectin. b.)Human scleral fibroblasts were employed as the indicator cells. Each point is the mean of 3 wells; bars are SEM. 30 ug/ml fibronectin was used as a positive control for human cells since optimal migration occurred in response to this dose.

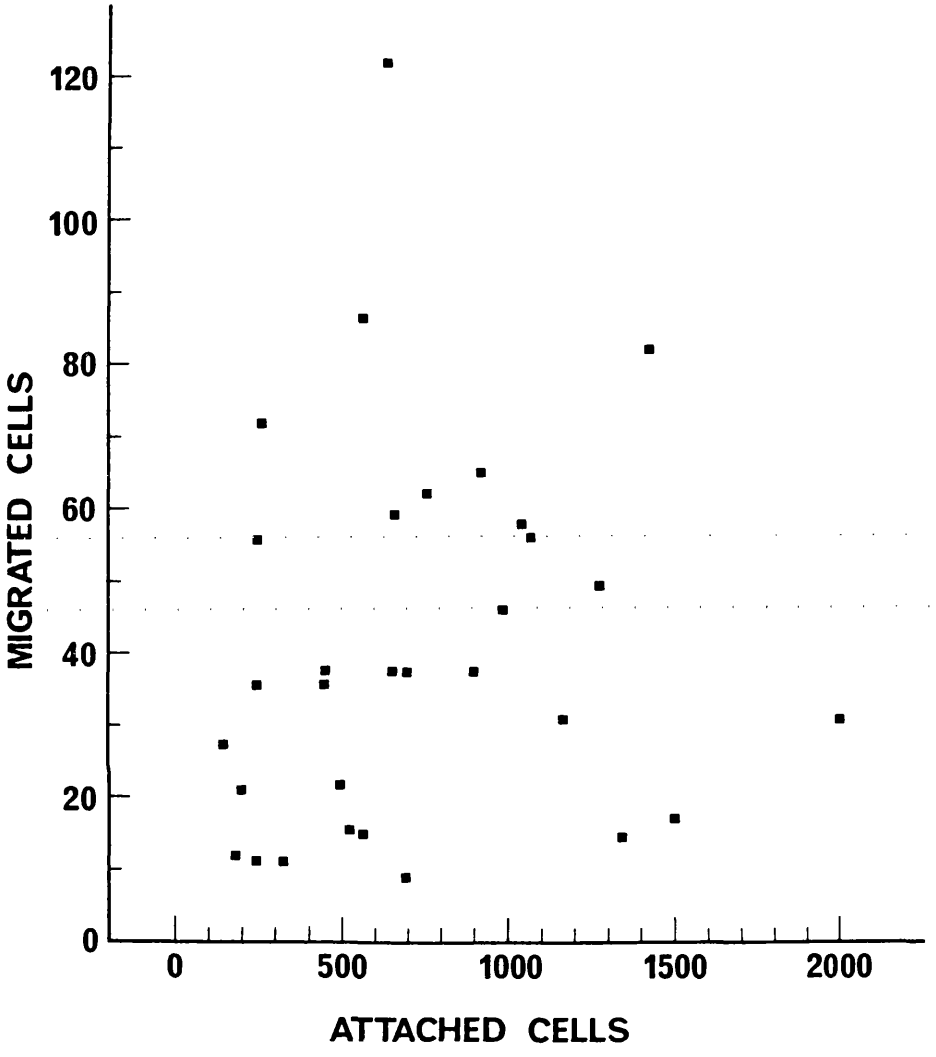


Fig.3.10. Scatter diagram comparing the number of cells attached to the membrane in 20 1000X fields (adding the number of cells migrated through the pores to the number of cells not migrated in each field) to the net number of cells migrating to 20 ug/ml fibronectin (number of cells migrating to fibronectin minus the negative control).
r = 0.117 Pearson correlation, $p > 0.5$.

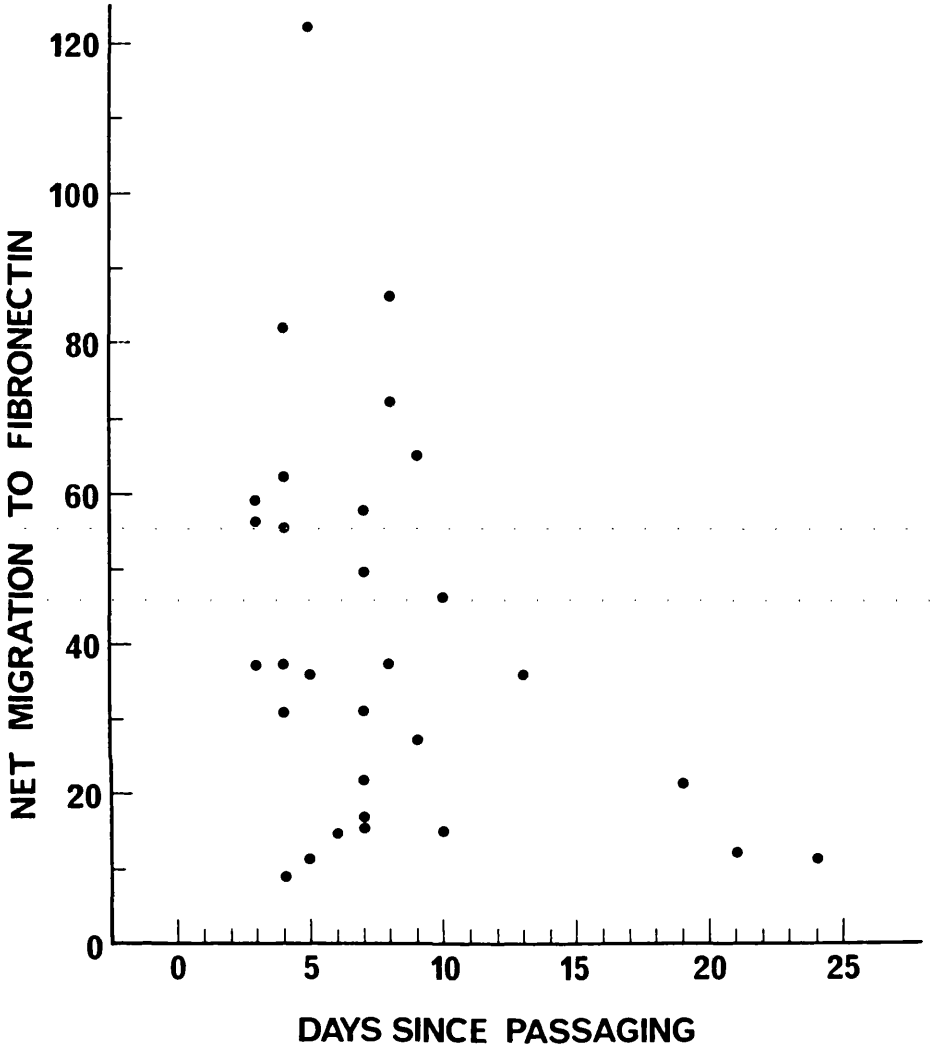


Fig.3.11. Scatter diagram comparing the net migration of rabbit Tenon's fibroblasts to 20 ug/ml fibronectin with the time elapsed since the last passage of the cells. Between 3 and 10 days the migration to fibronectin was independent of the time elapsed since passaging. Cells employed greater than 10 days after passaging migrate poorly. Consequently chemotaxis experiments were conducted with cells 4 to 7 days after passaging.

The plating efficiency of the fibroblasts appeared to be related to the passage number of the cells. With cells of second passage a mean of 20% of the cells attached to the membrane, while with fifth passage cells a mean of 52% of cells attached to the membrane within four hours (fig.3.12.). However, when the actual number of cells that migrated to 20 ug/ml fibronectin was compared to the passage number of the cells, it was apparent that in spite of a greater number of 5th passage cells attaching to the membrane than cells at lower passage numbers, fewer of these cells migrated (fig.3.13.). There was also no statistically significant difference in the mean cell migration to 20 ug/ml fibronectin for cells of passages 2 and 5 ($p > 0.05$ Student t test). Therefore, up to 5th passage, the passage number of the cells is unlikely to account for the day to day variability of fibroblast migration.

No other likely cause of the day to day variability could be identified. The variability of the response made direct quantitative comparison between experimental runs done on different days impossible. Qualitatively, however, responses on different days were similiar. If quantitative comparisons were to be made between multiple specimens then it was necessary to run all specimens simultaneously using if necessary up to four 48-well micro-chemotaxis chambers.

Until this point, fibronectin has been demonstrated to be chemoattractant to ocular fibroblasts, but it is not clear whether this attraction was due to chemotaxis, chemokinesis, or haptotaxis. The first two were differentiated by the use of a Zigmond-Hirsch chequer board analysis (Zigmond and Hirsch, 1973). When the cells were exposed only to a progressively increasing positive gradient of fibronectin, an increasing number of cells migrated. This indicated a chemotactic effect. When the cells were exposed not to a gradient of fibronectin, but merely to an increasing concentration, equal in both upper and lower wells of the chemotaxis apparatus, an increasing number of cells also migrated. However, the response was slightly less than with a positive gradient of fibronectin. Migration to an increasing concentration of fibronectin indicated a chemokinetic effect, but it was limited since an increasing number of cells did not migrate in the presence of a negative gradient of fibronectin alone. Both human and rabbit fibroblasts responded in a very similiar way to fibronectin. Thus, the migration of fibroblasts to fibronectin was due

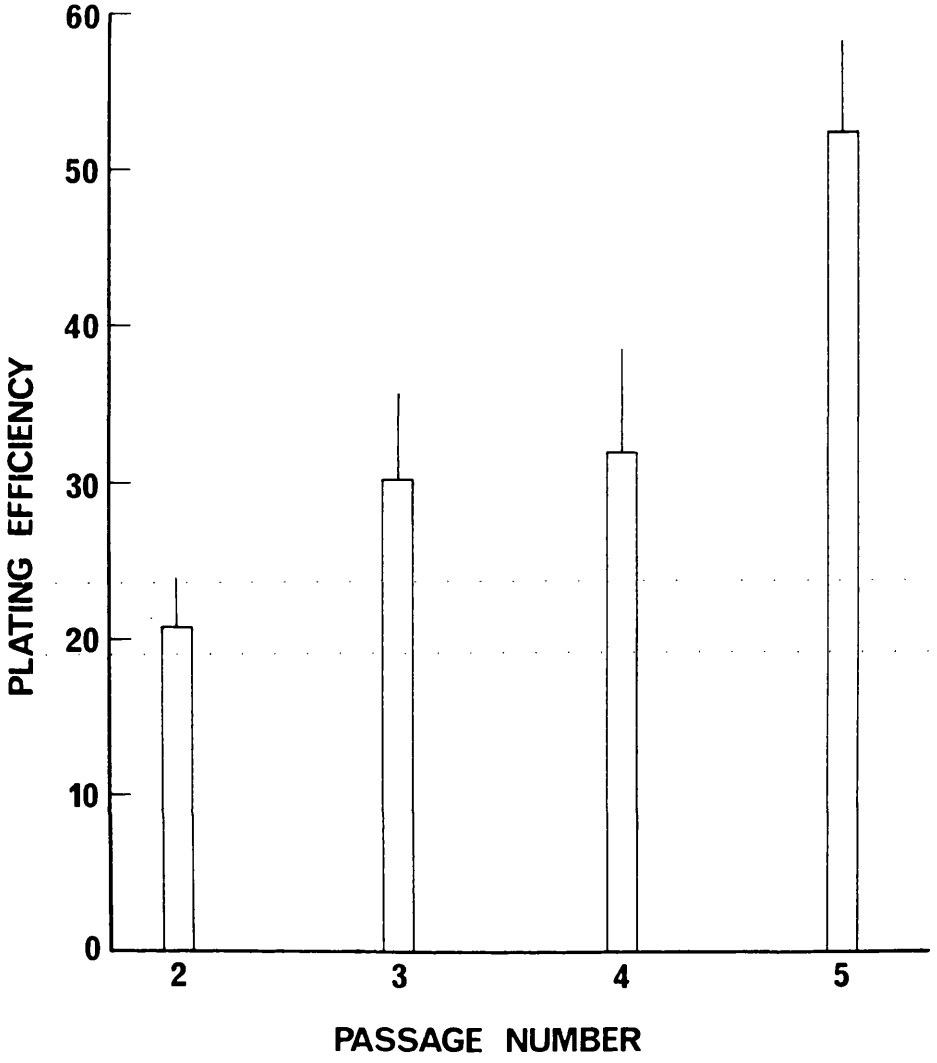


Fig.3.12. Histogram showing the number of cells attached to the membrane as a percentage of the cells added to each well (plating efficiency) for rabbit Tenon's fibroblasts at various passage numbers. Cells adhere better to the membrane with increasing passage number. Lines are SEM.

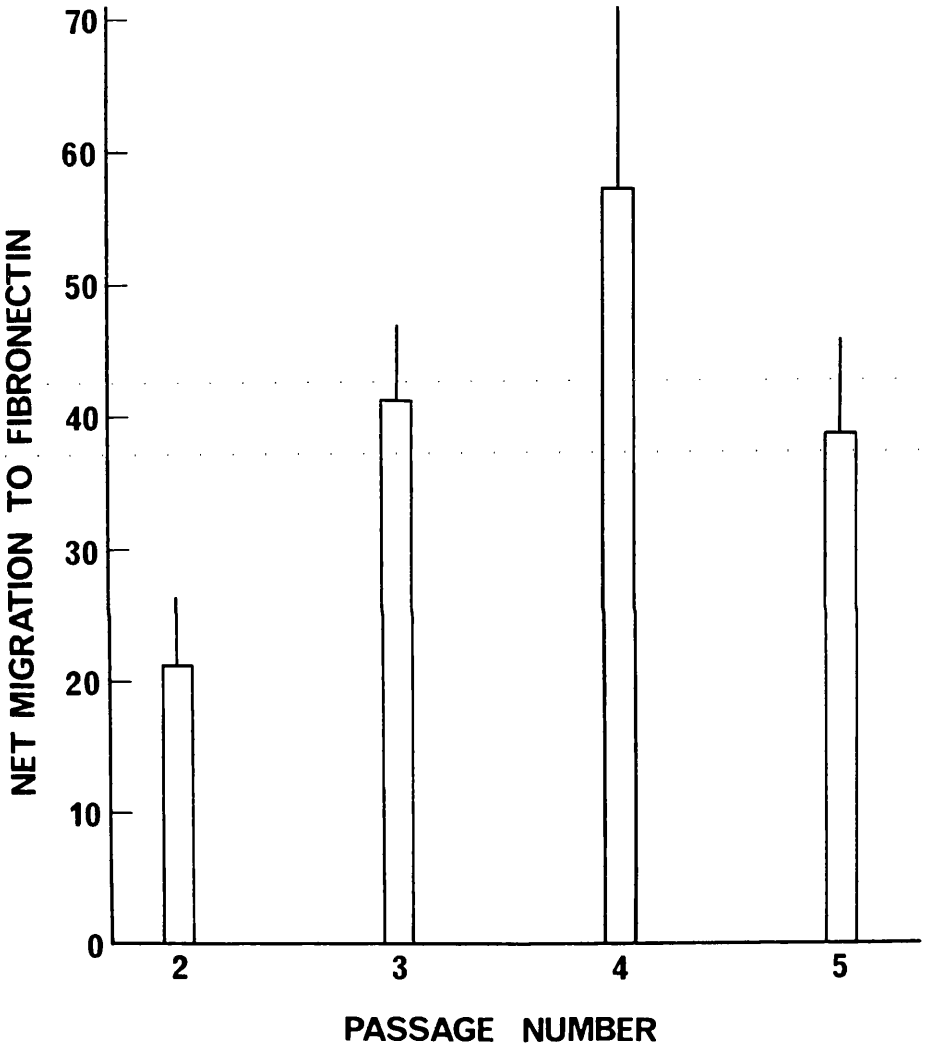


Fig.3.13. Histogram showing the net number of rabbit Tenon's fibroblasts migrating to 20 ug/ml fibronectin for cells at various passage numbers. The SEM's are large but there is a trend towards better migration of the cells with higher passage number, upto forth passage. However, at fifth passage there is a decline in the number of cells migrating.

primarily to chemotaxis, but chemokinesis also played an important role (fig.3.14.).

Fibronectin is known to bind avidly to numerous surfaces, including polycarbonate membranes and to aid the adherence of certain cell types to various substrates (Hynes and Yamada, 1982). It is therefore important to determine that the apparent increased migration of fibroblasts in response to increasing positive concentrations of fibronectin is not artefactually produced due to increasing cell adherence with increasing concentrations of fibronectin. Since adherence to the membrane is a prerequisite for migration, (because fibroblasts crawl rather than swim), a greater number of cells adherent to the membrane would then make a greater number of cells available for migration. Likewise fibroblasts may be induced to migrate by preferential adhesion to bound substances on a substrate. This directed migration of cells due to increasing adhesion gradients mediated by bound material is known as haptotaxis (Carter, 1967). Fibroblasts adherent to both surfaces of the membrane were accordingly counted in the fibronectin chequer-board experiment. Cells were counted in 5 fields at 1000X magnification in 3 wells for each concentration of fibronectin. No significant difference was found in the adherence of fibroblasts to any of the wells ($p > 0.05$ Student t test). Therefore, in these experiments, enhanced cell adherence to the membrane is not responsible for fibroblast migration to fibronectin.

3.2.3. Fibroblast conditioned medium

Rabbit scleral fibroblast conditioned medium was strongly chemoattractant to rabbit Tenon's fibroblasts. When the conditioned medium was first prepared it was 2 to 3 times as potent in its chemoattractant effect as an optimal dose of fibronectin. After storage for 28 days at -20^{o}C, fibroblast conditioned medium was approximately half as potent as fibronectin (fig.3.15.).

3.2.4. Rabbit aqueous humour.

All the specimens of normal rabbit aqueous humour were strongly chemoattractant to rabbit Tenon's capsule fibroblasts, and the optimal effect seen was always greater than that of an optimal dose of fibronectin. Using a small number of specimens at concentrations varying from 4 to 100%, the range of chemoattractant doses was defined (4% aqueous was used as the smallest dose as this represented 1 ul of

a

UPPER CHAMBERS

LOWER CHAMBERS: Fibronectin (µg/ml)	0	5	10	20
0	29	36	24	22
5	29	26	26	27
10	88	85	72	72
20	111	102	108	94

b

UPPER CHAMBERS

LOWER CHAMBERS: Fibronectin (µg/ml)	0	10	20	30
0	0	0	0	0
10	49	25	26	35
20	50	34	33	28
30	45	47	42	44

Fig.3.14. Chequer-board analysis of the chemoattractant activity of fibronectin a.)Rabbit Tenon's fibroblasts were used as the indicator

cells. Figures are the means of three identical chequer-boards run simultaneously. b.)Human scleral fibroblasts were used as the indicator cells in a single chequer-board. (In this particular run optimal migration of human scleral fibroblasts occurred at 20 ug/ml fibronectin, although in most other experiments 30 ug/ml proved to be the optimal chemoattractive concentration.) Similiar results are seen with both types of cells. Fibronectin was added to both upper and lower wells of the chambers such that the cells were exposed to i.)a positive gradient of fibronectin (seen in the second vertical column where fibronectin is present in the lower wells only) ii.)a negative gradient of the chemoattractant (seen in the second horizontal row where fibronectin is present in the upper wells only and F10 medium is present in the lower wells) and iii.)no gradient of fibronectin but merely an increasing concentration, equal in both the upper and lower wells (seen along the oblique parallel lines). Fibronectin is seen to be primarily chemotactic to fibroblasts since an increasing number of cells migrates to an increasingly positive gradient. However, there is also a moderate chemokinetic effect, shown by an increasing number of cells migrating in the presence of an increasing concentration of fibronectin. This effect is limited since an increasing number of cells do not migrate in the presence of a negative gradient alone.

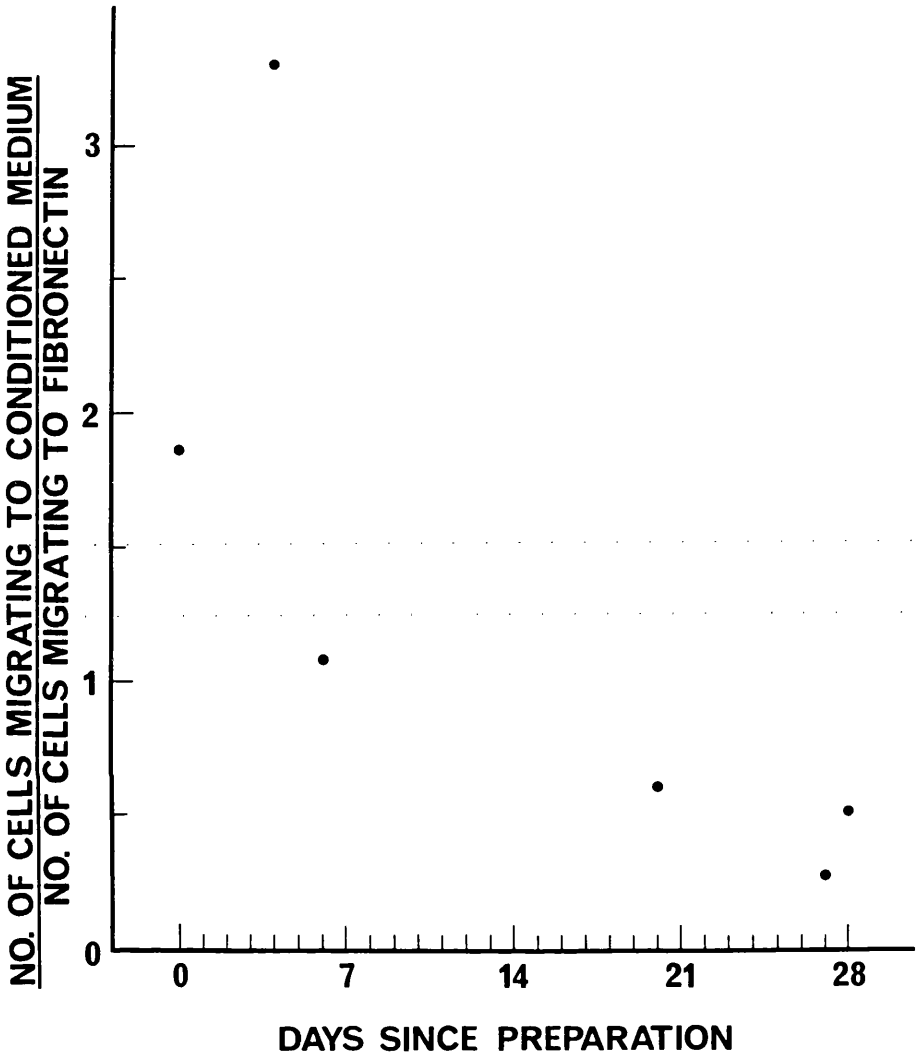


Fig.3.15. Graph showing the decline in the relative chemoattractant activity of fibroblast conditioned medium over time. The chemoattractant activity was measured as the ratio of the number of cells in 20 1000X fields migrating to fibroblast conditioned medium over the number of cells in 20 1000X fields migrating to 20 ug/ml fibronectin. Each point is the mean of three observations. Standard errors of the mean were all less than 16%.

aqueous in the 25 ul capacity lower wells of the chamber). There was a massive increase in cell migration with concentrations as low as 4% aqueous humour ($p < 0.001$ Student t test). The peak response was seen at about 10%. Beyond about 20%, there was a slow decline in chemoattractant activity upto 100% aqueous humour (fig.3.16.).

The chemoattractant activity of rabbit aqueous humour was submitted to chequer board analyses, repeated on 3 separate occasions. In the presence of an increasingly positive gradient of aqueous humour, an increasing number of fibroblasts migrated, indicating a potent chemotactic effect. When the cells were subjected to an increasing concentration of aqueous humour, equal in both the upper and lower wells of the chemotaxis chamber, an increasing number of cells migrated at low doses, but this then diminished at higher doses. This indicated a moderate chemokinetic response. Thus, rabbit aqueous humour primarily induced the chemotactic migration of rabbit Tenon's capsule fibroblasts, with a smaller chemokinetic component (fig.3.17.).

Rabbit aqueous humour boiled for 5 minutes retained approximately 35% of its original chemoattractant activity (fig.3.18.). Chequer-board analysis demonstrated that this activity was, like that of normal aqueous humour, primarily chemotactic (fig.3.19.). There thus appear to be at least 2 chemotactic components in normal rabbit aqueous humour, one heat stable and the other heat labile.

Pretreatment of rabbits with anti-inflammatory drugs before paracentesis caused no effect on the chemoattractant activity of their aqueous as compared to a normal untreated control (fig.3.20.). This indicated that the technique of aqueous humour sampling did not damage the blood-aqueous humour barrier during the procedure.

3.2.5. Human aqueous humour

A limited number of specimens of cataractous aqueous humour was used in chemoattraction assays to generate dose response curves, at concentrations varying from 4 to 100%. Once the range of greatest chemoattractant activity had been identified, twelve specimens were run simultaneously in a single 48-well chamber, using rabbit Tenon's fibroblasts as the indicator cells. The peak response was seen at

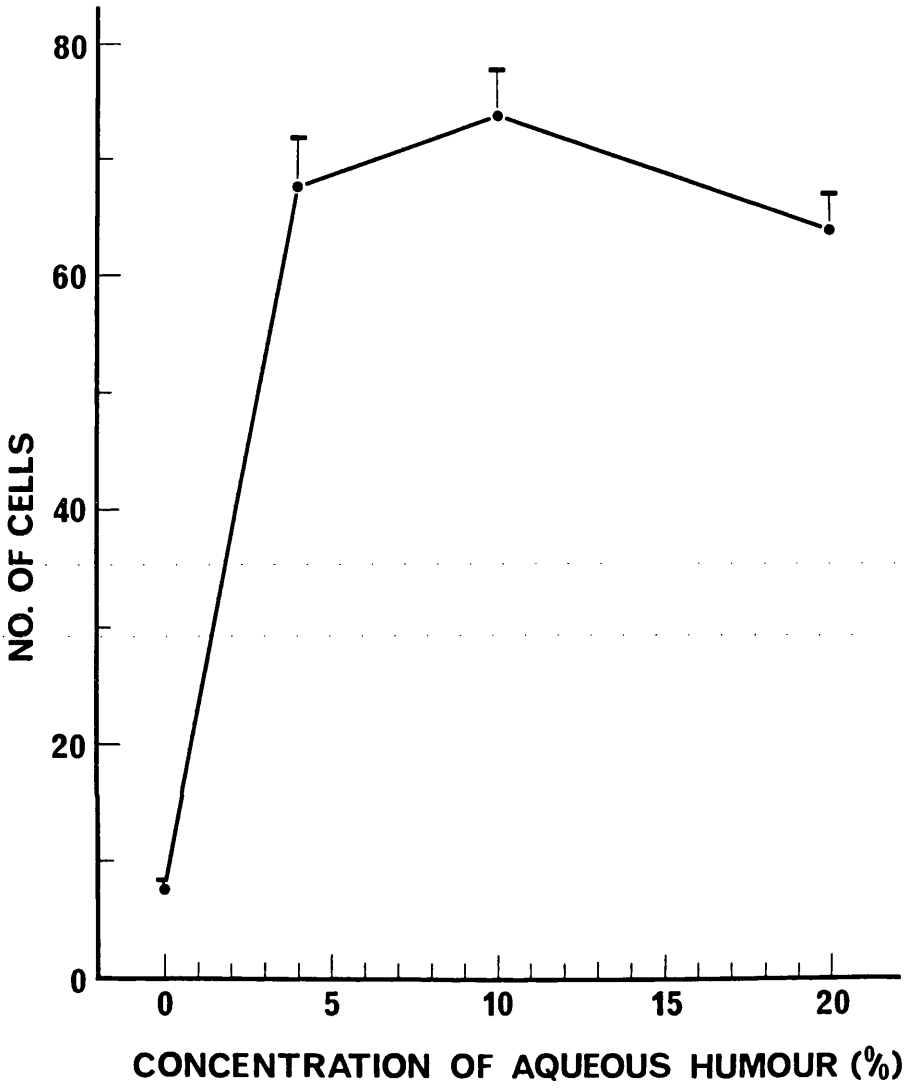


Fig.3.16. Dose-response curve of the chemoattractant activity of normal rabbit aqueous humour. At 0% aqueous (serum free F10 medium), point is the mean of three observations. Other points are the means of fourteen aqueous specimens taken from 8 rabbits and run simultaneously in the 48-well micro-chemotaxis chamber. Cells have been counted in 20 1000X fields for each sample. Bars are +SEM. 20 ug/ml fibronectin caused 45 cells to migrate. 4% aqueous caused a highly significant increase in the number of cells migrating across the membrane ($p < 0.001$ Student t test). There is no significant difference between the effect of 10% and 4% aqueous ($p > 0.05$) or between the effect of 20% and 4% aqueous ($p > 0.5$ Student t test).

UPPER CHAMBERS

LOWER CHAMBERS % Aqueous	0	4	10	20
0	26	25	16	6
4	87	63	40	20
10	118	55	38	16
20	152	88	54	15

Fig.3.17. Chequer-board analysis of the chemoattractant activity of rabbit aqueous humour. By examining the second vertical column it is apparent that normal rabbit aqueous humour is powerfully chemotactic to rabbit Tenon's capsule fibroblasts (cf.optimal dose of fibronectin which caused 62 cells to migrate), but at low doses there is a moderate chemokinetic effect which diminishes at higher doses (seen between the oblique parallel lines). A similiar result has been seen in chequer-boards done on 3 other aqueous specimens.

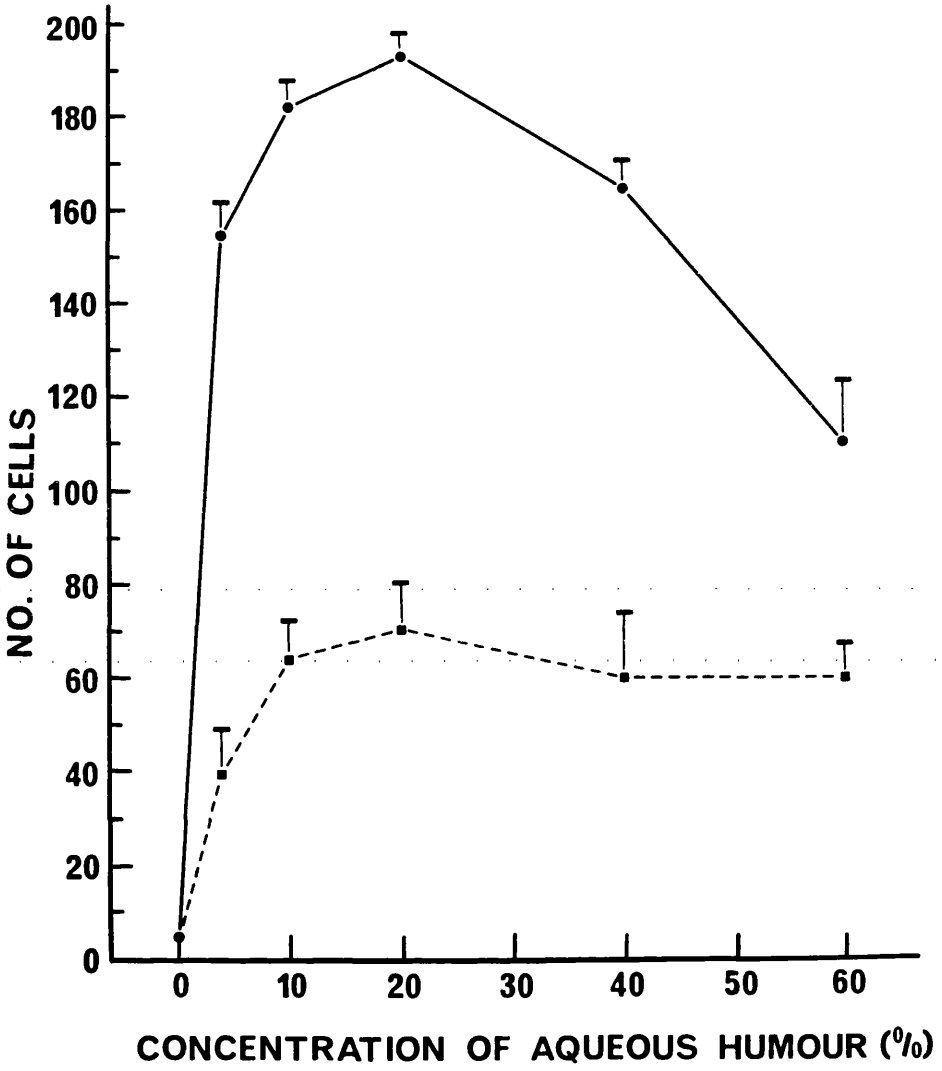


Fig.3.18. Graph showing the chemoattractant activity of normal rabbit aqueous humour (—●—) and of similiar aqueous humour that has been boiled for 5 minutes (- -■- -). The normal aqueous was taken from one eye of each of two rabbits, while the boiled aqueous was derived from their other eyes. Samples were run in duplicate, therefore each point is the mean of four wells. Bars are +SEM. 20 ug/ml fibronectin caused 90 cells to migrate. For 20% aqueous, boiling reduced the chemoattractant activity by about 65% ($p < 0.001$ Student t test).

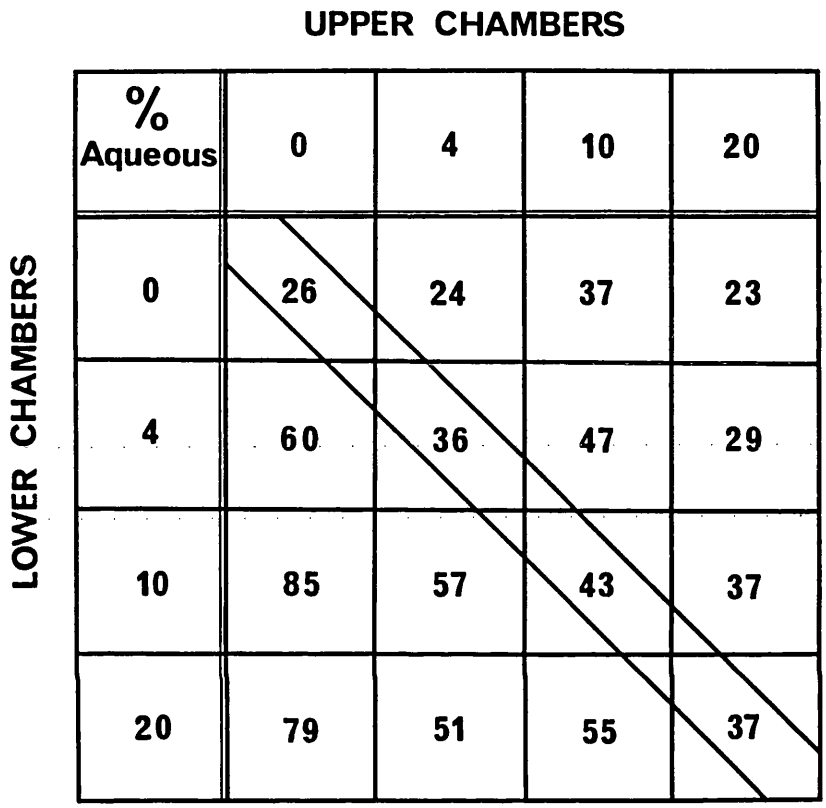


Fig.3.19. Chequer-board analysis of the chemoattractant activity of rabbit aqueous humour that had been boiled for five minutes. This specimen was derived from the other eye of the rabbit shown in figure 3.17. The aqueous retains much of its chemotactic activity with a moderate chemokinetic effect at low doses, which diminishes at higher doses.

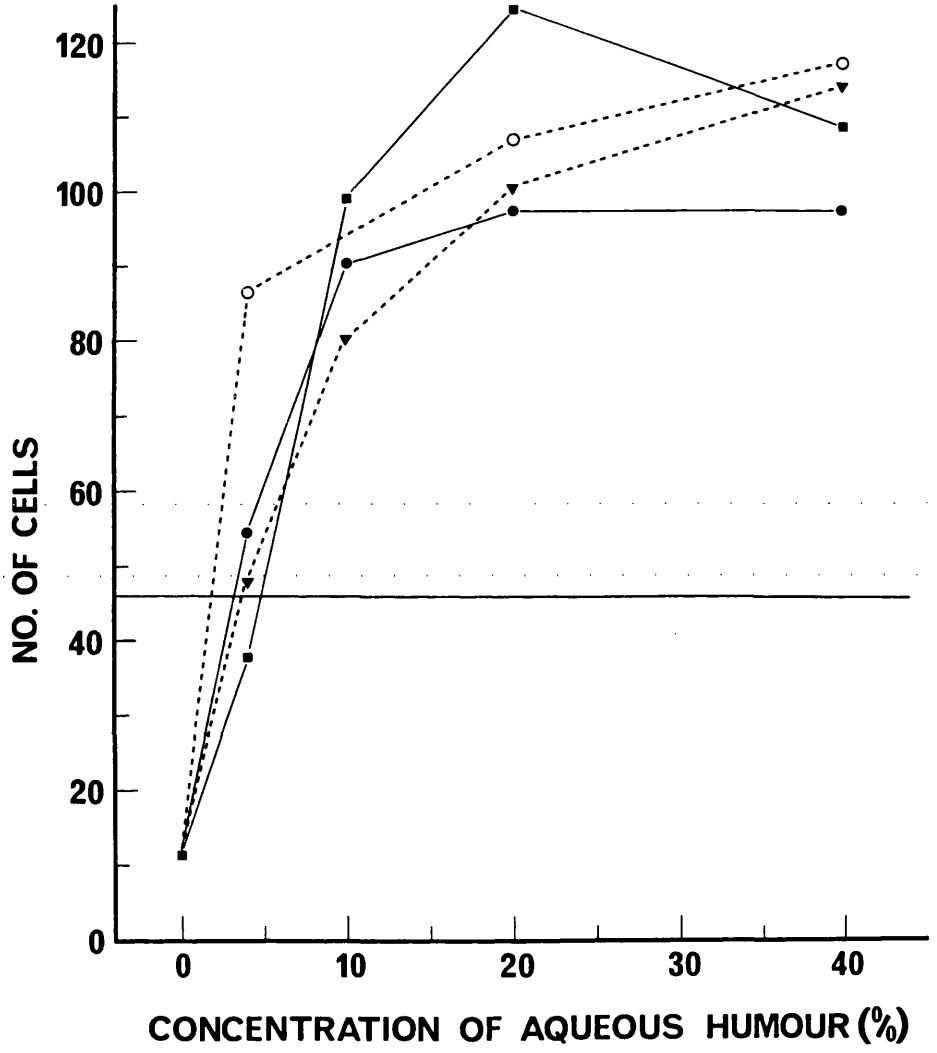


Fig.3.20. Graph showing the effect of pretreatment of rabbits with anti-inflammatory drugs on the chemoattractant activity of their aqueous humour. Two wells were run at each concentration for each rabbit. —■— untreated control; —●— BN52021; - - -▼- - - aspirin; - - -○- - - indomethacin. The horizontal line is the response of rabbit Tenon's fibroblast to fibronectin. Error bars have been omitted for the sake of clarity. There is no significant difference in the response of the four animals treated in different ways ($p > 0.05$ Student t test).

approximately 20% aqueous humour (fig.3.21.). Beyond 40% aqueous there was a slow decline in the number of migrated cells.

When human scleral fibroblasts were adopted as the indicator cells, in general fewer cells migrated than when rabbit cells were evaluated. An increasing number of cells also continued to migrate up to 60% aqueous, which was the highest concentration tested (fig.3.22.).

As with the rabbit aqueous humour, all the specimens of cataractous aqueous humour evaluated were chemoattractant for both rabbit and human ocular fibroblasts. The maximum response seen was also greater than that to an optimal concentration of fibronectin.

Twelve of the specimens of cataractous aqueous humour, taken by 3 separate surgeons, were further analysed to determine whether either the surgical approach or the surgical technique influenced the chemoattractant activity of the aqueous samples. Four specimens were provided by each surgeon.

The responses seen with the aqueous from the two surgeons who used an ab externo approach to cataract surgery, taking the specimens through a limbal groove after first elevating a fornix based conjunctival flap, were virtually identical. The mean numbers of cells migrating to the four specimens derived from the surgeon who used a corneal approach to cataract surgery, and therefore took the specimens through a partial thickness corneal groove, were generally lower than those of the other surgeons. However, there was no statistically significant difference (Wilcoxon two-sample test). It would therefore appear that neither the surgeon nor the surgical approach influences the chemoattractant activity of aqueous humour derived from patients undergoing cataract surgery, provided that the specimens are taken as the first intra-ocular manoeuvre, as close to the start of surgery as possible (fig.3.23.).

The pH of the wells of the micro-chemotaxis chamber when aqueous was used as the chemoattractant was measured after equilibration with air. The mean pH of the upper wells was 6.26+/-0.10 SD and of the lower wells was 6.33+/-0.08 SD. This difference is not significant (Wilcoxon two-sample test). These pH measurements are of the same order as

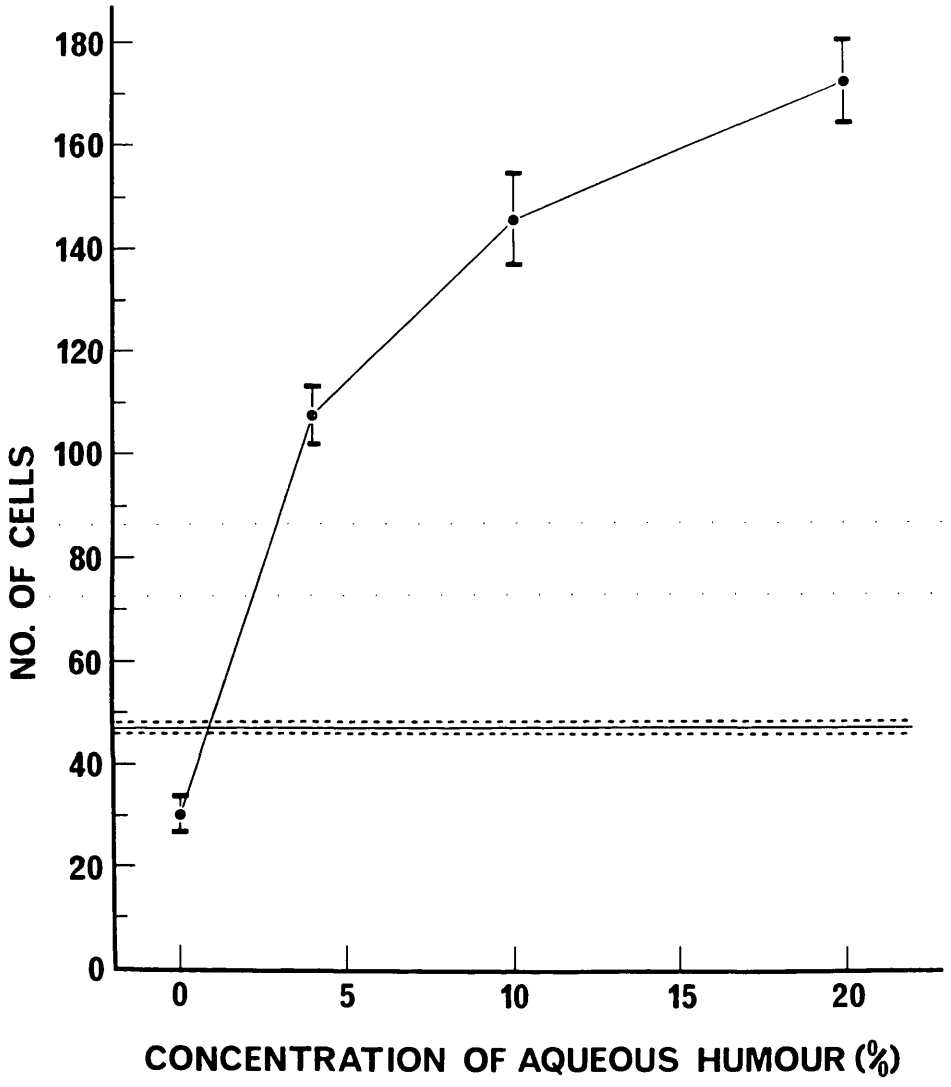


Fig.3.21. Dose-response curve for cataractous human aqueous humour. For 0% aqueous humour (serum free F10), n = 3. Other points show the mean of 12 specimens. The horizontal bar shows the response to an optimal dose of 20 ug/ml fibronectin +/-SEM. Rabbit Tenon's capsule fibroblasts have been counted in 20 1000X fields. Bars are SEM.

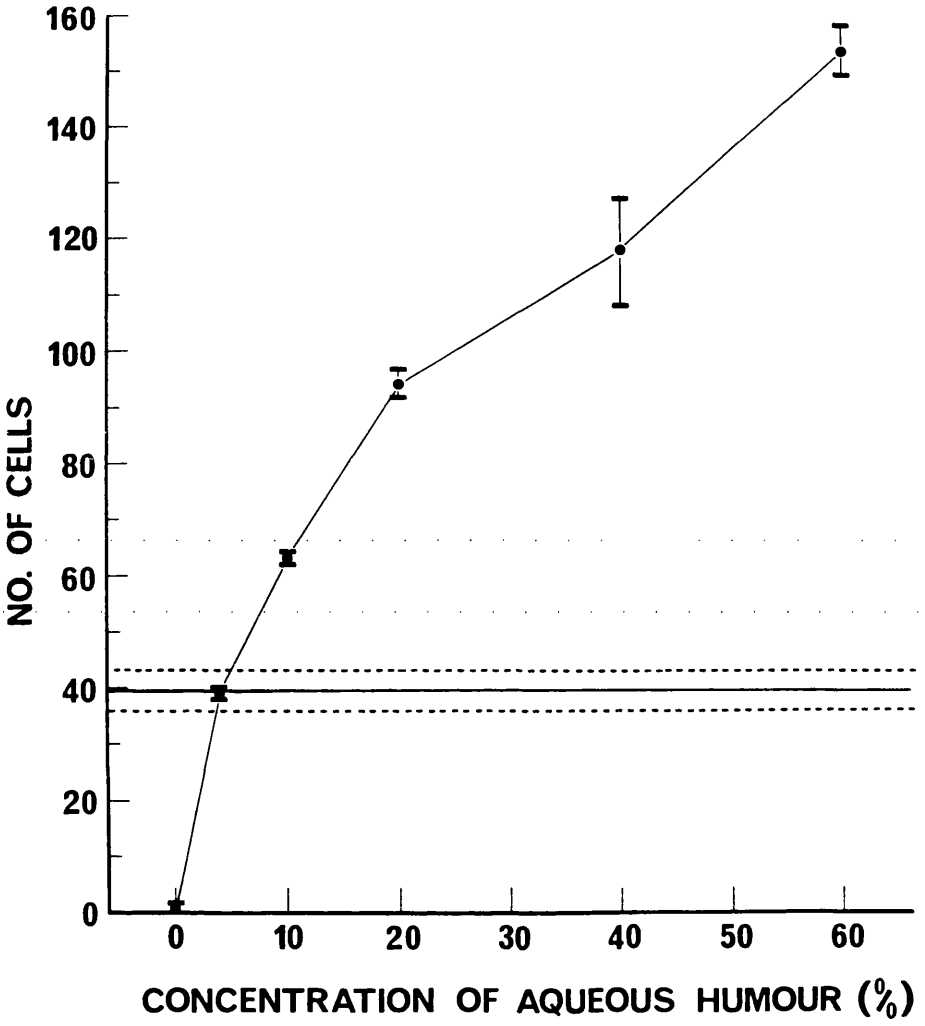


Fig.3.22. Graph showing the response of human scleral fibroblasts to aqueous humour taken from patients with cataracts. Horizontal line is the response of the cells to 30 ug/ml fibronectin +/-SEM. n = 3; bars are SEM.

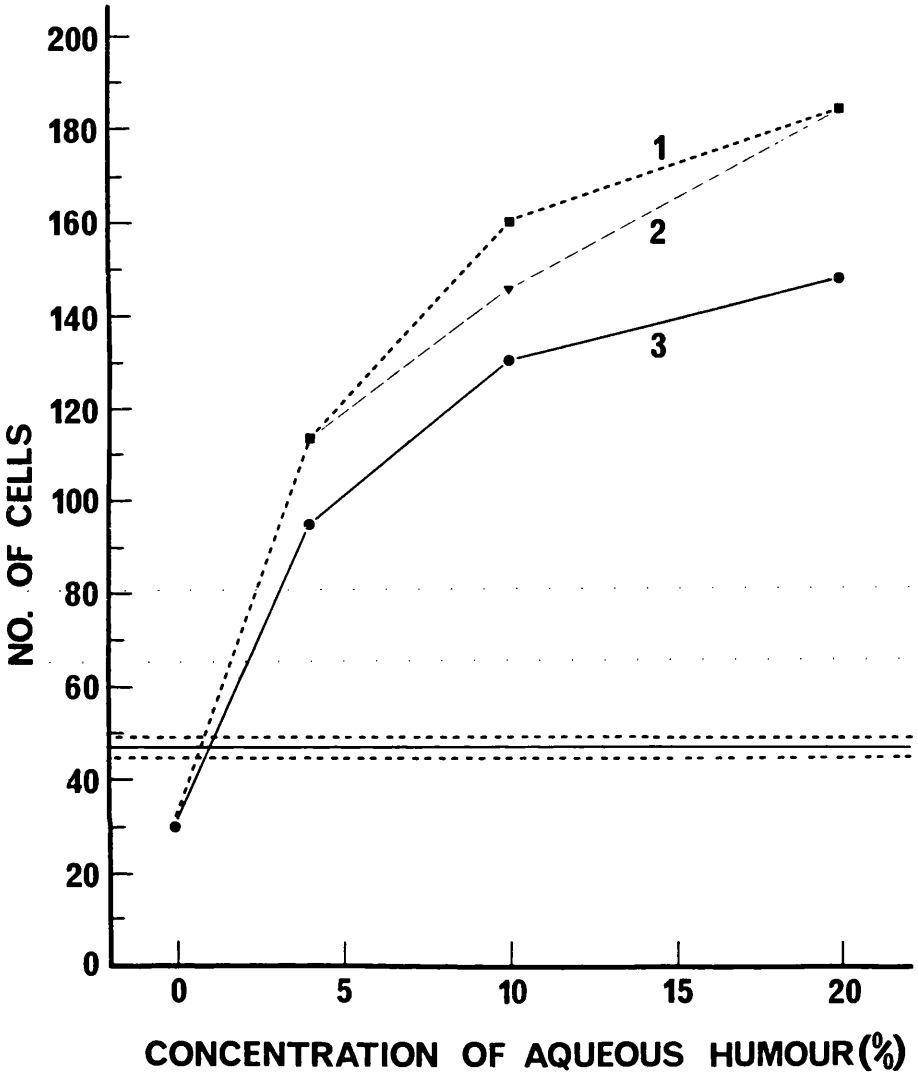


Fig.3.23. Graph showing the effect of different surgical approaches and different surgeons on the chemoattractant activity of aqueous humour taken from patients with cataracts. The data is the same as that shown in figure 3.21. Surgeons 1 and 2 used a limbal approach while surgeon 3 used a corneal section for cataract extraction. The responses from the specimens derived from surgeons 1 and 2 were virtually identical. The response from the corneal sections is generally lower than the limbal sections, but the difference was not significant (Wilcoxon two-sample test). The horizontal line is the response of the rabbit Tenon's fibroblasts to 20 ug/ml fibronectin +/-SEM.

intracellular pH measurements made in fibroblasts at the healing edge of a wound (Silver, 1973).

Chequer-board analyses were performed on cataractous aqueous humour using both rabbit and human ocular fibroblasts. These demonstrated that the aqueous humour was powerfully chemotactic to both rabbit and human cells, although in general fewer human cells migrated than rabbit cells. Part of the chemoattractant activity could also be attributed to a chemokinetic effect which was moderate at low concentrations of aqueous, but diminished at higher concentrations. A similiar response was seen with four samples of aqueous which were run on different occasions. The response seen is virtually identical to that described in section 3.2.4. for rabbit aqueous humour and rabbit Tenon's capsule fibroblasts (fig.3.24.).

Ultrafiltration of cataractous aqueous humour with a 30,000 molecular weight cut-off indicated that all chemoattractants in the aqueous were over this molecular weight (fig.3.25.). Unfortunately due to the unavailability of ultra-centrifuge devices with a higher molecular weight cut-off, no upper limit of the molecular weight was obtained.

Increasing the pH of cataractous aqueous humour to 11 and then returning it to 7.2 after 45 minutes barely effected the chemo-attractant activity. However, dropping the pH to 3 before returning to 7.2, markedly diminished the chemoattractant activity ($p < 0.01$ Wilcoxon two-sample test), (fig.3.26.).

Boiling reduces the chemoattractant activity of cataractous aqueous humour by about 50% ($p < 0.01$ Paired t test), (fig.3.27.). The remaining chemoattractant activity of boiled cataractous aqueous humour was shown by chequer-board analysis to be chemotactic.

Pretreatment of patients for 24 hours before cataract extraction with Guttae indomethacin 1% q.d.s. did not influence the chemoattractant activity of their aqueous humour when compared to untreated control patients ($p > 0.05$ Wilcoxon two-sample test), (fig.3.28).

The mean protein level of the nine specimens of cataractous aqueous humour assessed was 36.2 mg/100 ml (+/-4.0 mg/100 ml SEM) with a range from 25.5 to 63 mg/100 ml. This is in accordance with the normal range

UPPER CHAMBERS

LOWER CHAMBERS % Aqueous	0	4	10	20
0	17	36	28	30
4	62	136	89	62
10	174	129	130	50
20	215	190	153	94

UPPER CHAMBERS

LOWER CHAMBERS % Aqueous	0	4	10	20
0	0	0	2	0
4	1	1	3	3
10	13	9	3	4
20	22	10	9	7

Fig.3.24. Chequer-board analyses of cataractous human aqueous humour. Samples of aqueous were run in the chequer-boards at concentrations

varying from 0 to 20%. The predominant effect seen was chemotaxis ie. an increasing number of cells migrate in the presence of a positive gradient of aqueous (seen in the second vertical column), but there is also a chemokinetic effect which in a.) increases at low concentrations, but diminishes at higher concentrations of aqueous humour (seen between the oblique parallel lines). The indicator cells were rabbit Tenon's fibroblasts in a.) and human scleral fibroblasts in b.). Fewer human cells migrate than rabbit cells but the trends are similiar.

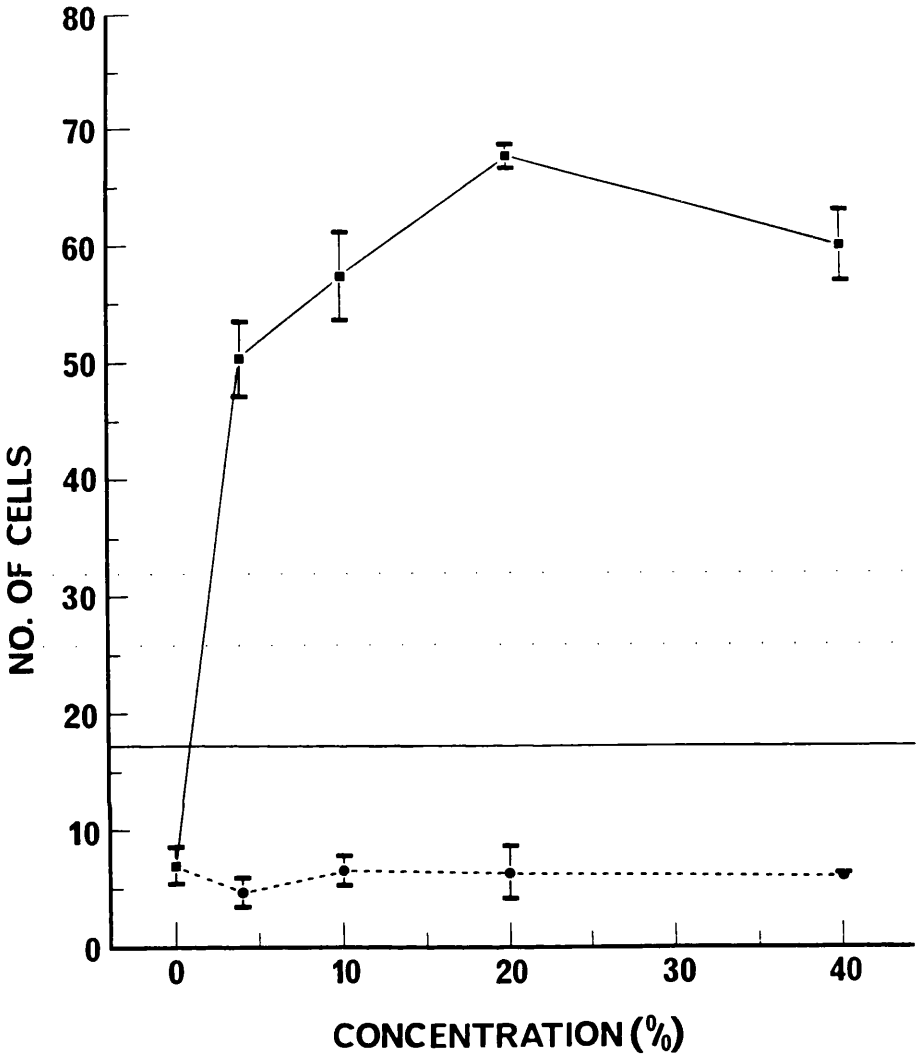


Fig.3.25. Graph showing the effect of ultrafiltration on a sample of aqueous humour taken from a patient with a cataract. The upper solid curve is the response to the unfiltered aqueous; the lower interrupted line is the response to the ultrafiltrate of less than 30 kiloDaltons; the horizontal line is the positive control of fibronectin. Bars are SEM. For each point n = 3. The chemoattractant activity of aqueous humour is attributable to substances with a molecular weight greater than 30,000.

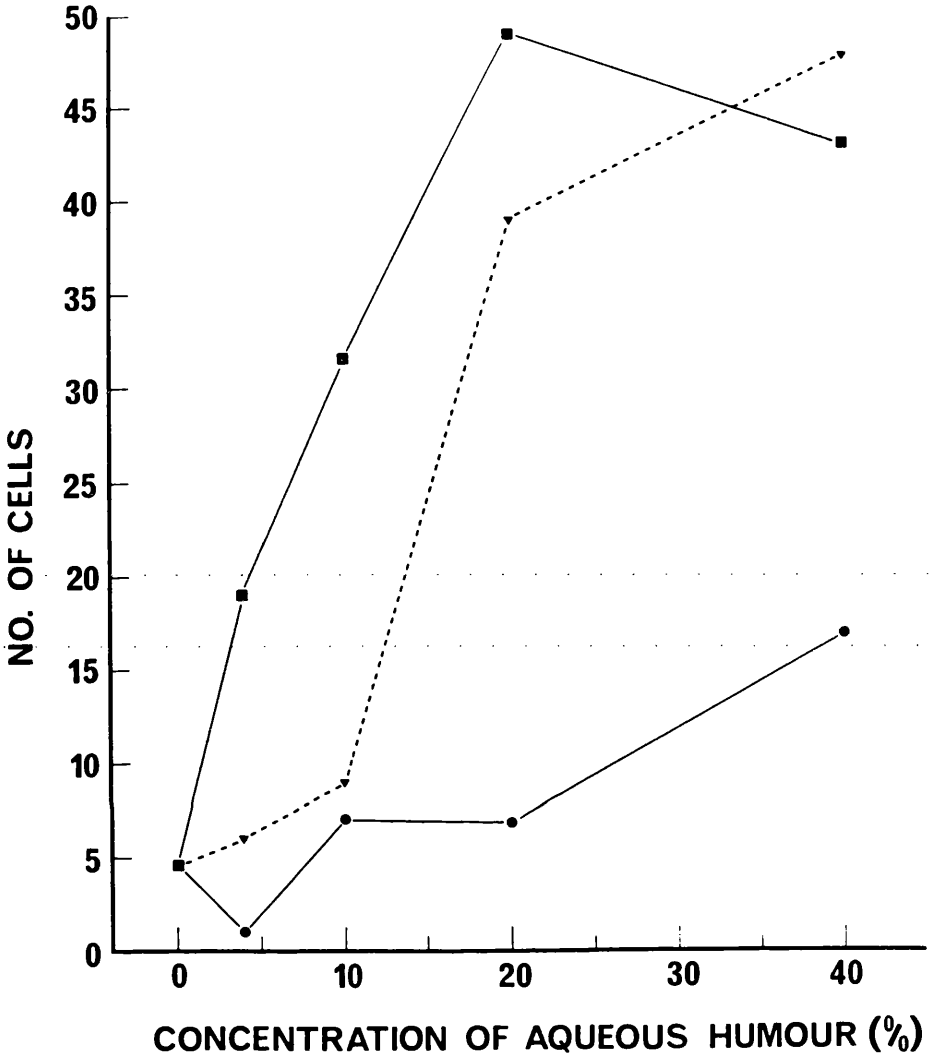


Fig.3.26. Graph showing the effect of pH changes on the chemoattractant activity of a sample of aqueous humour taken from a patient with a cataract. —■— untreated aqueous humour; - - -▼- - - initial pH 11; —●— initial pH 3. The chemoattractant constituents were sensitive to low pH which significantly reduced their activity ($p < 0.01$ Wilcoxon two-sample test).

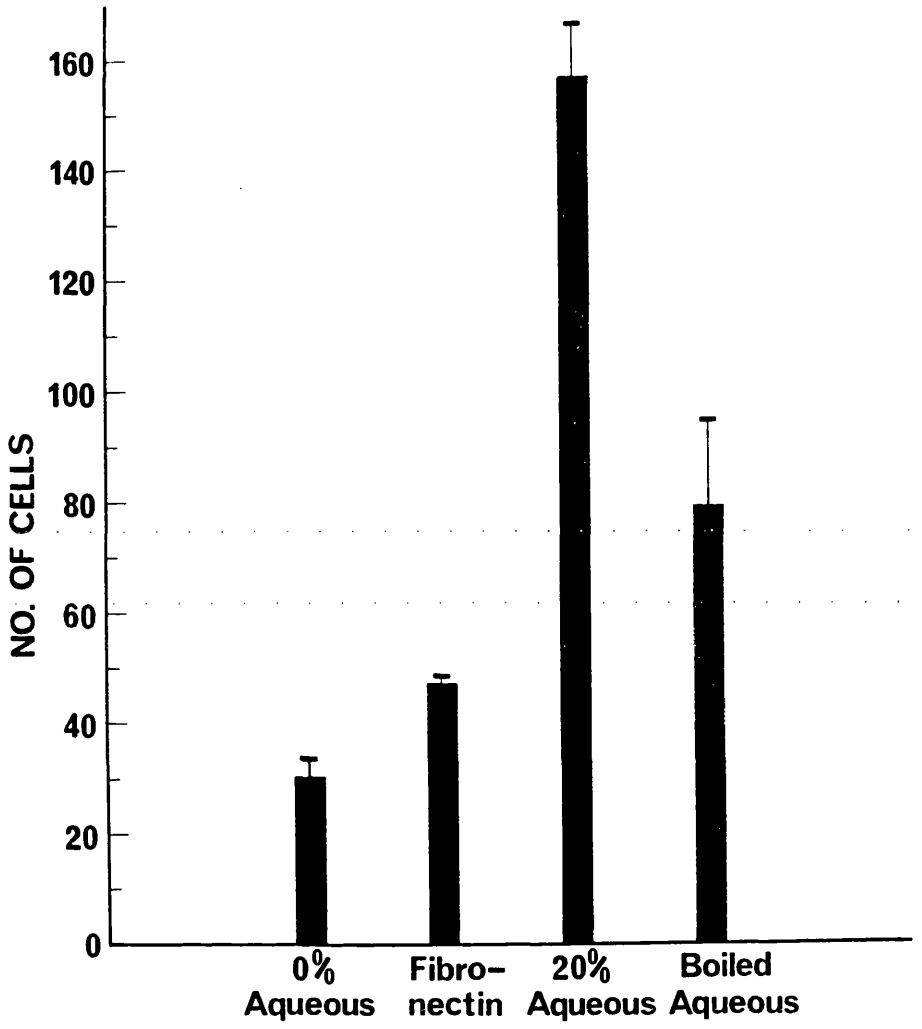


Fig.3.27. Histogram showing the effect of boiling on the chemoattractant activity of aqueous humour taken from patients with cataracts. Aliquots of 6 specimens of aqueous were boiled for 5 minutes. Chemoattractant activity was then compared with the unboiled specimens. Bars are SEM. Boiling reduced the chemoattractant activity by about 50% ($p < 0.01$ Paired t test).

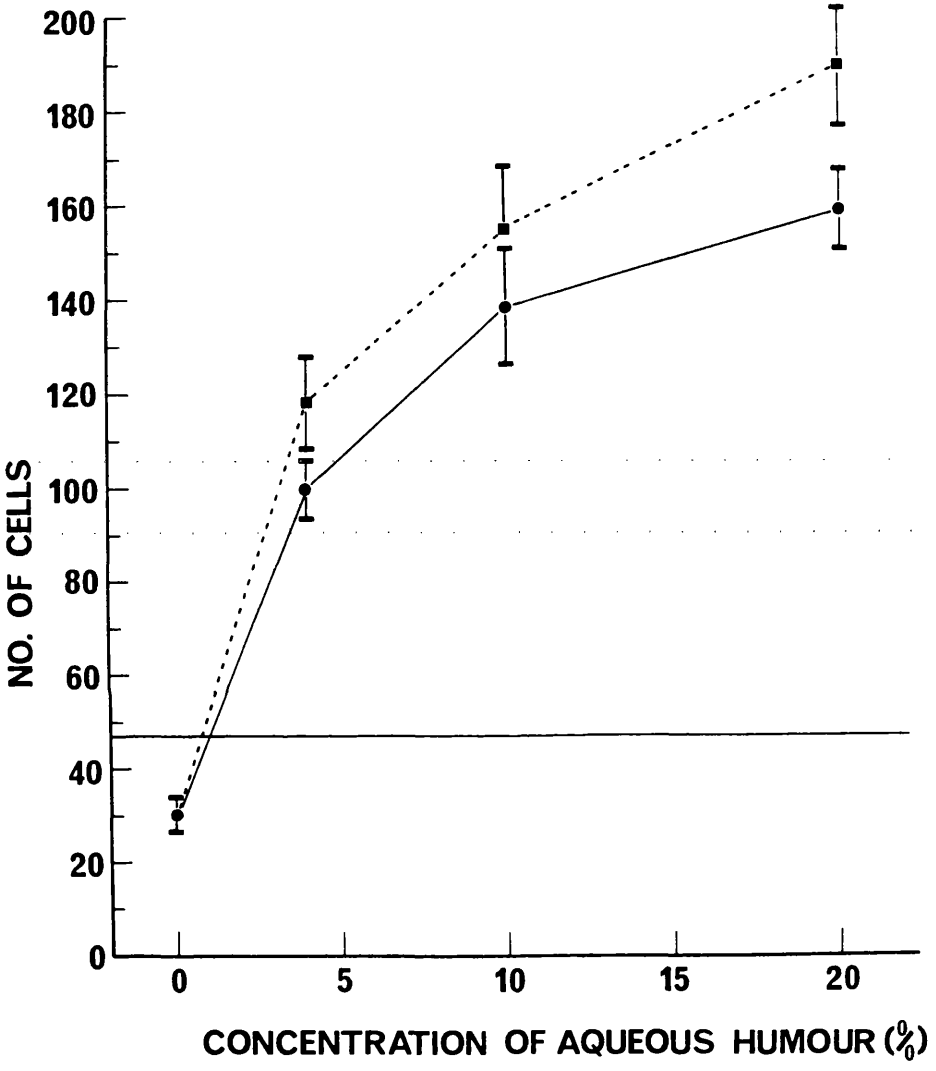


Fig.3.28. Graph showing the effect of pretreatment of patients with Guttae Indomethacin 1% q.d.s. for 24 hours before cataract extraction. The data is the same as that used in figure 3.24. in which 12 aqueous samples were evaluated. Five of the 12 patients were treated with indomethacin.- - -■- - -on indomethacin;——●——off indomethacin. There is no significant difference at any concentration between the chemoattractant activity of the aqueous humour of the two groups ($p > 0.05$ Wilcoxon two-sample test).

of 30 to 50 mg/100 ml as described by Cole, (1984). The small number of samples assessed precludes detailed statistical analysis. However, there was a small but statistically non-significant trend of increased chemoattractant activity with increased aqueous protein concentration. High protein levels did not appear to be related to a particular surgeon or surgical technique.

Initial experiments using aqueous humour derived from patients with glaucoma, at concentrations varying from 4 to 100%, showed that the dose-response curve for the migration of fibroblasts was similiar to that of aqueous humour derived from patients with cataracts.

The chemoattractant activity of cataractous and glaucomatous aqueous humour was compared. Of the 58 specimens of aqueous humour shown in table 2.1. (page 135), forty two specimens were run simultaneously in a comparative assay using rabbit Tenon's fibroblasts as the indicator cells. Samples were run at 4, 10, and 20% concentrations. In another experiment human scleral fibroblasts and a total of forty specimens of aqueous humour were run at a 40% concentration, in duplicate. All the specimens assessed were powerfully chemoattractant. The responses seen with rabbit and human cells were qualitatively very similiar, but there were quantitative differences in that fewer human cells migrated than rabbit cells. The mean number of cells migrating to the specimens derived from the patients undergoing reoperation for glaucoma was significantly greater than the mean number migrating to the control cataractous specimens ($p < 0.01$ Analysis of variance). The specimens derived from patients undergoing primary or secondary trabeculectomies did not differ significantly from the control cataractous specimens (fig.3.29.). There was also no significant difference between the chemoattractant activities of the aqueous specimens obtained from patients undergoing secondary trabeculectomies at Addenbrooke's Hospital and Moorfields Eye Hospital.

Chequer-board analysis of the aqueous taken from patients undergoing glaucoma surgery, having previously sustained failed drainage surgery, demonstrated a similiar response to that seen with both cataractous human aqueous humour and with rabbit aqueous humour, ie. the chemoattractant activity was primarily due to chemotaxis with a moderate chemokinetic effect at low doses, which diminished at higher doses (fig.3.30.)

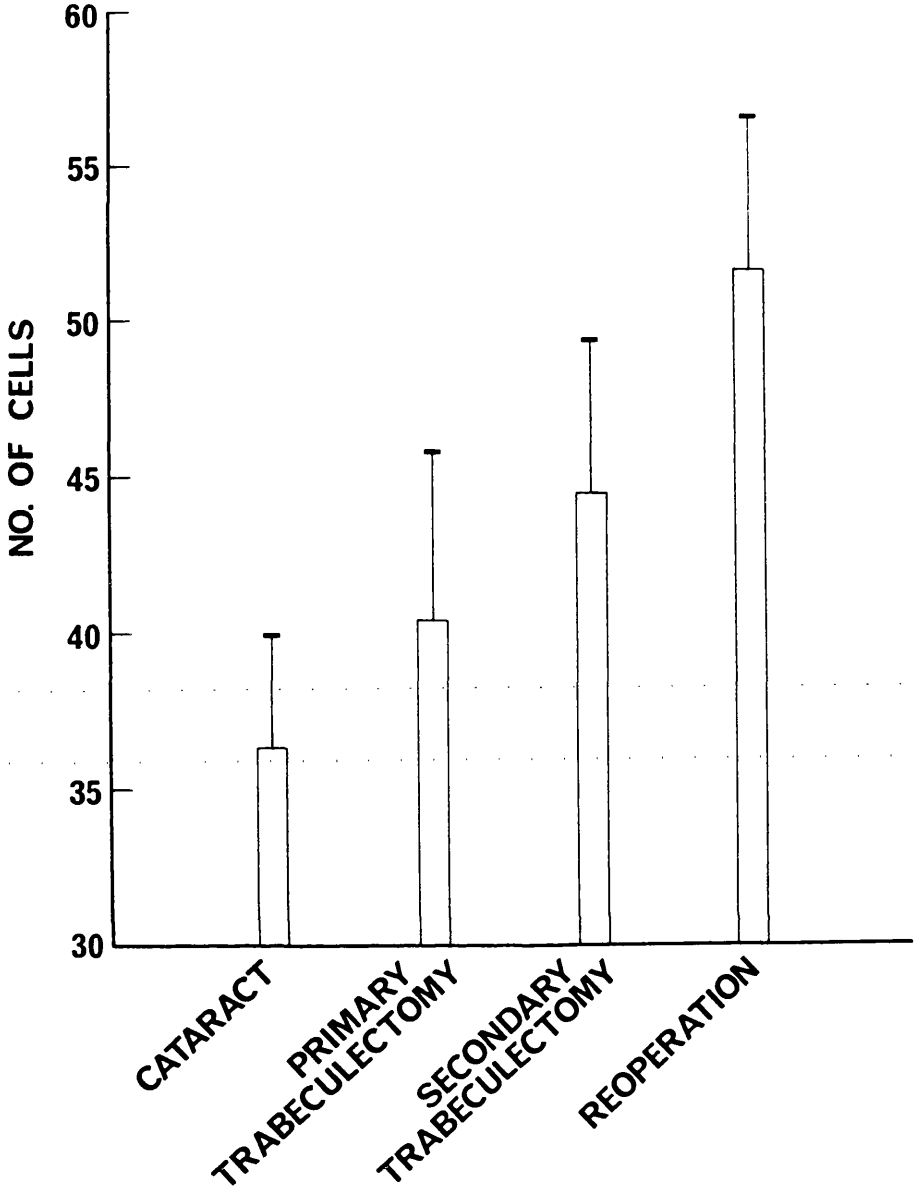


Fig.3.29. Histogram showing the chemoattractant activity of various types of glaucomatous aqueous humour, run in duplicate at a 40% concentration. For cataractous aqueous humour (control) n = 13; primary trabeculectomies n = 6; secondary trabeculectomies n = 13; reoperations n = 8. Human scleral fibroblasts have been counted in 20 1000X fields. Bars are SEM. Aqueous derived from patients undergoing reoperation attracts significantly more fibroblasts than the control cataractous specimens ($p < 0.01$ Analysis of variance). Primary and secondary trabeculectomy specimens did not differ significantly from the control aqueous.

UPPER CHAMBERS

LOWER CHAMBERS % Aqueous	0	4	10	20
0	18	35	26	25
4	223	213	105	73
10	246	201	164	92
20	306	283	93	115

Fig.3.30. Chequer-board analysis of the chemoattractant activity of aqueous humour derived from a patient undergoing implantation of a tube and gutter, having previously sustained failed glaucoma drainage surgery. The aqueous humour is powerfully chemotactic, and like cataractous aqueous humour (fig.3.24.) there is a moderate chemokinetic effect at low doses, which is diminished at higher doses. Similiar results have been seen on two other occasions. Rabbit Tenon's capsule fibroblasts were used in this instance, but similiar results are seen with human cells, although the total number of cells migrating is less.

The patients in the group undergoing reoperation had a lower mean age (41.8 years +/-12.8 SD) than the control patients with cataracts (70.8+/-12.8 SD). In order to determine whether the lower ages of the patients undergoing reoperation might account for their increased chemoattractant activity, the age of patients was correlated with the chemoattractant activity of their aqueous humour. For the patients undergoing cataract surgery there was no correlation ($r = -0.063$). Likewise, for 22 patients undergoing trabeculectomies for the first time, there was no correlation ($r = 0.270$). For the 9 patients undergoing reoperation, there was a trend towards increasing chemoattractant activity with increasing age ($r = 0.652$), however this was not statistically significant ($p > 0.05$). If the younger mean age of the reoperation group accounted for the increased chemoattractant activity of their aqueous, one would expect to see a negative correlation of age with chemoattractant activity. The fact that a positive correlation was obtained indicates that the observed chemoattractant activities were unlikely to be an artefact due to the ages of the patients.

The clinical data of the 23 patients who underwent a first trabeculectomy is shown in Table 3.1. Of these patients followed for a mean of 9.6 months (range 5 to 14 months), all but 2 attained an intraocular pressure less than 21 mmHg without additional medication. Of the remaining 2 patients one attained an intraocular pressure of 18 mmHg with the addition of Guttae Timolol 0.25% b.d.; the other patient's pressure was 28 mmHg on Guttae Pilocarpine 1% q.d.s. However, there was no progression of field loss at this pressure. Both of these patients had previously undergone YAG-laser iridotomies, one underwent a primary trabeculectomy and the other a secondary trabeculectomy. There did not appear to be an association between the level of chemoattractant activity in their aqueous humour and the fact that they had not had a totally successful surgical result. The other patient in this group who had previously undergone a surgical iridectomy had a successful surgical result with an intraocular pressure of 7 mmHg at 13 months post-operatively.

Table 3.1. Summary of the clinical data of the 23 patients undergoing a first trabeculectomy

Patient Number	Age	Follow-up (months)	Intra-ocular Pressure Diagnosis	Pre-op	Post-op	Medications	Visual Acuity Pre-op	Visual Acuity Post-op	Optic Disc Cupping Pre-op	Optic Disc Cupping Post-op	Visual Fields Defect Pre-op	Visual Fields Defect Post-op	Additional Medical Conditions
1	42	12	29	43	28	G.Pilo 1%	6/5	6/5	Normal	Normal	Normal	Normal	Nil
2	82	14	27	28	7	Nil	6/9	6/18	Mild	Mild	Mild	Not done	Nil
3	61	12	37	48	14	Nil	6/9	6/9	Mild	Mild	Normal	Normal	Nil
4	73	11	20	24	18	Nil	6/9	6/12	Moderate	Moderate	Severe	Not done	Nil
5	74	13	23	29	16	Nil	6/9	6/9	Severe	Severe	Severe	Severe	Nil
6	67	12	35	26	10	Nil	Unknown	6/9	Moderate	Moderate	Unknown	Unknown	Unknown
7	66	9	32	22	10	Nil	6/9	6/9	Mild	Mild	Moderate	Not done	Renal stones
8	73	7	24	19	10	Nil	Unknown	Unknown	Severe	Unknown	Moderate	Not done	Nil
9	67	13	25	22	18	Nil	6/12	6/12	Severe	Severe	Moderate	Moderate	Nil
10	79	12	28	22	10	Nil	6/5	6/9	Moderate	Unknown	Mild	Mild	Mild diabetes
11	66	12	36	27	16	Nil	4/60*	6/18	Severe	Severe	Severe	Severe	Hypertensive
12	75	3	26	27	17	Nil	6/9	6/9	Moderate	Moderate	Severe	Not done	Emphysema
13	83	15	24	24	18	Nil	6/24	6/18	Severe	Unknown	Severe	Not done	Angina pectoris
14	78	6	25	30	8	Nil	6/9	6/6	Severe	Severe	Mild	Mild	Hypertensive
15	93	10	26	20	17	Nil	6/24*	6/12	Severe	Severe	Moderate	Not done	Myocardial infarct
16	85	11	24	38	10	Nil	6/18*	6/9	Moderate	Moderate	Mild	Not done	Myocardial infarct
17	76	10	28	19	12	Nil	6/60*	6/9	Severe	Severe	Mild	Mild	Rheumatoid arthritis
18	52	9	31	23	14	Nil	6/6	6/6	Moderate	Moderate	Moderate	Moderate	Nil
19	74	12	28	27	12	Nil	6/12	6/18	Severe	Severe	Moderate	Moderate	Nil
20	65	9	42	27	18	G.Timolol	6/9	6/24	Severe	Severe	Moderate	Severe	Hypertensive
21	75	9	26	21	13	Nil	6/24*	6/12	Severe	Severe	Severe	Severe	Nil
22	55	12	22	22	16	Nil	6/36	6/60	Severe	Severe	Moderate	Moderate	Nil
23	58	15	27	23	10	Nil	6/4	6/4	Severe	Severe	Moderate	Not done	Nil

*Simultaneous cataract extraction.
Patients 1 to 8 - Primary trabeculectomies; patients 9 to 16 - Secondary trabeculectomies (Cambridge); patients 17-23 - Secondary trabeculectomies (London).

3.2.6. Drugs

Drugs with a related mode of action were assessed in groups as inhibitors of fibroblast migration.

Drugs with activity against the cytoskeleton

Taxol, cytochalasin B and colchicine strongly inhibited the migration of rabbit Tenon's fibroblasts to fibronectin when the cells were exposed to the drugs for the four hour duration of the assay. The optimally effective doses were 10^{-6}M, 10^{-5}M, and 10^{-3}M respectively. All three drugs reduced the response of the cells to the background level of migration or below ($p < 0.001$ Student t test). The dose of taxol required to reduce fibroblast migration to 50% of the maximum inhibition attained (ID_{50}) was approximately $3x10^{-8}$M. The ID_{50} for cytochalasin B was $2x10^{-7}$M and for colchicine, $2x10^{-6}$M (fig.3.31.).

Although the drugs had marked morphological effects on the cells, counting the number of cells adherent to the membrane for each dose of the drug did not reveal a significant effect on cell adherence which could account for the reduction in cell migration ($p > 0.05$ Student t test). Taxol and cytochalasin B were solubilised in dimethyl-sulphoxide. Controls were incorporated where DMSO was added to the cells at the same concentration as that used in the lowest dilution of the active drugs (0.17%). This concentration of DMSO did not inhibit fibroblast migration and had no morphological effects on the cells.

When rabbit aqueous humour was used as the chemoattractant at a 10% concentration, all three drugs inhibited fibroblast migration. Taxol reduced the response to the baseline at 10^{-6}M ($p < 0.001$ Student t test); cytochalasin B at 10^{-5}M reduced the response below the baseline ($p < 0.001$), but at 10^{-6}M this was reduced to about 78% of the response to the control ($p > 0.05$); colchicine at 10^{-3}M reduced the response to 54% of the control ($p < 0.01$) and at 10^{-4}M to 68% ($p < 0.02$), (fig.3.32.).

Drugs which increase intracellular cyclic-AMP levels

Theophylline at concentrations from 10^{-12} to 10^{-3}M had no effect on the migration of rabbit Tenon's fibroblasts. Dibutyryl cyclic-AMP caused a marginal inhibition of migration to fibronectin. The response was bell-shaped with maximum inhibition seen at both 10^{-12}

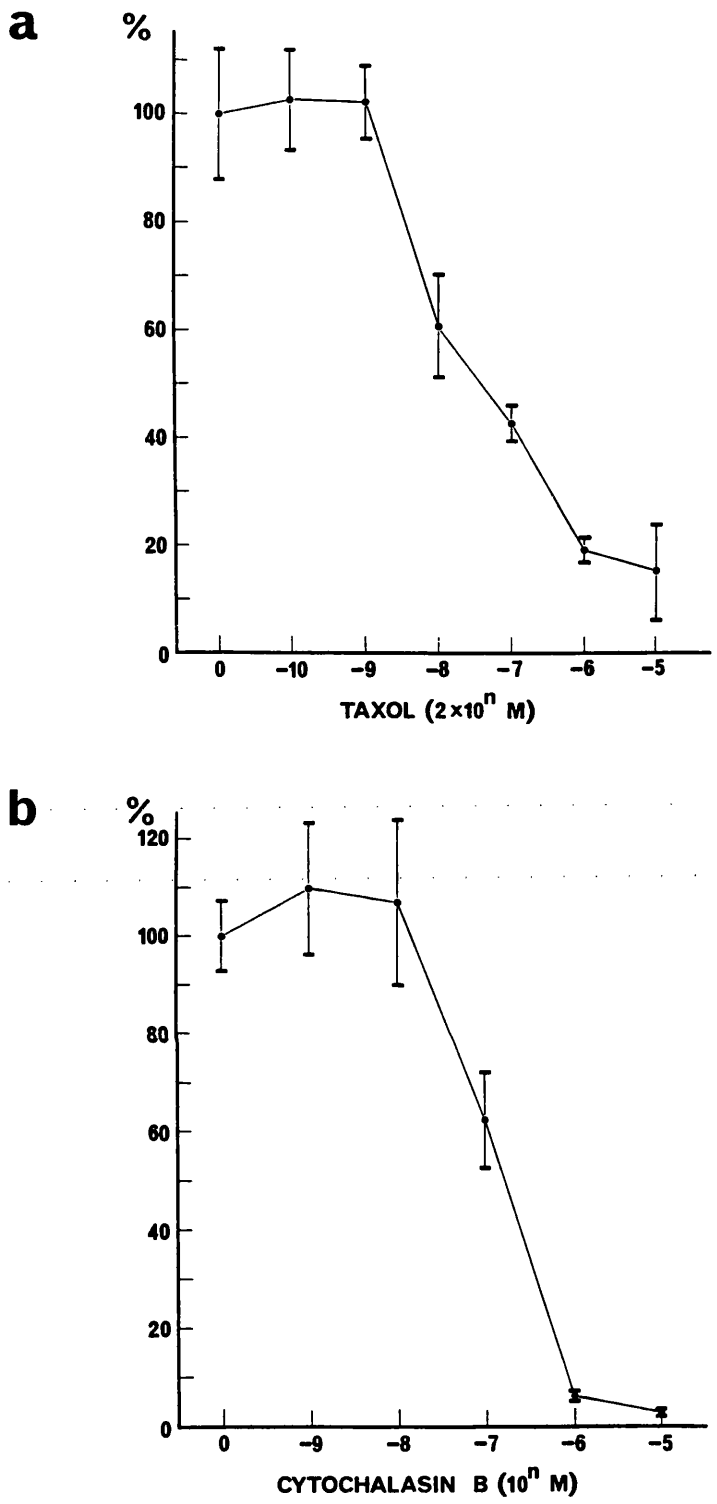


Fig.3.31a. and b. Graphs showing the effect of a.)Taxol and b.)Cytochalasin B on the migration of rabbit Tenon's capsule fibroblasts. The migration of the cells has been expressed in terms of the migration to 20 ug/ml fibronectin being 100% (this varied between 51.8 and 58.5 cells per 20 1000X fields in the different experiments). For each drug dose 4 wells were counted. At the optimally effective doses fibroblast migration is highly significantly reduced ($p < 0.001$ Student t test).

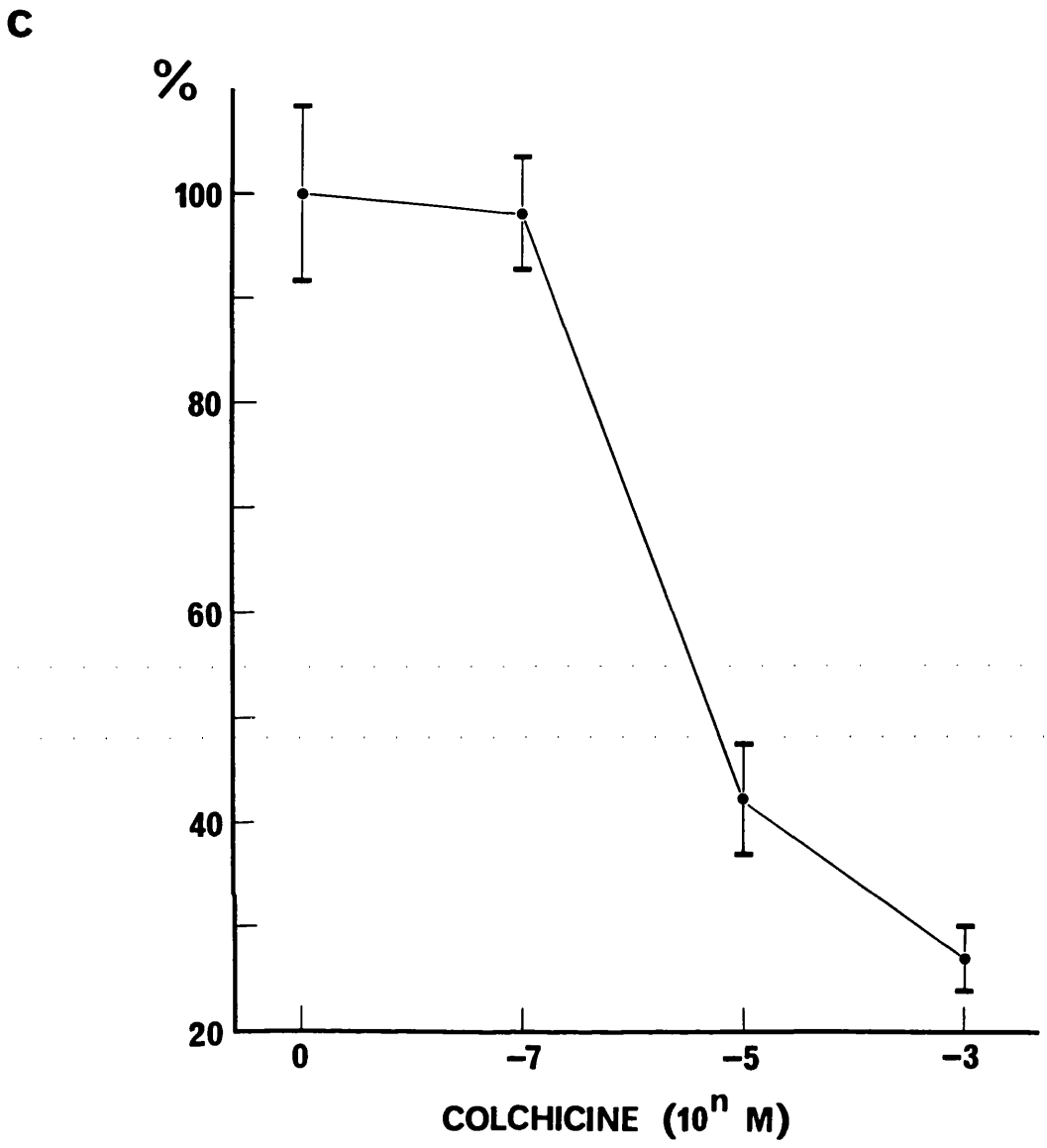


Fig.3.3lc. Graph showing the effect of Colchicine on the migration of rabbit Tenon's capsule fibroblasts to fibronectin. The migration of the cells has been expressed in terms of the migration to 20 ug/ml fibronectin being 100% (this varied between 51.8 and 58.5 cells per 20 1000X fields in the different experiments). For each drug dose 8 wells were counted. At the optimally effective dose fibroblast migration is highly significantly reduced ($p < 0.001$ Student t test).

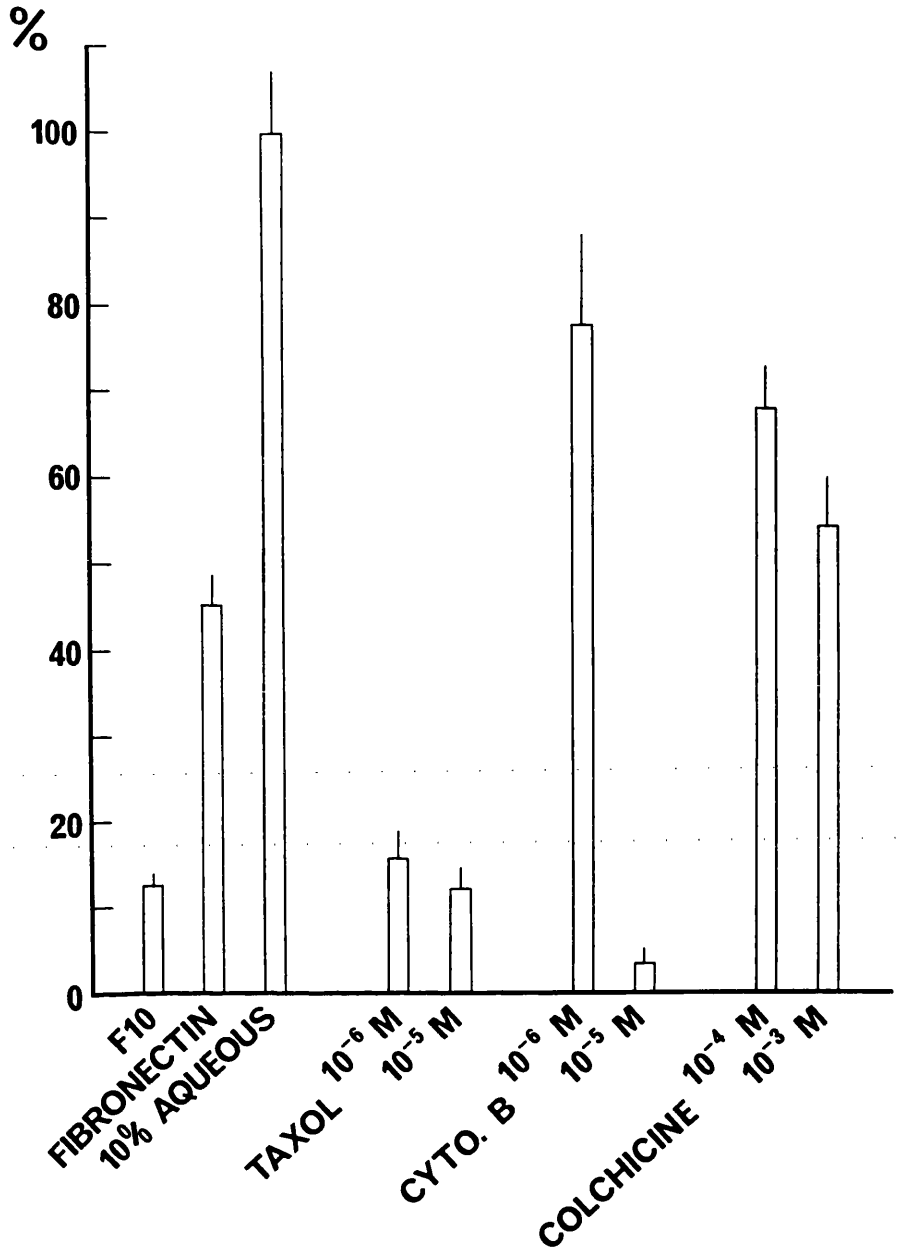


Fig.3.32. Histogram showing the effect of taxol, cytochalasin B and colchicine on inhibiting the migration of rabbit Tenon's fibroblasts to 10% rabbit aqueous humour. For each drug concentration four wells were counted. Results are expressed in terms of the migration to 10% aqueous humour being 100% (145.5+/-10.6 [Mean+/-SEM] cells migrated to 10% rabbit aqueous humour). Bars are SEM. Taxol at 10^{-6}M caused a highly significant reduction in fibroblast migration ($p < 0.001$ Student t test). Cytochalasin B at 10^{-6}M reduced fibroblast migration to 78% of the control ($p > 0.05$), but at 10^{-5}M this was reduced to about 5% ($p < 0.001$ Student t test). Colchicine at 10^{-4}M reduced the response to 68% of the control ($p < 0.02$) while at 10^{-3}M the response was reduced to 54% of the control ($p < 0.01$ Student t test).

and 10^{-3}M ($p < 0.01$). Two ID_{50} doses were therefore obtained; 10^{-6} and $4x10^{-4}$M (fig.3.33a.). Prostaglandin E_2 profoundly inhibited migration to well below the baseline at 10^{-3}M ($p < 0.001$). The ID_{50} was $5x10^{-5}$M (fig.3.33b.).

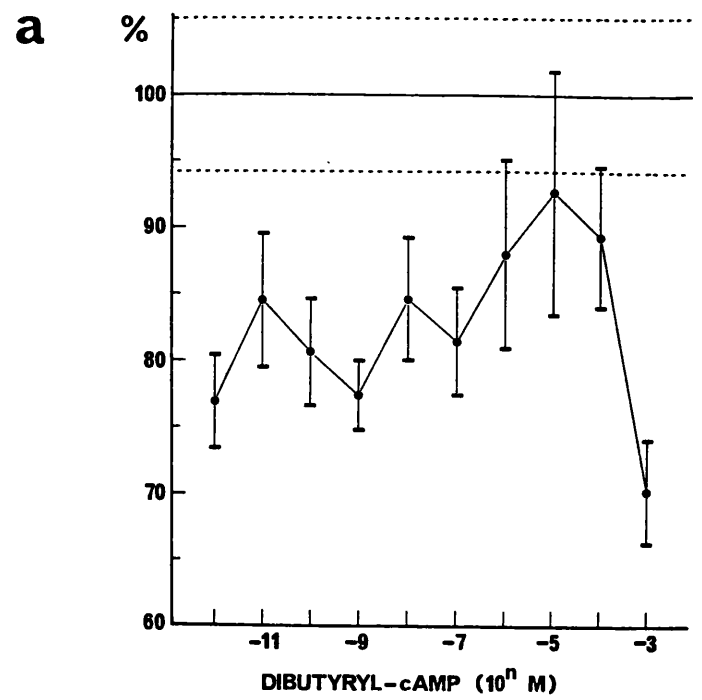
When 10% rabbit aqueous humour was used as a chemoattractant, inhibition of migration was less marked. 10^{-3}M dibutyryl cyclic-AMP reduced fibroblast migration by 21% ($p < 0.05$); at 10^{-4}M migration was only reduced by an insignificant 4.2%. PGE_2 at 10^{-3}M reduced migration by 48% ($p < 0.001$); at 10^{-4}M migration was reduced by 21% ($p < 0.05$), (fig.3.34.). Cell adherence to the membrane was not affected by these drugs.

Calmodulin antagonist

When trifluoperazine was added to the cell suspension at the start of the assay, adherence of the cells to the upper surface of the membrane was markedly impaired. Consequently, the cells were allowed to settle for 1 hour, whereupon the drug was added. However, the adherence of the cells to the membrane was still impaired in the presence of 10^{-4}M trifluoperazine ($p < 0.01$ Student t test) but surprisingly, not in the presence of 10^{-3}M trifluoperazine. 10^{-4}M also caused a greater number of fibroblasts to adhere to the periphery of the membrane in each well. Both concentrations of the drug caused marked morphological effects on the cells which will be discussed in section 3.4.2. Trifluoperazine markedly affected the migration of rabbit Tenon's fibroblasts to both fibronectin and 10% rabbit aqueous humour. 10^{-3}M was the optimally effective dose and reduced fibroblast migration to under 20% of the control ($p < 0.001$ Student t test). When 20 ug/ml fibronectin was used as the chemoattractant, the ID_{50} was about $7x10^{-5}$M (fig.3.35.).

Calcium antagonist

Nifedipine had no effect on fibroblast migration at concentrations varying from 10^{-11} to 10^{-3}M. Nifedipine was solubilised in ethanol. The maximum concentration of ethanol was 2% in the 10^{-3}M dilution of nifedipine. This dose of ethanol had no effect on migration nor the morphology of the cells.



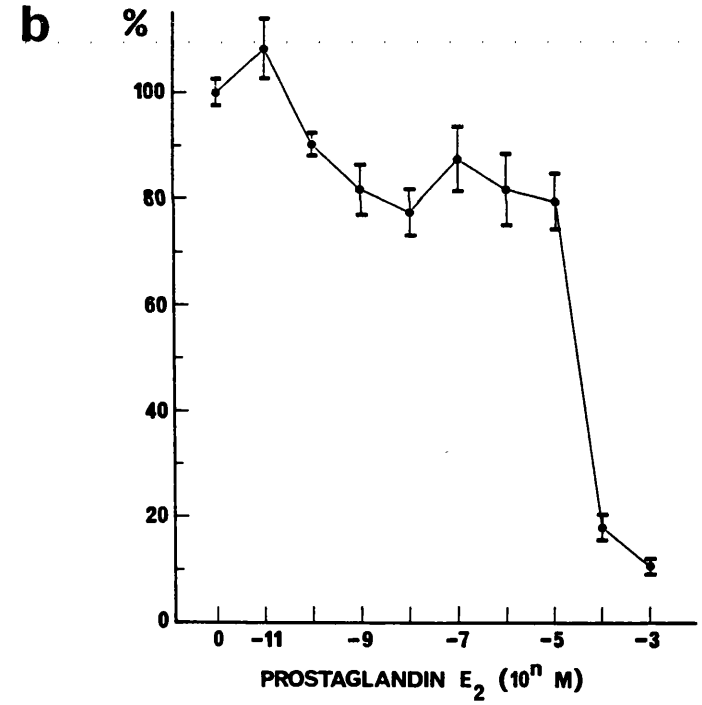


Fig.3.33. Graphs showing the effect of a.)Dibutyryl cyclic-AMP and b.)Prostaglandin E_2 on the migration of rabbit Tenon's capsule fibroblasts to 20 ug/ml fibronectin. Results have been expressed in terms of the migration to fibronectin being 100%. For each drug concentration four wells were counted. Bars are SEM. Dibutyryl c-AMP at 10^{-12} and 10^{-3}M significantly reduced the migration of fibroblasts ($p < 0.01$ Student t test). Prostaglandin E_2 at 10^{-3}M also significantly reduced fibroblast migration ($p < 0.001$ Student t test).

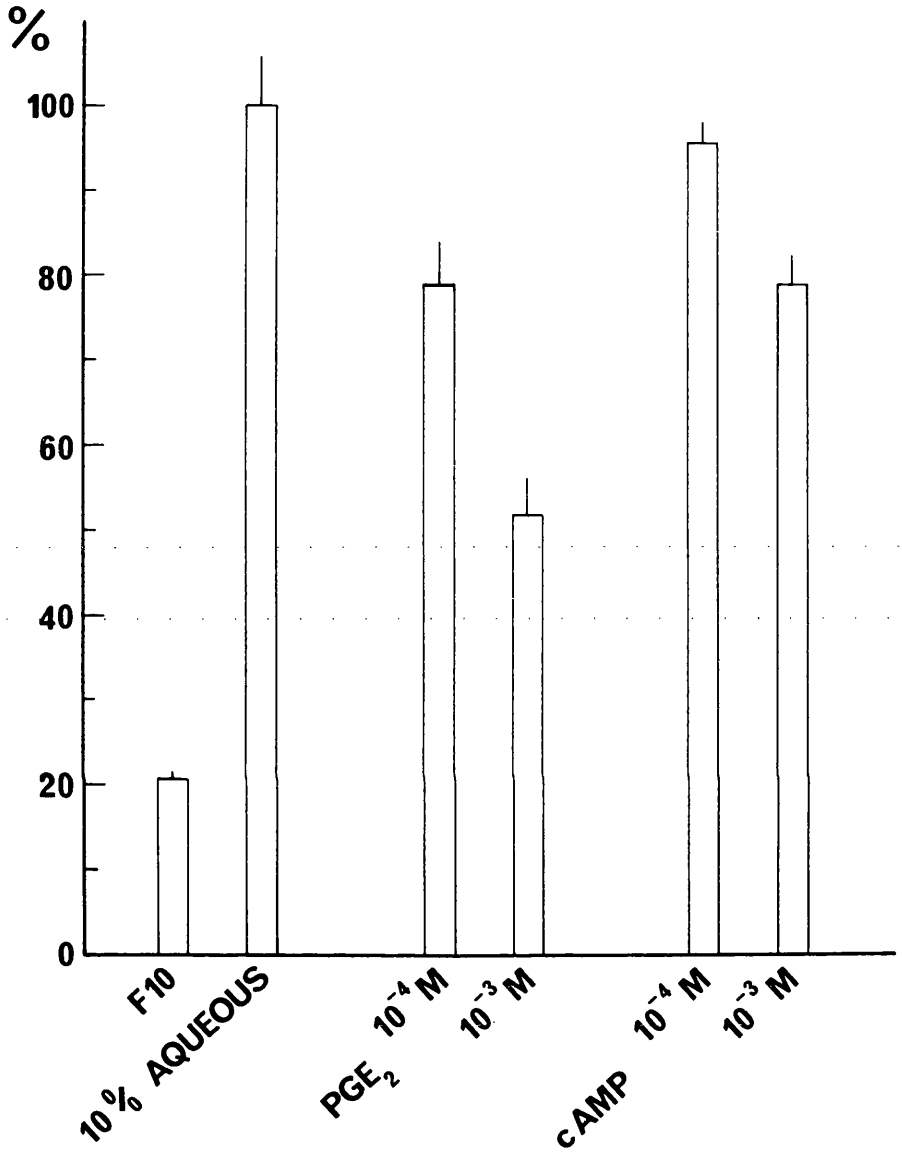
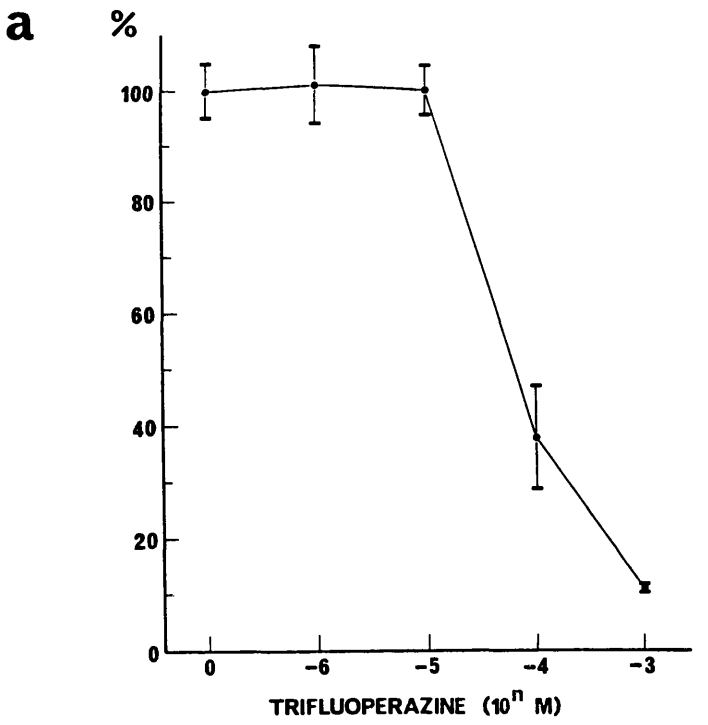


Fig.3.34. Histogram showing the effect of prostaglandin E_2 and dibutyryl cyclic-AMP on the migration of rabbit Tenon's fibroblasts to 10% rabbit aqueous humour. Results have been expressed in terms of the migration to 10% aqueous humour being 100%. For each drug concentration four wells have been counted. Bars are SEM. PGE_2 at 10^{-4}M reduced migration by 21% ($p < 0.05$ Student t test) and at 10^{-3}M by 48% ($p < 0.001$ Student t test). Dibutyryl cyclic-AMP at 10^{-4}M reduced fibroblast migration by an insignificant 4.2%, but at 10^{-3}M, migration was reduced by 21% ($p < 0.05$ Student t test).



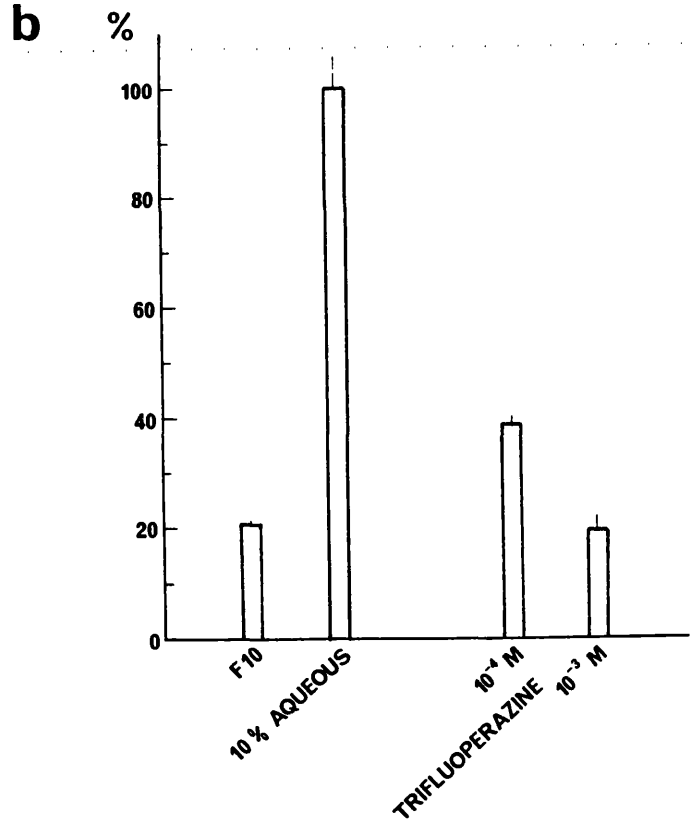


Fig.3.35. Graphs showing the effect of trifluoperazine on the migration of rabbit Tenon's capsule fibroblasts to a.)20 ug/ml fibronectin, and b.)10% rabbit aqueous humour. Both graphs show the responses in terms of the maximal migration being 100%. Trifluoperazine significantly reduces fibroblast migration at 10^{-4}M ($p < 0.001$ Student t test).

Cholinergic antagonist

Ipratropium bromide at doses from 10^{-9} to 10^{-4}M had no effect on fibroblast migration.

Beta$_2$-adrenoceptor stimulants

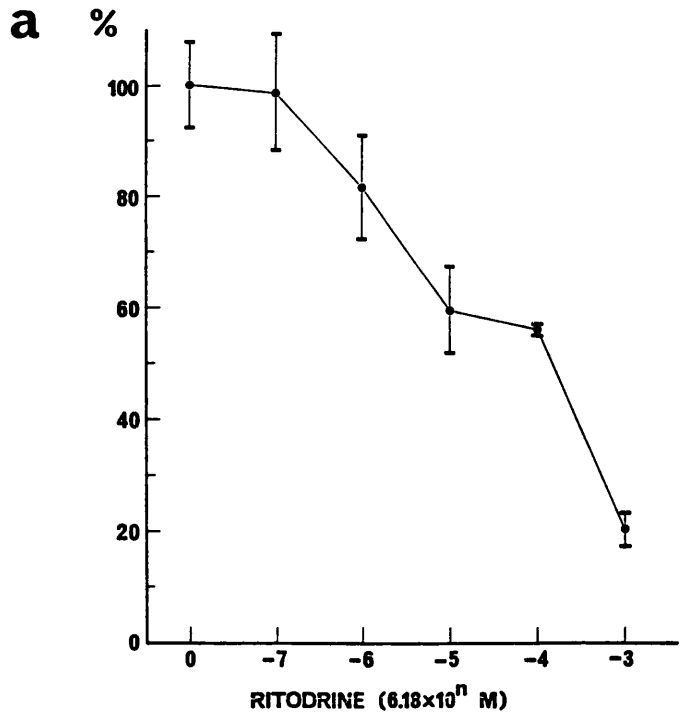
Ritodrine HCl in both the intravenous preparation and the pure powder preparation, markedly reduced the migration of both rabbit and human ocular fibroblasts to fibronectin. The optimally effective dose was 6.18×10^{-3}M, and reduced fibroblast migration to 20% of the control ($p < 0.02$ Student t test). The ID_{50} was approximately 6.18×10^{-5}M (fig.3.36a.). Salbutamol caused a moderate decrease in the migration to fibronectin. The maximum effect was seen at 10^{-4}M, and fibroblast migration was reduced to 73% of the control ($p < 0.02$ Student t test). The ID_{50} could not be accurately determined but was less than 10^{-7}M (fig.3.36b.).

When 10% rabbit aqueous humour was used as the chemoattractant, 6.18×10^{-3}M ritodrine HCl (intravenous preparation) reduced the migration of rabbit Tenon's fibroblasts by 83% ($p < 0.001$). However, this reduction was not sustained at a slightly lower dose, since 10^{-3}M ritodrine reduced migration only by 8% ($p > 0.1$). Salbutamol caused only a marginal reduction in migration in that 10^{-4}M salbutamol reduced migration by 12.6% and 10^{-5}M salbutamol caused a 17% reduction ($p > 0.1$), (fig.3.37.). Cell adherence to the membrane was normal in the presence of these drugs.

Retinoids

Unlike the previous drugs which were added to the upper wells of the micro-chemotaxis chamber, thus exposing the cells to them for four hours, retinol and retinoic acid were added to the cells three days before the assay (Hein, et al., 1984). No further drug was added to the cells during the assay.

Pretreatment of rabbit Tenon's fibroblasts with retinol at concentrations varying from 10^{-12} to 10^{-4}M significantly increased the background random migration of the cells ($p < 0.001$ Student t test). In the absence of retinol there was a random migration of 4.2+/-0.8 (mean+/-SEM) cells per 20 1000X fields across the membrane in four hours. The response to retinol was U-shaped such that pretreatment with 10^{-4}M retinol, for example, increased the random migration to



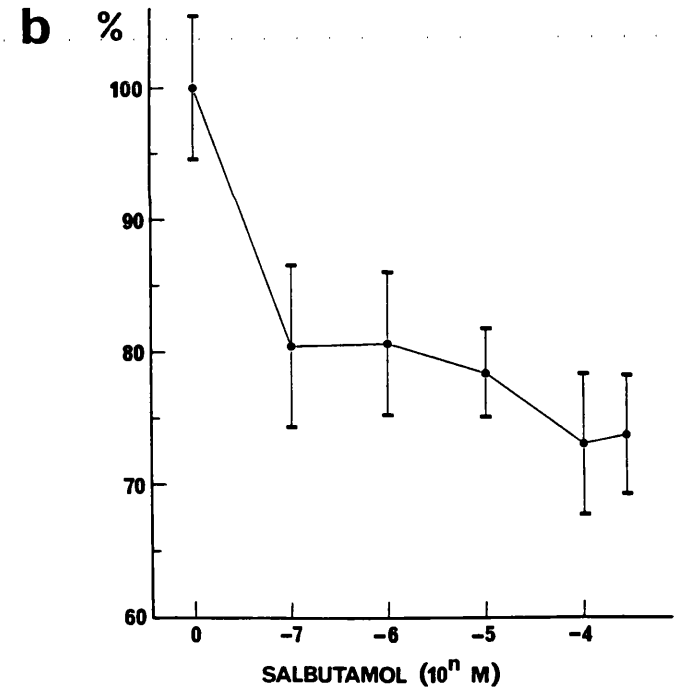


Fig.3.36. Graphs showing the effects of a.)Ritodrine HCl, and b.)Salbutamol on the migration of rabbit Tenon's capsule fibroblasts to 20 ug/ml fibronectin. For each drug the results are shown in terms of the migration to fibronectin being 100%. Bars are SEM. For ritodrine four wells were counted per dose, for salbutamol 3 wells. 6.18×10^{-3}M ritodrine reduced migration by 80% ($p < 0.02$ Student t test); 10^{-4}M salbutamol reduced migration by 27% ($p < 0.02$ Student t test).

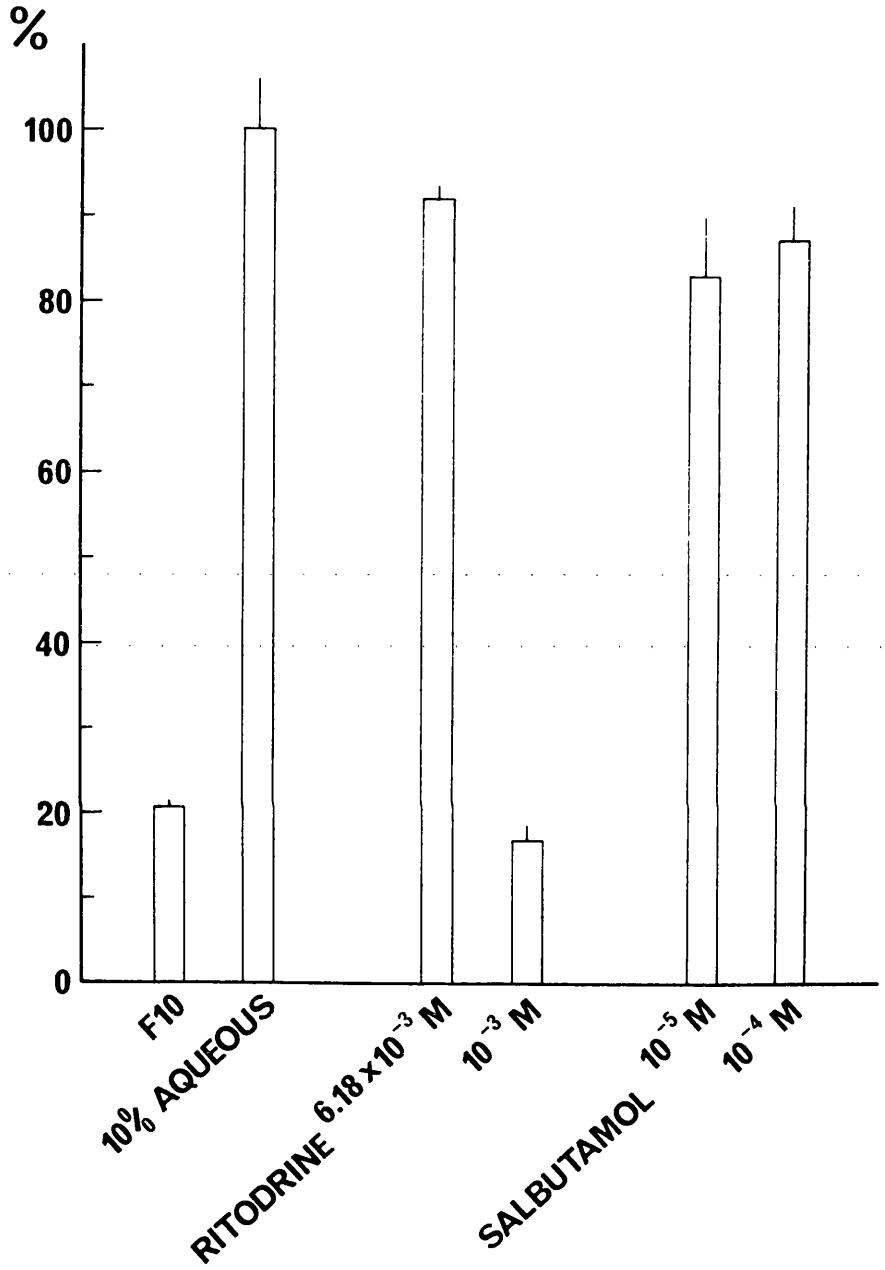


Fig.3.37. Histogram showing the effect of ritodrine HCl and salbutamol on inhibiting the migration of rabbit Tenon's fibroblasts to 10% rabbit aqueous humour. Results have been expressed in terms of the maximal migration being 100%. 6.18×10^{-3}M ritodrine reduced fibroblast migration to 17% of the control ($p < 0.001$ Student t test). 10^{-3}M ritodrine did not significantly reduce fibroblast migration, nor did 10^{-3} and 10^{-4}M salbutamol ($p > 0.1$ Student t test).

34.8+/-3.0 cells ($p < 0.001$); at 10^{-8}M the random migration was 14.5+/-0.5 cells, while at 10^{-12}M this was increased to 30.5+/-1.4 cells per 20 1000X fields. When an optimal dose of 20 ug/ml fibronectin was used as the chemoattractant, the response was somewhat different. At 10^{-4}M retinol a similiar number of cells migrated as in the retinol free control. However, with progressively decreasing doses of retinol down to the minimum tested of 10^{-12}M, there was a progressive increase in the number of cells migrating, so that 100.8+/-3 cells per 20 1000X fields migrated when pretreated with 10^{-12}M retinol, compared to 51+/-9.1 cells in the absence of retinol ($p < 0.01$ Student t test), (fig.3.38.).

Retinoic acid at 10^{-5}M doubled the random migration of rabbit Tenon's fibroblasts in the presence of F10 ($p < 0.01$ Student t test). However, a similiar effect was not seen when fibronectin was used as the chemoattractant.

These retinoids were not tested with aqueous humour as the chemo-attractant. The diluent of ANALAR grade ethanol constituted 0.02% of the solution at the strongest concentrations of retinoids tested. Even at a 2% concentration ethanol failed to affect the migration of the cells.

The effects of the numerous drugs tested are summarised in Table 3.2.

3.2.7. Error check

The percentage error for the accuracy of cell migration through the pores in the membrane depended on the number of cells that had migrated and were therefore available for counting. Thus when very few cells were counted in 20 1000X fields, the percentage error was high eg. for a negative control migration of human cells to F10 medium, the first count of 4 identical wells showed 0.8+/-0.5 cells per 20 1000X fields (mean+/-SEM). Another count by the same observer 3 months later showed 0.5+/-0.3 cells per 20 1000X fields. Thus the intra-individual error for this count was 23.1%. The inter-individual error for the same wells was 60%.

When greater numbers of cells were available for counting, the percentage errors were much reduced. For example, at 20% aqueous humour, the first count by one observer revealed 94+/-2.6 migrated

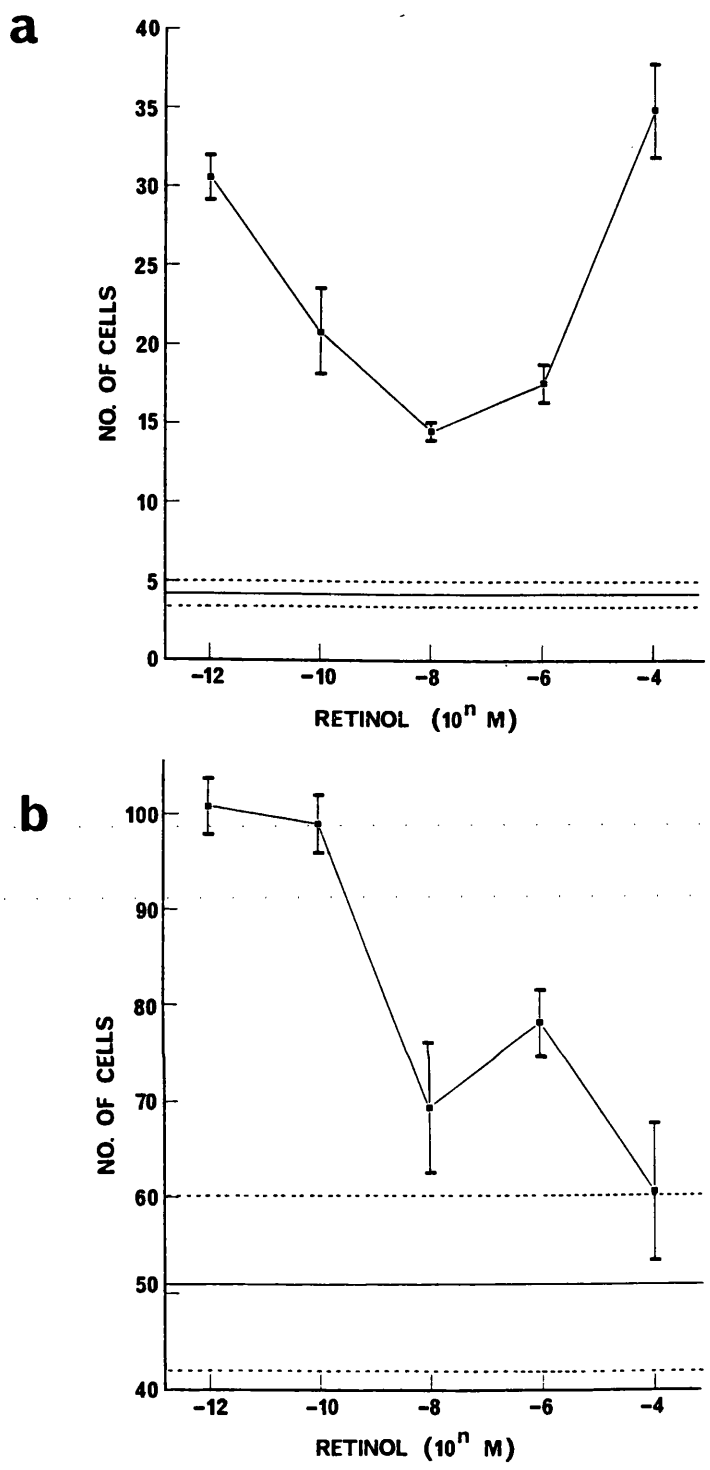


Fig.3.38. Graphs showing the effect of retinol on the migration of rabbit Tenon's capsule fibroblasts. a.)Shows the effect of retinol on the random migration of the cells in the presence of serum free F10 medium. The random migration was enhanced at all doses tested ($p < 0.001$ Student t test). b.)Shows the effect of retinol when 20 ug/ml fibronectin was used as the chemoattractant. Migration is significantly increased at 10^{-12}M and 10^{-10}M ($p < 0.01$) and at 10^{-6}M retinol ($p < 0.05$ Student t test). For each dose 4 wells have been counted. Bars are SEM. Cells have been counted in 20 fields at 1000X magnification.

cells in 20 1000X fields, while a second count showed 91.3+/-5.8 cells. The intra-individual error was therefore 1.4%. The inter-individual error was 4.9% for the same count. For cell counts over 40 cells per 20 1000X fields, the intra-individual error ranged from 0.4 to 4.1%, while the inter-individual error ranged from 0.2 to 11.4%. Errors of this magnitude indicate reasonable reproducibility, particularly since even a 60% error at very low cell counts implies only a fractional difference between counts, which is not meaningful.

These observations justify counting 20 1000X fields per well, which represents 1/15th of the area of each well. They also justify the choice of 35 000 cells per well or $7x10^5$ cells per ml added to the upper wells of the 48-well chemotaxis chamber since reasonably high cell numbers migrated to appropriate stimuli, thereby allowing accurate and reproducible cell counts to be performed.

Table 3.2. Summarising the effects of drugs on the migration of ocular fibroblasts

DRUG	DOSES TESTED	OPTIMAL DOSE	EFFECT ON MIGRATION: Migration to 20 ug/ml fibronectin	p value	Migration to 10% aqueous humour	p value
Colchicine	10^{-9} - 10^{-3}M	10^{-3}M	[a]-73.0%	p<0.001	-45.6%	p<0.01
Cytochalasin B	10^{-10} - 10^{-5}M	10^{-5}M	-97.4%	p<0.001	-96.6%	p<0.001
Taxol	10^{-12} - 10^{-5}M	10^{-6}M	-80.8%	p<0.001	-84.1%	p<0.001
Theophylline	10^{-12} - 10^{-3}M	Ineffective	Unchanged	-	Not tested	-
Dibutyryl-cAMP	10^{-12} - 10^{-3}M	10^{-3}M	-29.8%	p<0.01	-21.0%	p<0.05
Prostaglandin E_2	10^{-11} - 10^{-3}M	10^{-3}M	-94.5%	p<0.001	-48.2%	p<0.001
Trifluoperazine	10^{-12} - 10^{-3}M	10^{-3}M	-89.1%	p<0.001	-80.5%	p<0.001
Nifedipine	10^{-11} - 10^{-3}M	Ineffective	Unchanged	-	Not tested	-
Ritodrine HCl	10^{-8}-6.18x10^{-3}M	6.18x10^{-3}M	-79.7%	p<0.02	-83.2%	p<0.001
Ipratropium Bromide	10^{-9} - 10^{-4}M	Ineffective	Unchanged	-	Not tested	-
Salbutamol	10^{-7} - 10^{-4}M	10^{-4}M	-27.0%	p<0.02	-12.6%	p>0.1
Retinol	10^{-12} - 10^{-4}M	10^{-12}M	+97.6%	p<0.01	Not tested	-
Retinoic Acid	10^{-13} - 10^{-5}M	Ineffective[b]	Unchanged	-	Not tested	-

[a]Minus sign indicates inhibition of migration; plus sign shows enhancement.

[b]Random migration enhanced at 10^{-5}M by 109.7% ($p < 0.01$)

3.3. CELL CONTRACTION

3.3.1. Triton X-100 cytoskeletons

Fibroblasts treated with Triton X-100 form cytoskeletons. The normal shape of the cells is lost to a degree, with a tendency for the cells to round up. This makes precise identification of the cell boundary difficult and consequently evaluation of the cell area may be subject to inaccuracies. The fibroblast cytoskeletons contract on exposure to ATP (fig.3.39.). The optimal dose of ATP was 0.1 mM and this elicited a contraction to about 65% of the initial area at 60 minutes (fig.3.40.). The contractile process was slow and was essentially complete by 10 minutes, although there was a further slight contraction up to 1 hour (fig.3.41.)

3.3.2. Whole cells

Whole fibroblasts underwent a marked area change on application of ATP (fig.3.42. and 3.43.). This involved a retraction of some of the cell processes with rounding up of the cell body. Many cells remained tethered to the substrate by fine filamentous processes, which were clearly demonstrated by scanning electron microscopy. Unlike the cytoskeletons, the cell membranes were intact. Thus the cells were well deliniated and accurate measurement of the cell areas was possible using the image analyser. The optimally effective dose of ATP was 15 mM (cf. 0.1 mM for the cytoskeletons). The response was also far greater than seen with the cytoskeletons in that the cells reduced to about 20% of their initial size (fig.3.44.). The dramatic difference in the response of the whole cells is contrasted to that of the fibroblast cytoskeletons in Table 3.3. Fifty percent of the contraction of whole cells was completed within about 90 seconds of the addition of ATP. The response was essentially complete within 3 minutes, with a further slight reduction in size up to 10 minutes (fig.3.45.). From 10 to 60 minutes there was little change.

Whole fibroblasts derived from rabbit sclera, conjunctiva, Tenon's capsule and skin all demonstrated a similiar contraction when exposed to exogenous ATP. However, there was some day to day variability in the response of the fibroblasts. Thus, the dose of ATP which produced a half maximal contraction of the cells varied between 5 and 7 mM. When the effects of ATP were explored or when drugs were evaluated as potential inhibitors of the contractile response, the dose of ATP

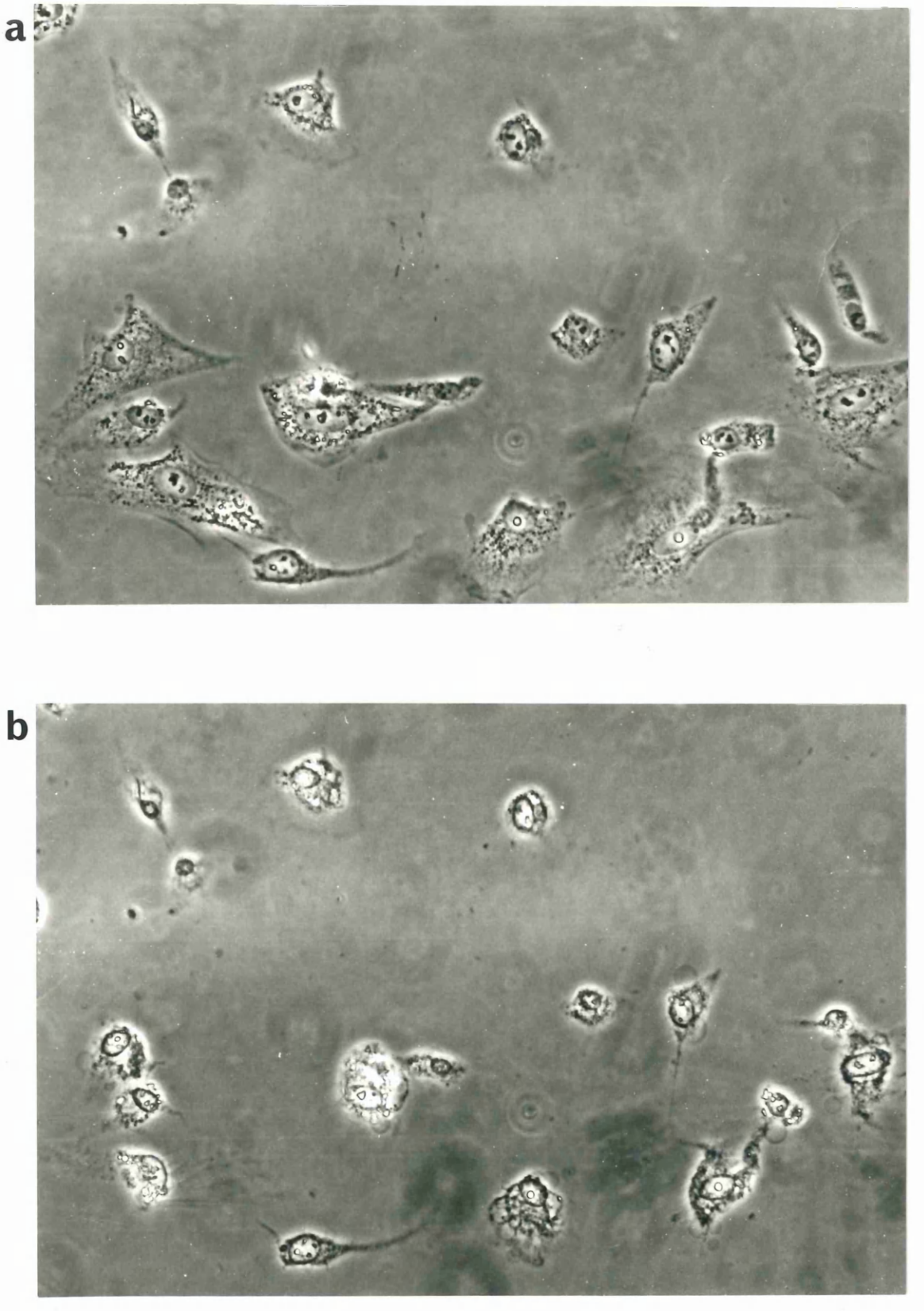


Fig.3.39. Phase contrast micrographs of fibroblast cytoskeletons produced by exposure to the detergent Triton X-100. a.)The cytoskeletons at time 0. b.)The same field after 60 minutes in 0.1 mM ATP. (Magnification X300).

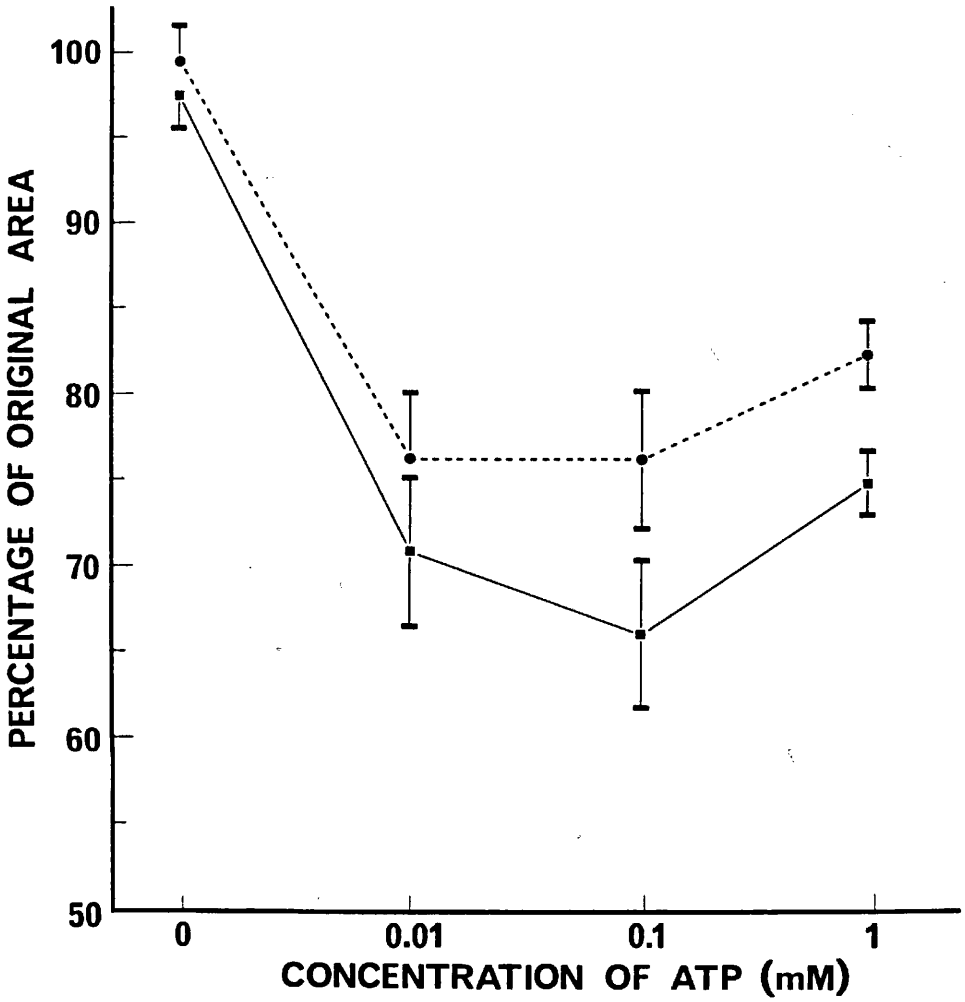


Fig.3.40. Dose-response curve of fibroblast cytoskeletons. The dashed line shows the situation at 10 minutes; the solid line the same cytoskeletons at 60 minutes. The optimal dose is 0.1 mM ATP. The change in area at this dose compared to the baseline with 0 ATP is highly significant at both 10 and 60 minutes ($p < 0.001$ Student t test). Bars are SEM; between 21 and 23 cytoskeletons were measured at each dose.

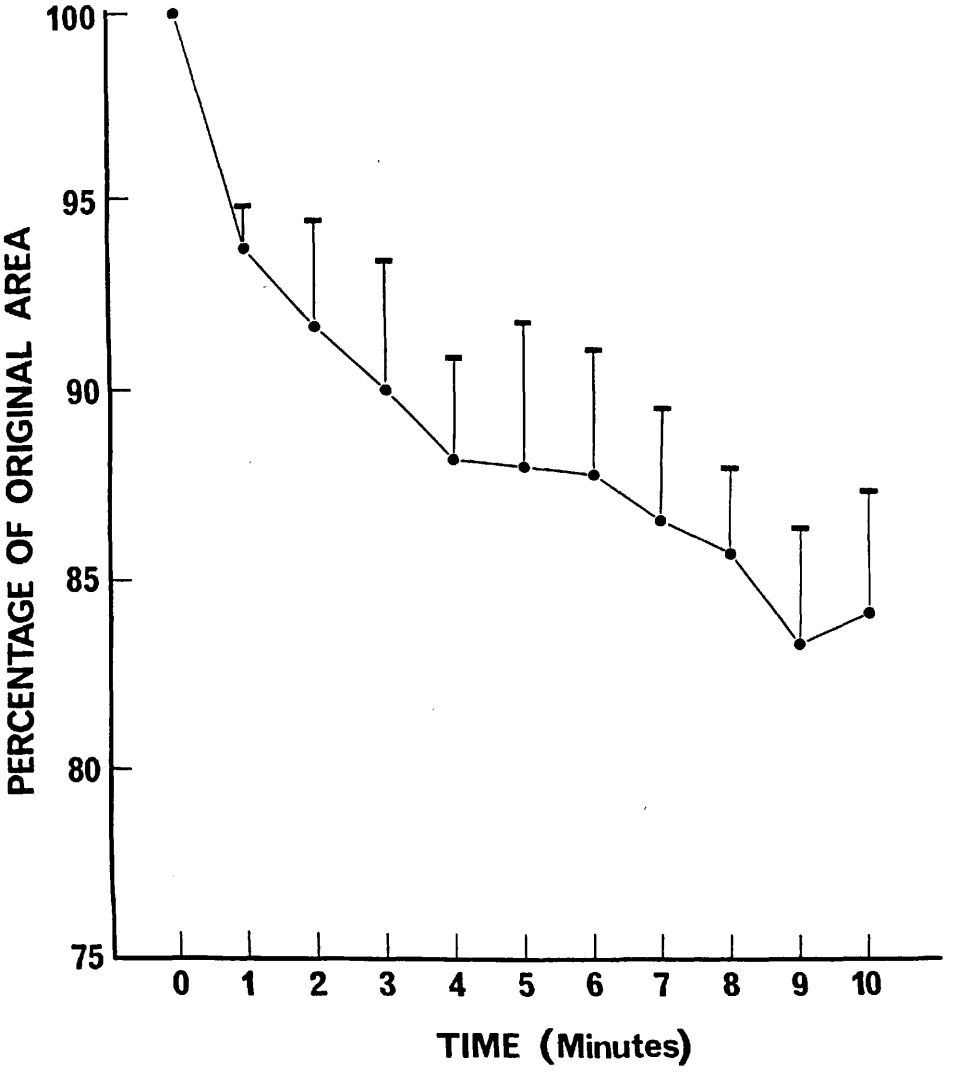


Fig. 3.41. Time function of fibroblast cytoskeletons treated at time 0 with 1 mM ATP. Bars are SEM; 10 cytoskeletons were measured for each point. Beyond 10 minutes there is a further slow reduction in the mean area to 82.1% of the original area (+/-3.0% SEM).

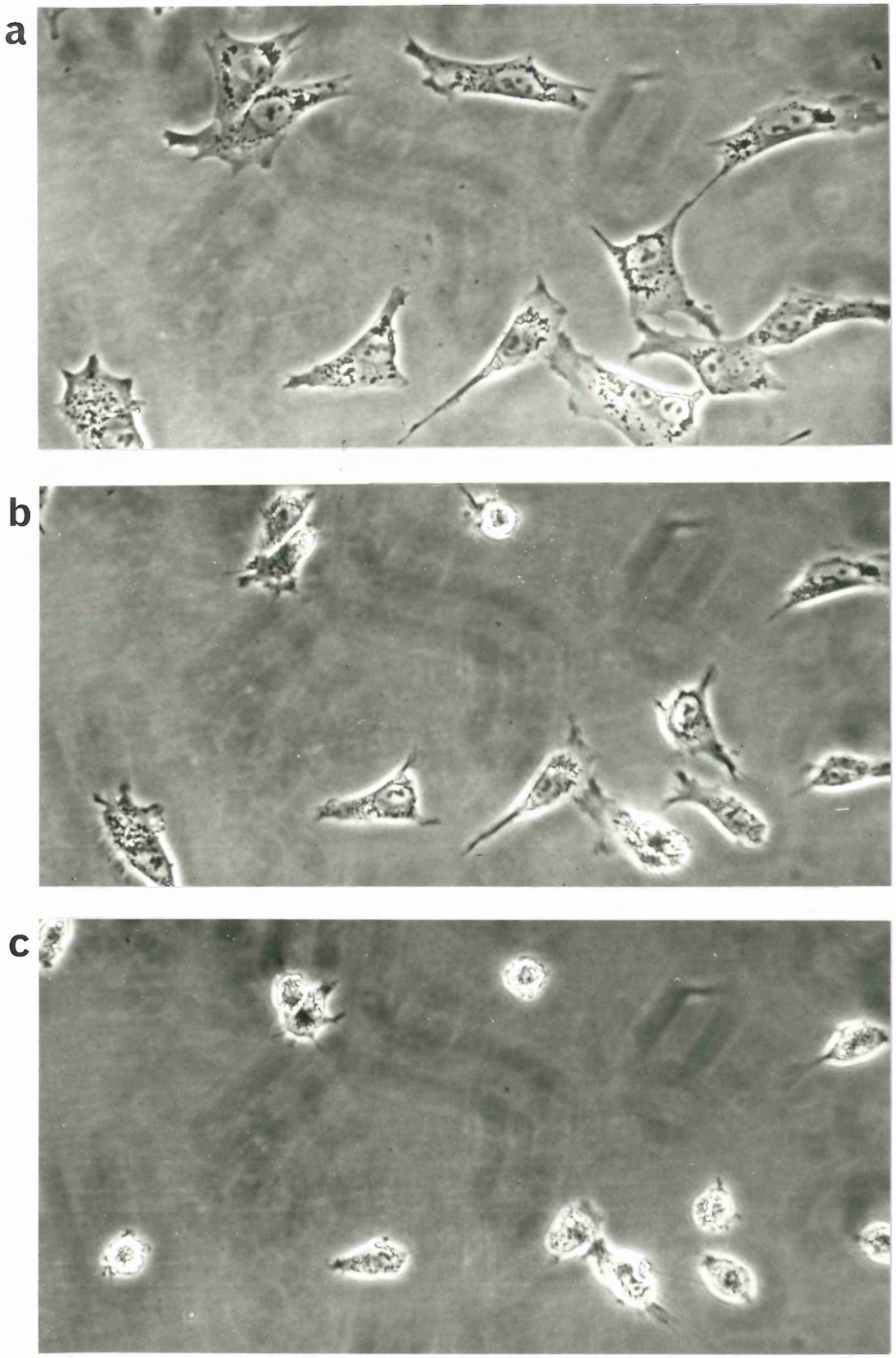


Fig.3.42. a.)Intact fibroblasts in Dulbecco's phosphate-buffered saline with calcium and magnesium at time 0. b.)The same cells, at 3 minutes, after aspiration of PBS and addition of 15 mM ATP in F10 medium. c.)Same cells, at 10 minutes, having contracted to about 20% of their initial area. (Magnification X300)

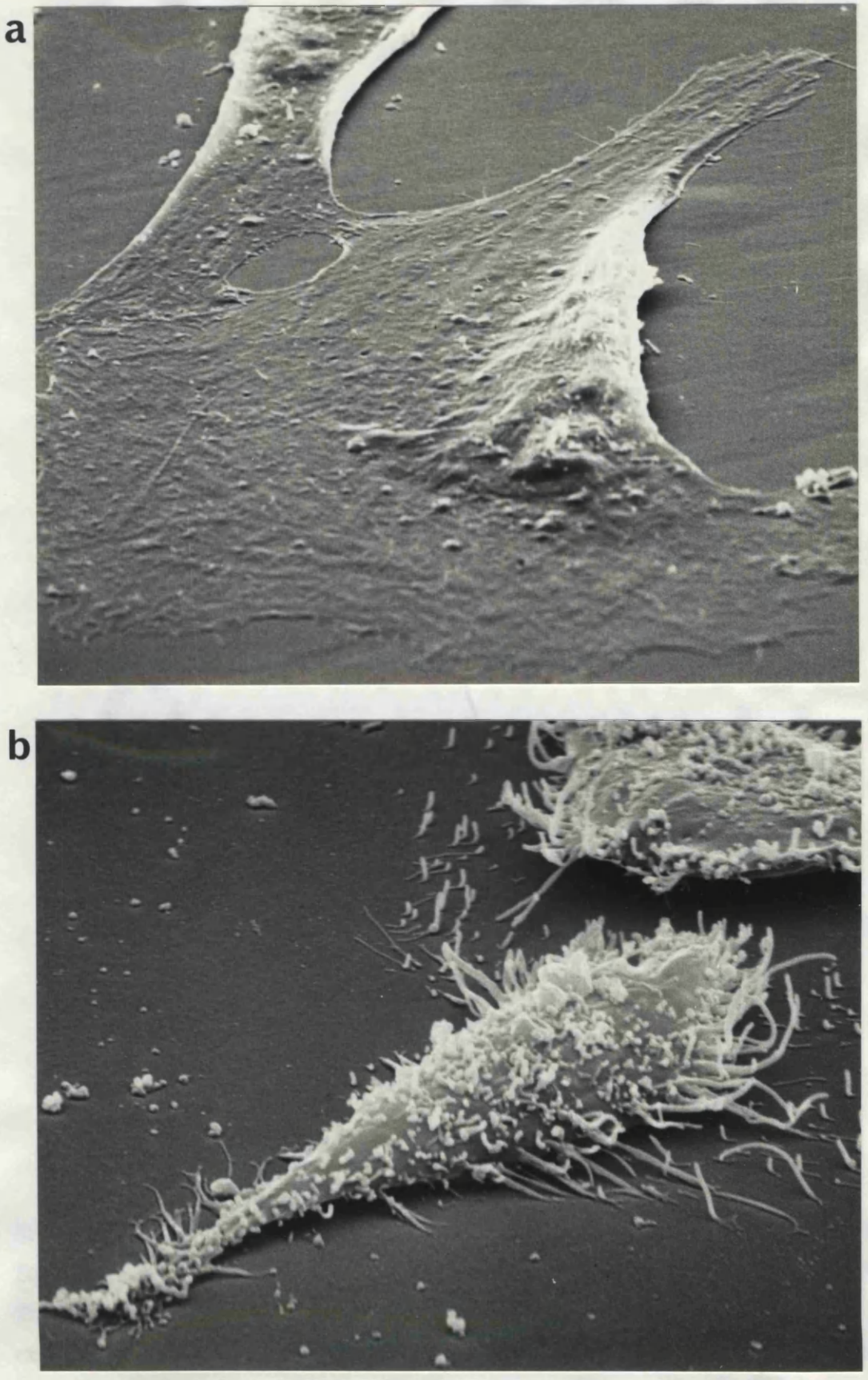


Fig.3.43. a.)Scanning electron micrograph showing fibroblasts that had been growing in F10 medium (X2 700). b.)Scanning electron micrograph of a similiar fibroblast to that seen in (a.) that had been exposed to 15 mM ATP for 10 minutes (X3 700).

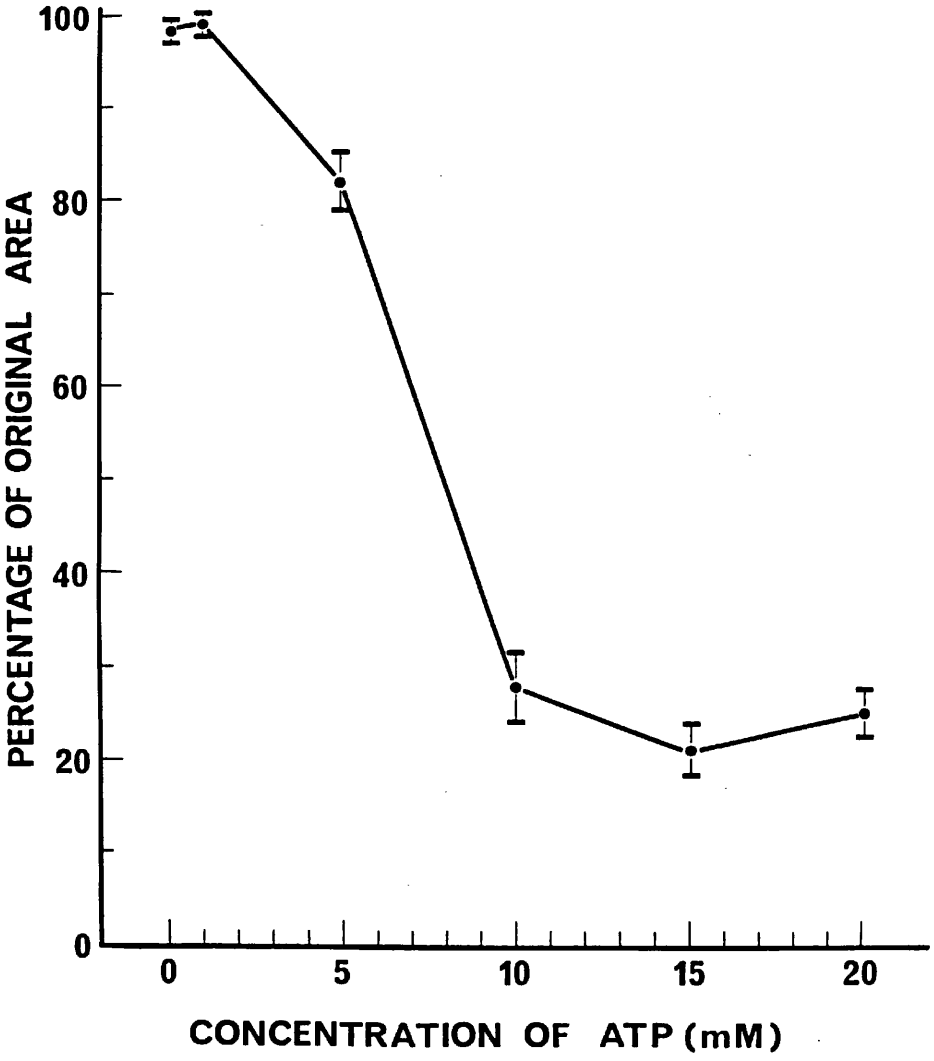


Fig.3.44. Dose-response curve of intact fibroblasts treated with ATP for 10 minutes. 20 cells were measured for each point. Bars show SEM. The optimal dose is 15 mM ATP. The reduction in area at this dose compared to the baseline with 0 ATP is highly significant ($p < 0.001$ Student t test). This curve was generated using rabbit Tenon's fibroblasts. Very similiar results were achieved using conjunctival, scleral and skin fibroblasts from rabbits.

Table 3.3. A comparison of the reduction in area of fibroblast Triton X-100 cytoskeletons and whole cells in response to ATP. Optimal doses of ATP have been compared.

TYPE OF PREPARATION	NUMBER OF CELLS	CONCENTRATION OF ATP	TIME [MINUTES]	AREA OF CELLS [um^2] (Mean+/-S.E.M.)	% OF ORIGINAL AREA	PAIRED t-TEST
TRITON X-100	22	0.1 mM	0	1328.3+/-172.7	100%	-
CYTOSKELETONS	22	0.1 mM	10'	913.8+/-91.7	68.8%	p<0.001
	22	0.1 mM	60'	754.6+/-65.8	56.8%	p<0.001
WHOLE CELLS	20	15 mM	0	1960.6+/-202.0	100%	-
	20	15 mM	10'	445.0+/-132.9	22.7%	p<0.001
	20	15 mM	60'	317.3+/-30.3	16.2%	p<0.001

The smaller size of the cytoskeletons is due to the partial loss of the cell membrane produced by exposure to Triton X-100.

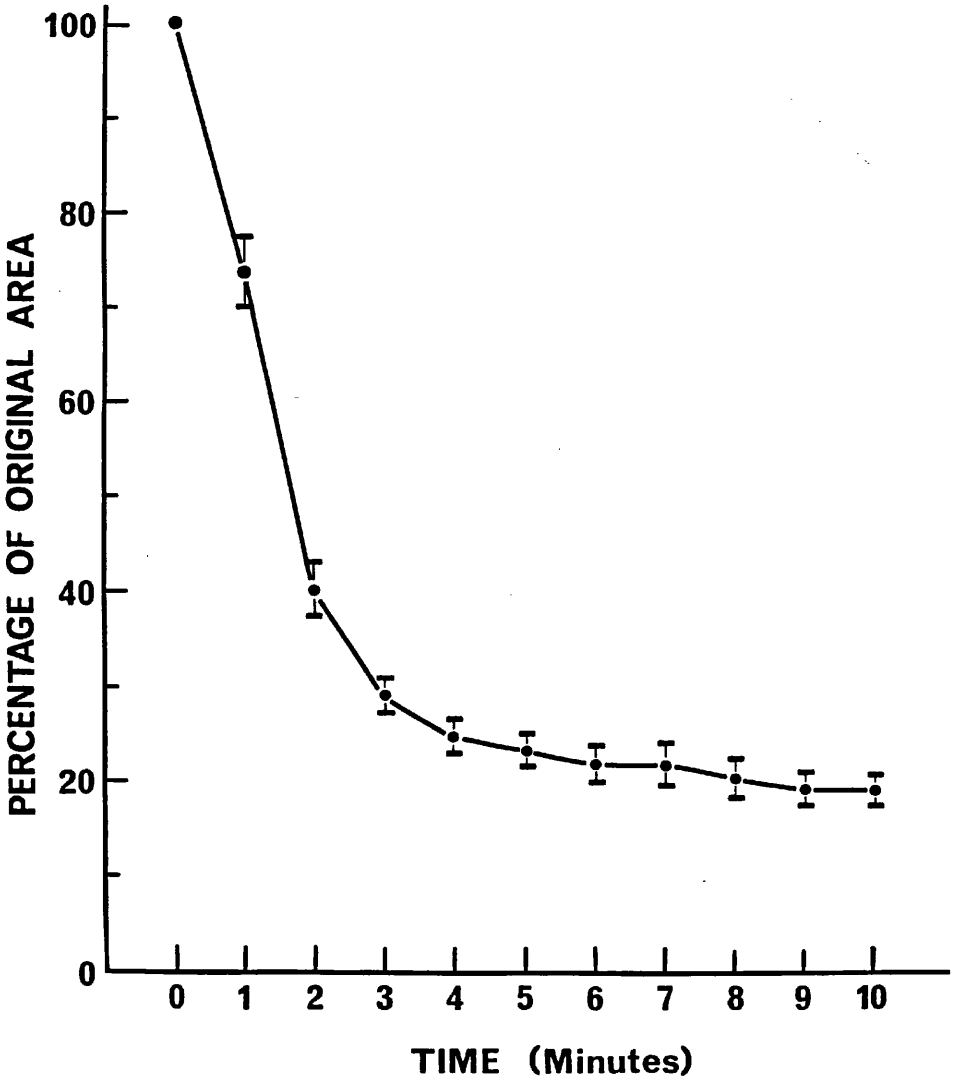


Fig.3.45. Time function of intact rabbit Tenon's fibroblasts treated with 15 mM ATP; 17 cells were measured for each point. Bars show SEM. Beyond 10 minutes there was a further slow reduction in the mean area to 15.2% of the original (+/-0.9% SEM). 50% of the contraction was completed within about 90 seconds of the addition of ATP.

required to elicit 50% of the maximal response was therefore established at the start of each series of experiments.

The process of fibroblast contraction is also reversible and repeatable (fig.3.46.). This was dramatically demonstrated by time-lapse cine photography and was shown to occur over at least 7 cycles of contraction and relaxation during an eight hour period. When the cells relaxed they tended to assume their original shape. Close examination showed that the rounded up cell bodies spread out along the fine tendrils that had anchored them to the substrate in the contracted state. The process of contraction and relaxation showed a degree of hysteresis which was partially contributed to by the loss of some cells that contracted particularly markedly. These cells maintained only tenuous attachments to the substrate in the contracted state and some were lost due to turbulence in the culture medium when it was exchanged between cycles.

Cell division was noted to occur virtually throughout the contraction and relaxation cycles. Newly formed daughter cells also underwent contraction when exposed to ATP. This indicates that exposure to ATP in F10 medium was not harmful to the cells and that the dramatic change in cell shape produced was unlikely to be due to a non-specific toxic effect.

Indirect immunofluorescence using a monoclonal anti-vinculin antibody demonstrated that normal fibroblasts had lines of vinculin most evident near the periphery of the cells. After treatment with 15 mM ATP for 10 minutes the normal staining pattern was markedly distorted with the vinculin pulled centripitally (fig.3.47.).

The osmolality of the solutions used in the contractile assay was as follows:

Dulbecco's PBS	282 mosmol/kg
F10 medium with Hepes	315 mosmol/kg
5 mM ATP in F10	315 mosmol/kg
15 mM ATP in F10	325 mosmol/kg
25 mM ATP in F10	339 mosmol/kg

There was a 2% error inherent in the osmometer machine.

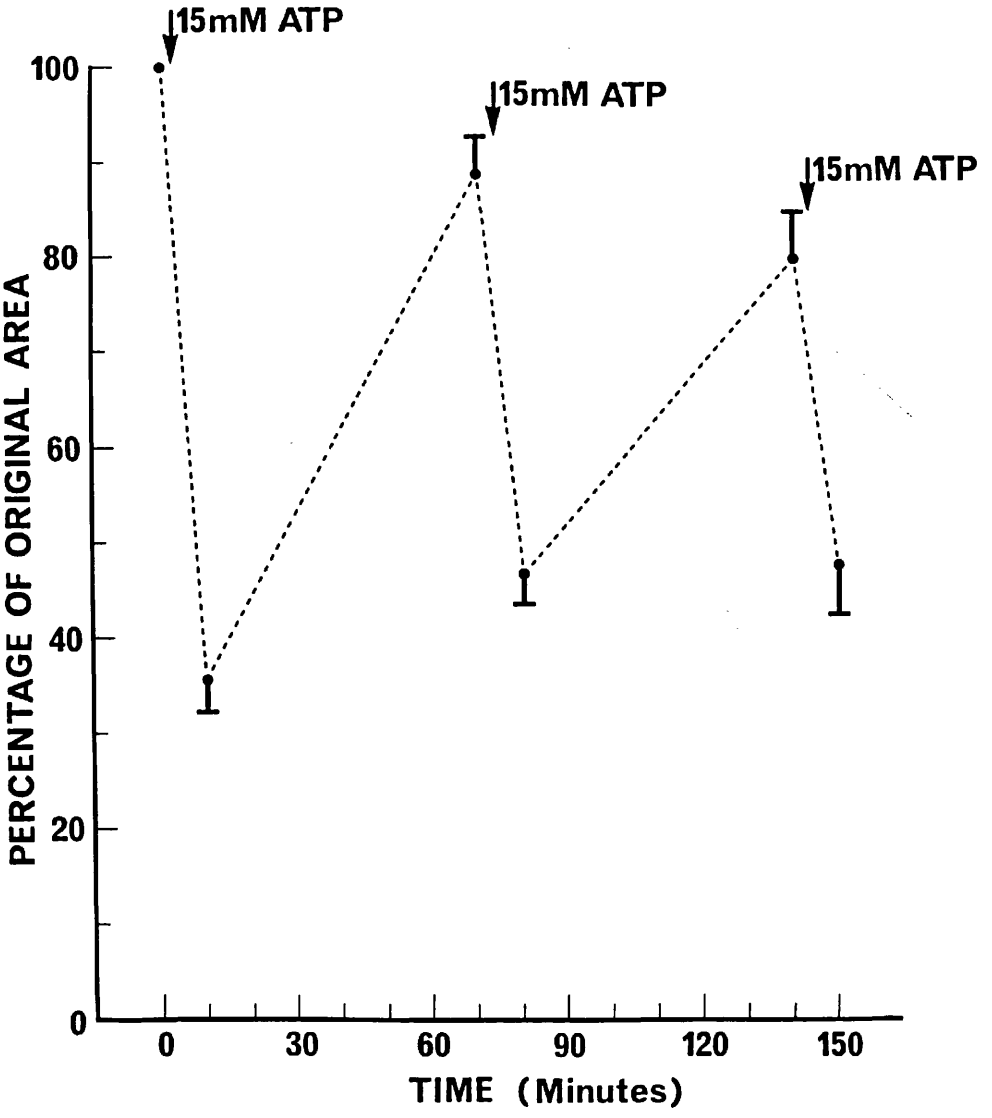


Fig.3.46. Graph showing the reversibility and repeatability of the contraction produced by the addition of 15 mM ATP to intact rabbit Tenon's fibroblasts. Relaxation of the cells was achieved by washing the cells with F10 medium 3 times and then leaving them in this medium for 60 minutes. Further ATP was then added to the cells. For the first two cycles 29 cells were measured; for the third cycle 20 cells; bars are SEM. The cells which contract maximally have tenuous attachments to the substrate when contracted. They may become detached and therefore washed out when the culture medium is exchanged between cycles. This partially accounts for the hysteresis. The dashed line has been included for the sake of clarity and is not intended to show the shape of the cells with time.

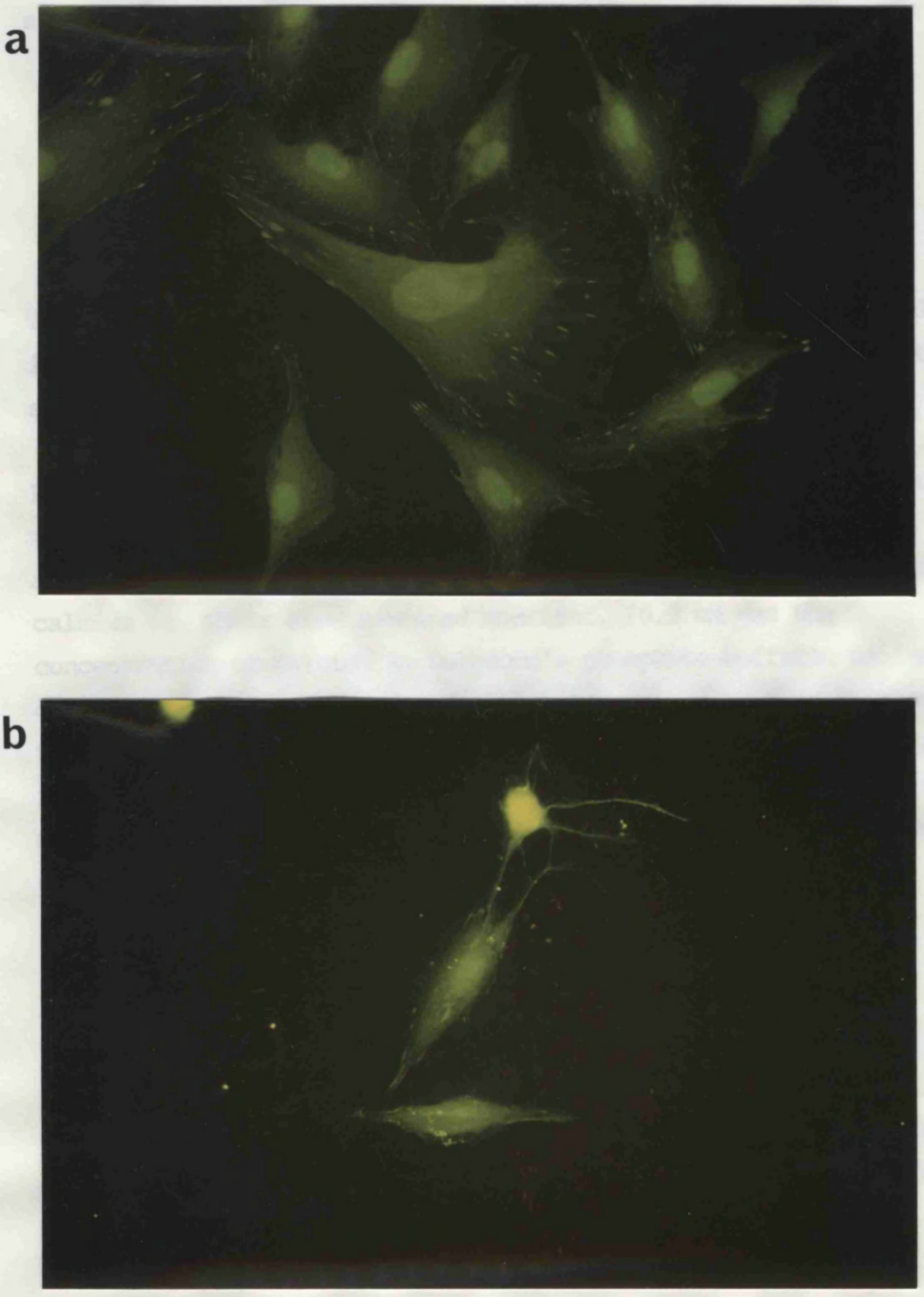


Fig.3.47. Rabbit Tenon's fibroblasts have been labelled with a monoclonal vinculin antibody. a.)Control cells with lines of vinculin evident peripherally. b.)Cells exposed to 15 mM ATP for 10 minutes. Vinculin has been pulled centripitally as the cells have contracted.

Similar concentrations of adenosine, which is a partial agonist (see section 3.3.3.) produce similar osmolality changes:

F10 medium with Hepes	304 mosmol/kg
5 mM Adenosine	310 mosmol/kg
10 mM Adenosine	312 mosmol/kg
15 mM Adenosine	315 mosmol/kg
20 mM Adenosine	320 mosmol/kg

Therefore, although there was an increase in osmolality produced by increasing concentrations of ATP, this was not responsible for the shape change induced in the fibroblasts.

Placing the cells in phosphate-buffered saline with no calcium or magnesium led to their contraction. Replenishing the calcium in the absence of magnesium led to the stability of the cells at 0.9 mM calcium ie. their size remained constant. (0.9 mM was the concentration of calcium in Dulbecco's phosphate-buffered saline with calcium and magnesium, and when the cells were placed in this solution they maintained a stable size). Likewise, replenishing the magnesium in the absence of calcium led to the stability of the cells at 0.6 mM magnesium (the concentration of magnesium in F10 medium, in which the cells were stable), (fig.3.48).

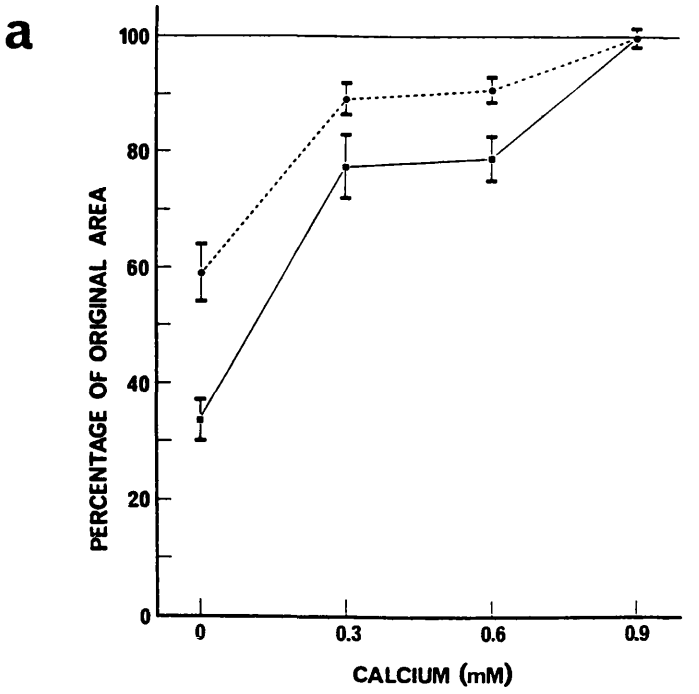
3.3.3. Drugs

a.)Adenosine.

The addition of adenosine to the cells resulted in a slow contraction, clearly evident by 60 minutes. The maximum response occurred at about 15 mM but the cells only reduced their areas to about 80% of the original area ($p < 0.02$ Student t test). Adenosine is thus a partial agonist (fig.3.49.)

b.)$ANAPP_3$.

For the series of experiments using $ANAPP_3$, 5 mM ATP produced a half maximal contraction of the fibroblasts. After treatment of the cells with various doses of $ANAPP_3$ for 40 minutes (Hogaboom, et al., 1980), their areas were virtually unchanged. Addition of 5 mM ATP to the cells then led to a greater contraction of the cells exposed to $ANAPP_3$ than the untreated controls. For example, at the optimally effective dose of 10^{-4}M $ANAPP_3$, the fibroblasts reduced their areas to 51.4+/-6.4% (Mean+/-SEM) of the initial area. This was a significantly



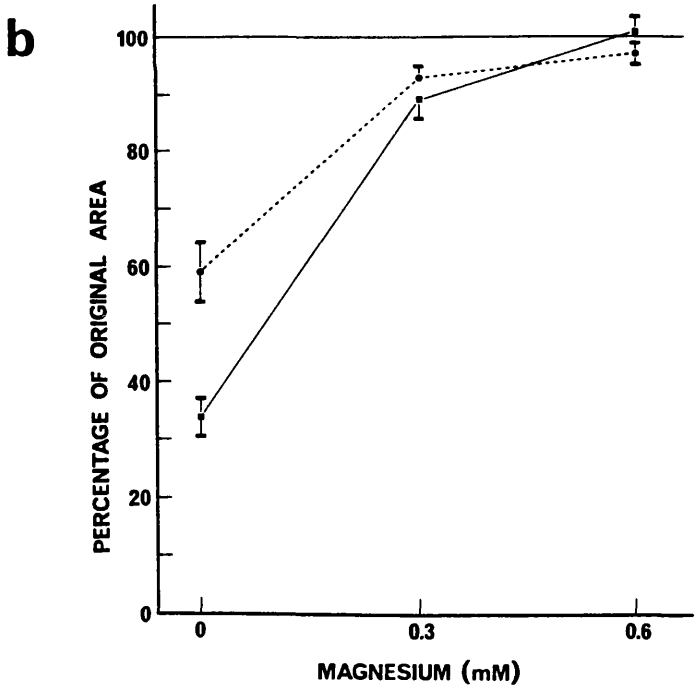


Fig.3.48. Graphs showing the calcium and magnesium dependence of intact rabbit Tenon's fibroblasts for the maintainance of normal cell

shape. The dashed line shows the size of the cells at ten minutes; the solid line that at 60 minutes. Placing the cells in a calcium- and magnesium-free environment (phosphate-buffered saline) leads to the contraction of the cells. a.)Shows how the cells attain a stable shape as the calcium is gradually replenished to 0.9 mM, in the absence of magnesium. This is the level found in Dulbecco's phosphate-buffered saline with calcium and magnesium. There is 0.3 mM calcium in F10 medium. b.)Shows how the cells attain a stable shape as the magnesium is gradually replenished to 0.6 mM, in the absence of calcium. The level of magnesium in F10 medium is 0.62 mM, and in Dulbecco's PBS is 0.49 mM. Twenty cells were measured for each point; bars are SEM.

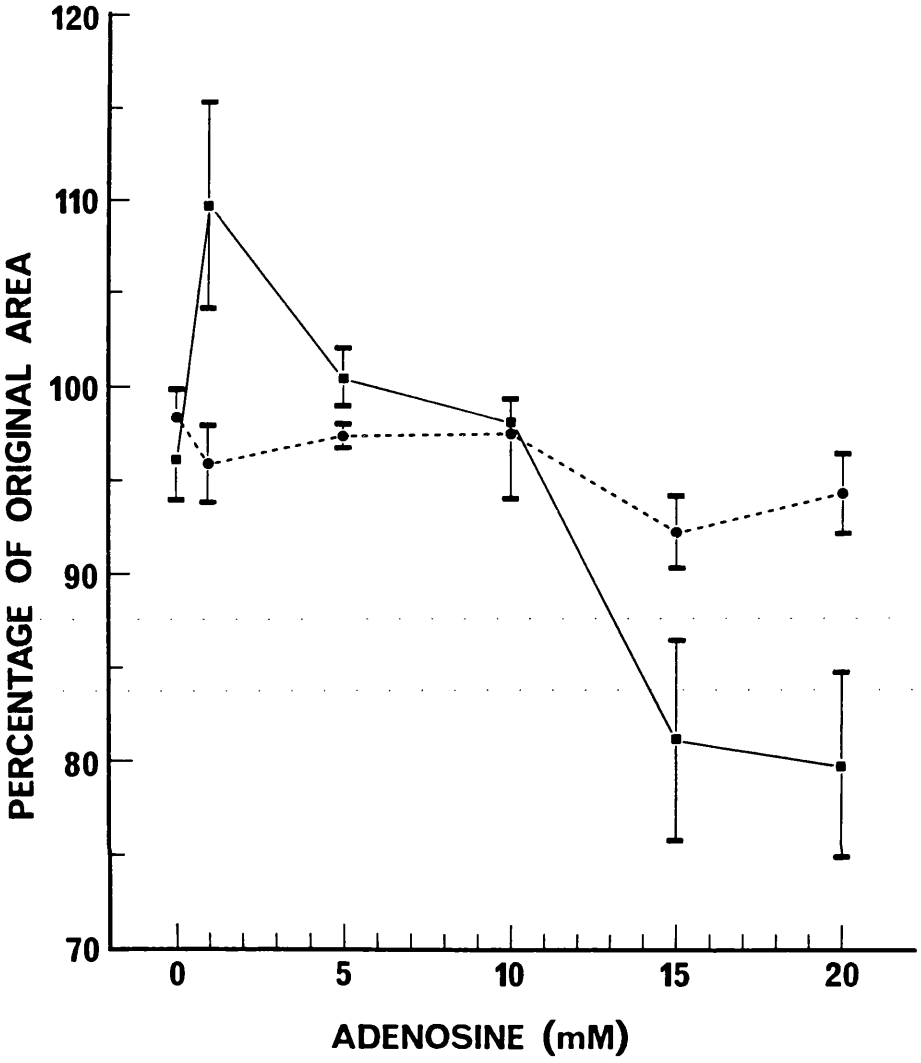


Fig.3.49. Dose-response curve of intact fibroblasts treated with adenosine for 60 minutes. The dashed line shows the situation at 10 minutes; the solid line that at 60 minutes. For 15 mM adenosine there is a significant reduction in the mean area at 10 minutes ($p < 0.02$ Student t test). At 60 minutes the mean reduction in area caused by 15 mM adenosine is significant ($p < 0.02$) as is that caused by 20 mM adenosine ($p < 0.01$ Student t test). Twenty cells were measured at 0, 15, and 20 mM adenosine; six cells at 1 mM; and twelve cells at 5 mM. Bars show SEM.

greater contraction than that produced by ATP alone on the control cells, which reduced their areas to 79.5+/-4.4% of the initial area ($p < 0.001$ Student t test), (fig.3.50.).

c.)Reactive Blue 2.

The dose of ATP producing a half maximal contraction of the fibroblasts proved to be 7 mM in this series of experiments. Exposure to Reactive Blue 2 for 10 minutes led to a progressive decrease in cell area with progressively increasing doses. At 10^{-2}M the mean area was 81.9+/-1.5% of the initial area ($p < 0.001$ Paired t test). Reactive Blue 2 was therefore a partial agonist. After exposure to RB2 for 10 minutes, 7 mM ATP was then added to the cells, which resulted in a further contraction. The optimally effective dose was 10^{-4}M RB2, which resulted in a contraction of the cells to 22.5+/-1.7% (Mean+/-SEM) of the initial area. This was a significantly greater contraction to 49.9+/-5.5% of the initial area than that elicited on the control cells by ATP alone ($p < 0.001$ Student t test), (fig.3.51.)

d.)Taxol.

No effect of taxol on cell contractility was observed with incubation periods upto two hours. After 24 hours incubation, taxol strongly inhibited the contraction of rabbit Tenon's capsule fibroblasts, reducing the response of the cells to ATP to about 18% of the control, with the optimal effect seen at 10^{-5}M ($p < 0.001$ Student t test). At 10^{-6} M (which optimally inhibits chemotaxis), contraction was reduced to 30% of the control ($p < 0.001$ Student t test). The dose of taxol causing a 50% inhibition of contraction (ID_{50}) was about $2x10^{-7}$M (fig.3.52).

e.)Cytochalasin B.

When tested after 30 minutes exposure, cytochalasin B did not inhibit fibroblast contraction at any of the doses tested from 10^{-12} to 10^{-5}M. At 10^{-5}M a further contraction beyond that shown by the controls was produced. A contraction inhibition assay was not attempted beyond thirty minutes exposure to the drug because it was felt that the cell rounding produced by the drug would make the results impossible to interpret.

f.)Colchicine.

No inhibition of cell contraction by colchicine could be demonstrated

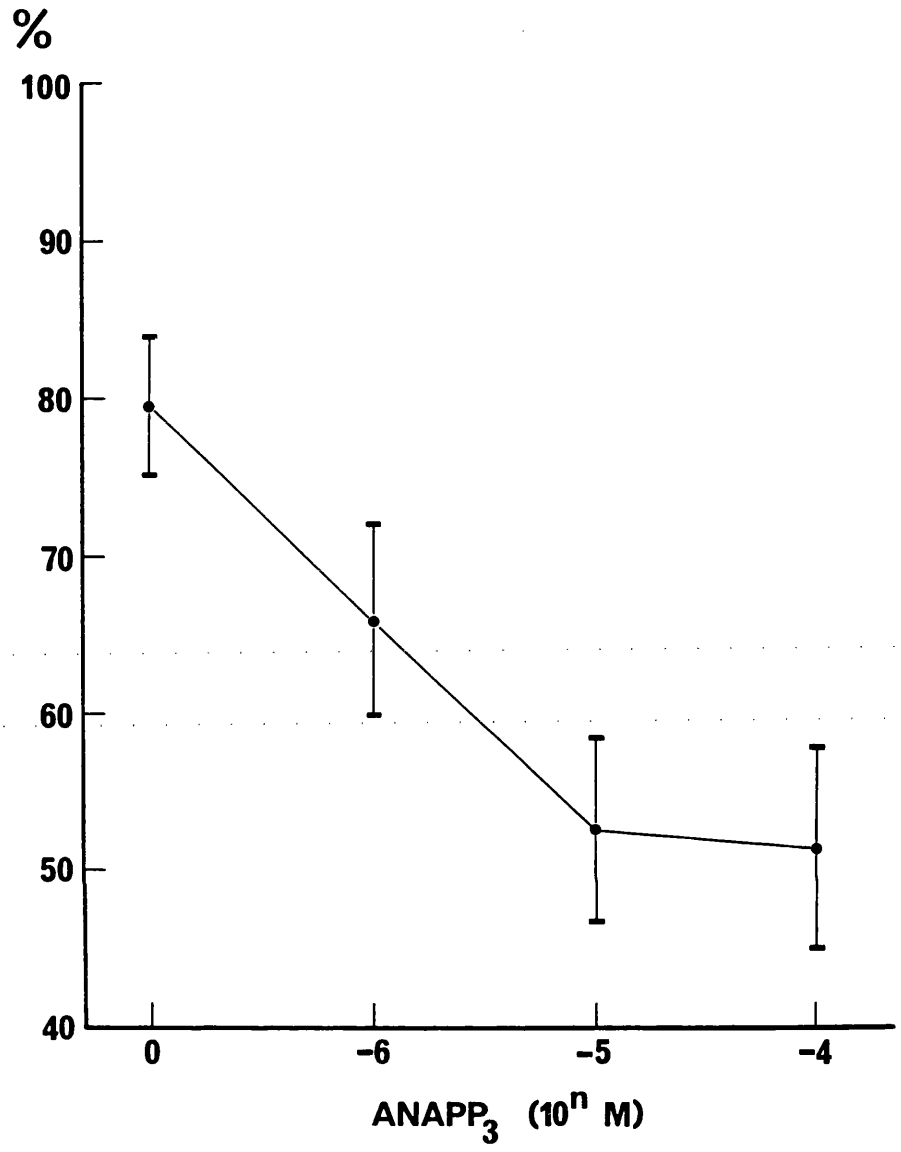


Fig.3.50 Dose-response curve of fibroblasts exposed to $ANAPP_3$ for 40 minutes and then treated with 5 mM ATP for 60 minutes. 0 on the abscissa shows the response of control cells not exposed to $ANAPP_3$, but treated only with 5 mM ATP. For each point 20 cells were measured except for 10^{-4}M $ANAPP_3$ where 18 cells were measured. Bars show the SEM. The areas of the cells after treatment with 10^{-5} and 10^{-4}M $ANAPP_3$ are significantly less than the controls ($p < 0.001$ Student t test).

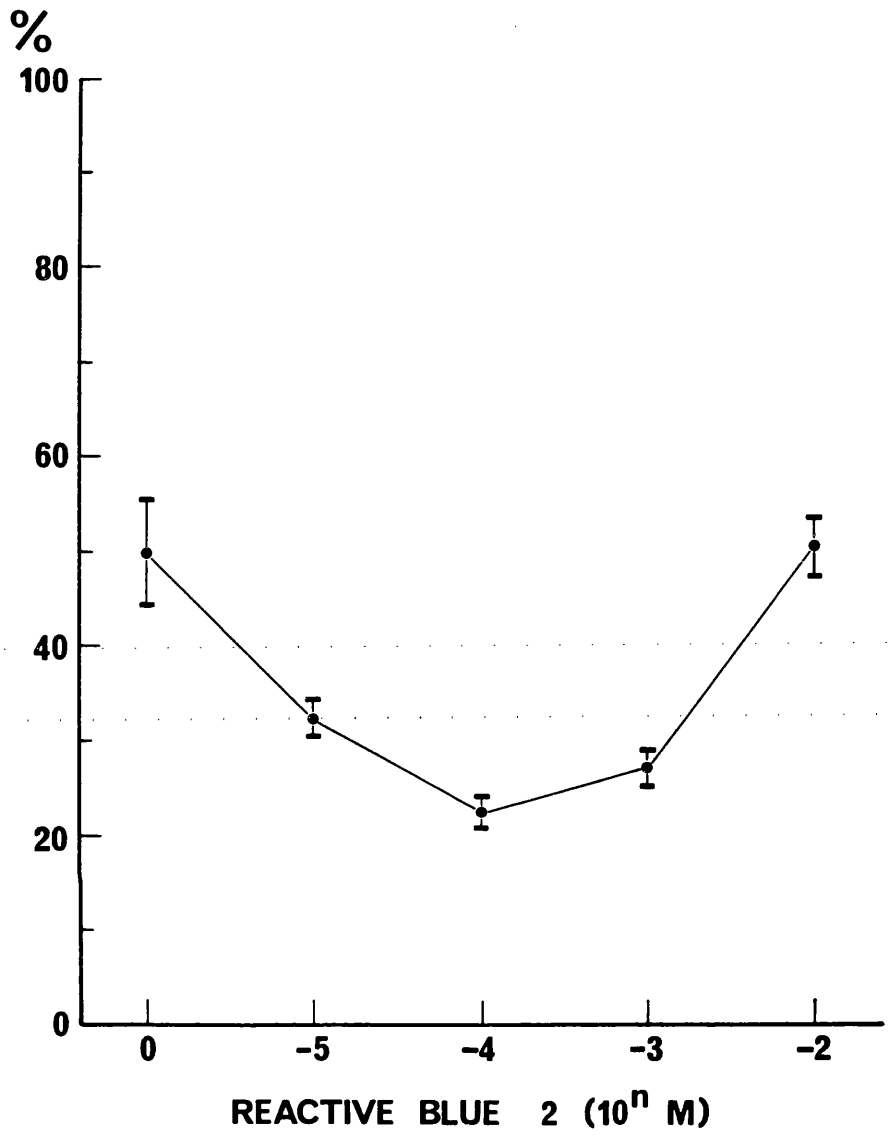


Fig.3.51. Dose-response curve of fibroblasts exposed to Reactive Blue 2 for 10 minutes and then 7 mM ATP for 60 minutes. 0 on the abscissa shows the response of control cells not exposed to RB2, but treated only with 7 mM ATP. For each point 10 cells have been measured. Bars are SEM. The areas of the cells after treatment with RB2 and ATP are significantly less than the controls (for 10^{-5} and 10^{-3}M, $p < 0.01$; for 10^{-4}M RB2, $p < 0.001$ Student t test).

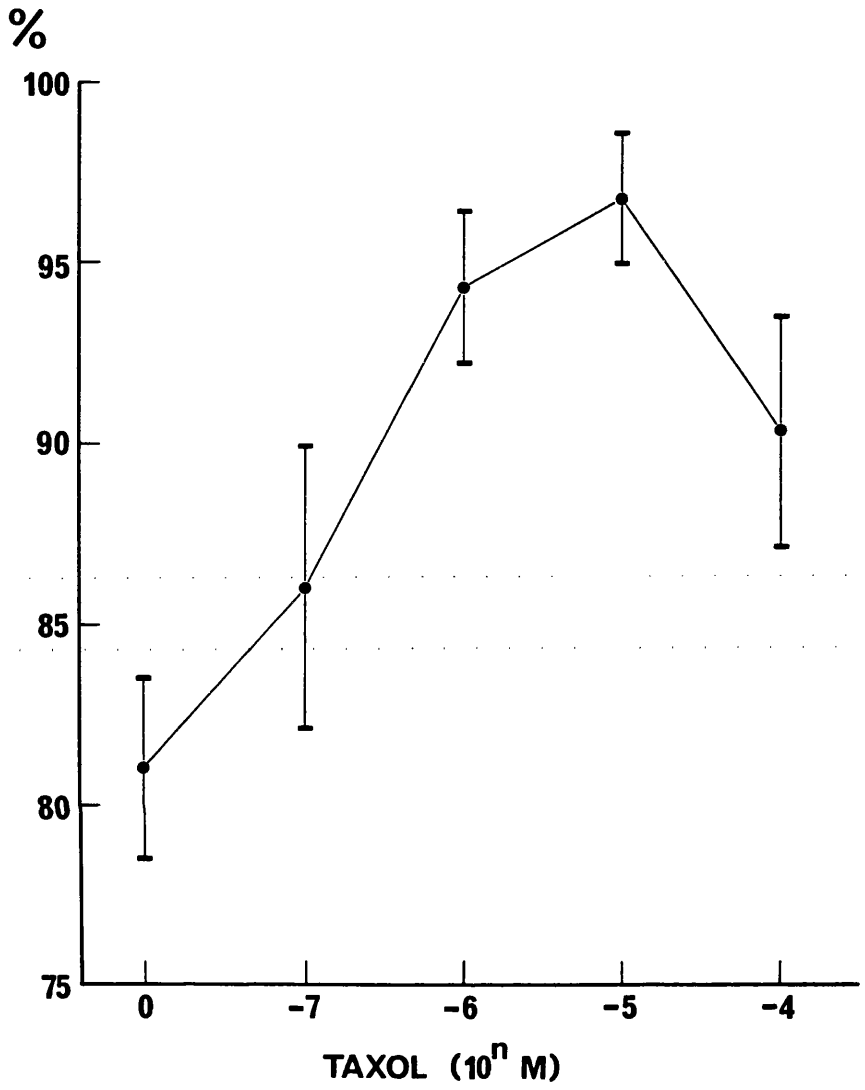


Fig.3.52. Graph showing the effect of taxol on inhibiting the contraction of rabbit Tenon's fibroblasts elicited by the application of 5 mM ATP. 0 on the abscissa shows the contraction produced by 5 mM ATP in the absence of taxol. For each point 10 cells were measured. Bars are SEM. Taxol at 10^{-5}M significantly reduced the contraction of the cells ($p < 0.001$ Student t test).

with incubation periods upto 3 hours. Therefore, longer incubation periods were evaluated. After 24 hours incubation, many cells were "trapped" in mitosis. At this time 10^{-3}M colchicine inhibited fibroblast contraction to 20% of the untreated control ($p < 0.001$ Student t test). No inhibition was seen at other doses tested between 10^{-9} and 10^{-5}M.

g.)Trocinate.

Placing the cells for 30 minutes in trocinate at varying concentrations, before exposure to 5 mM ATP did not inhibit the contraction of the cells caused by ATP alone. In fact, after treatment with 10^{-5}M trocinate, the cells contracted 2.6 times as much as the controls ($p < 0.01$ Student t test). At 10^{-4}M the cells contracted twice as much as the controls but this was not statistically significant, due to the large standard error of the response (fig.3.53).

g.)Trifluoperazine.

Pretreatment of the cells with trifluoperazine for 30 minutes before the application of 5 mM ATP for 60 minutes, markedly reduced the contractile activity of the cells. 5 mM ATP produced a contraction of the control cells to 28.5+/-5.5% (Mean+/-SEM) of the initial area. Pretreatment with 10^{-4} and 10^{-3}M trifluoperazine significantly reduced the contraction of the cells to 64.2+/-10.2% ($p < 0.01$) and 87.5+/-4.6% ($p < 0.001$ Student t test) of the initial area, respectively (fig.3.54.).

i.)Prostaglandin E_2.

Prostaglandin E_2, applied to the cells for 24 hours, at concentrations varying from 10^{-10} to 10^{-4}M, did not alter the contractile response of rabbit Tenon's fibroblasts to ATP.

j.)Ritodrine HCl.

10^{-3} and 6.18×10^{-3}M Ritodrine HCl caused a significant reduction in the cell area after application for 30 minutes ($p < 0.001$ Student t test). After subsequent application of 5 mM ATP to the fibroblasts for 60 minutes, those cells pretreated with ritodrine HCl at 10^{-3} and 6.18×10^{-3}M underwent a further significant reduction in the cell area ($p < 0.05$ and $p < 0.001$ respectively, Student t test), (fig.3.55).

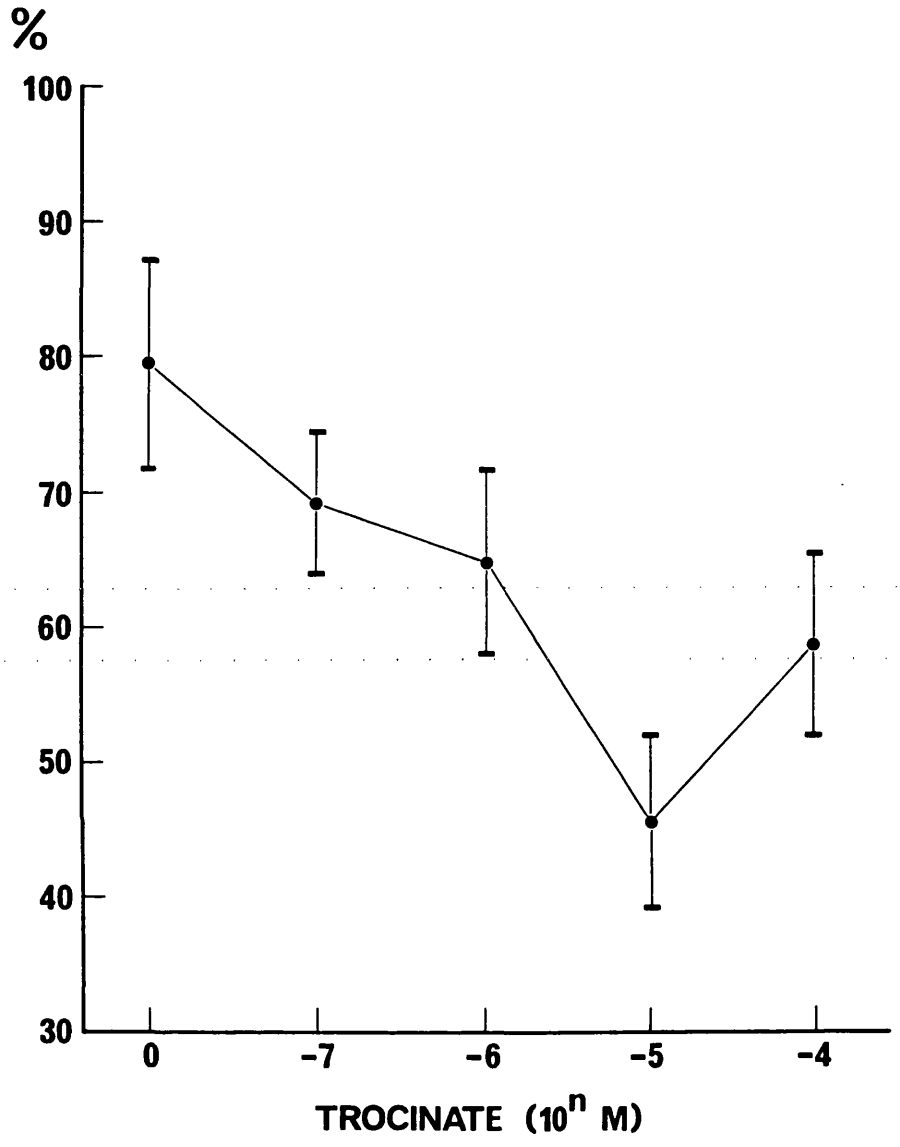


Fig 3.53. Graph showing the effect of pretreatment for 30 minutes of rabbit Tenon's fibroblasts with trocinate, followed by 5 mM ATP for 60 minutes. 0 on the abscissa shows the contraction elicited by exposure of the cells to 5 mM ATP without pretreatment with trocinate. For each point 10 cells have been measured. Bars are SEM. Cells pretreated with 10^{-5}M trocinate contracted 2.6 times as much as the controls ($p < 0.01$ Student t test).

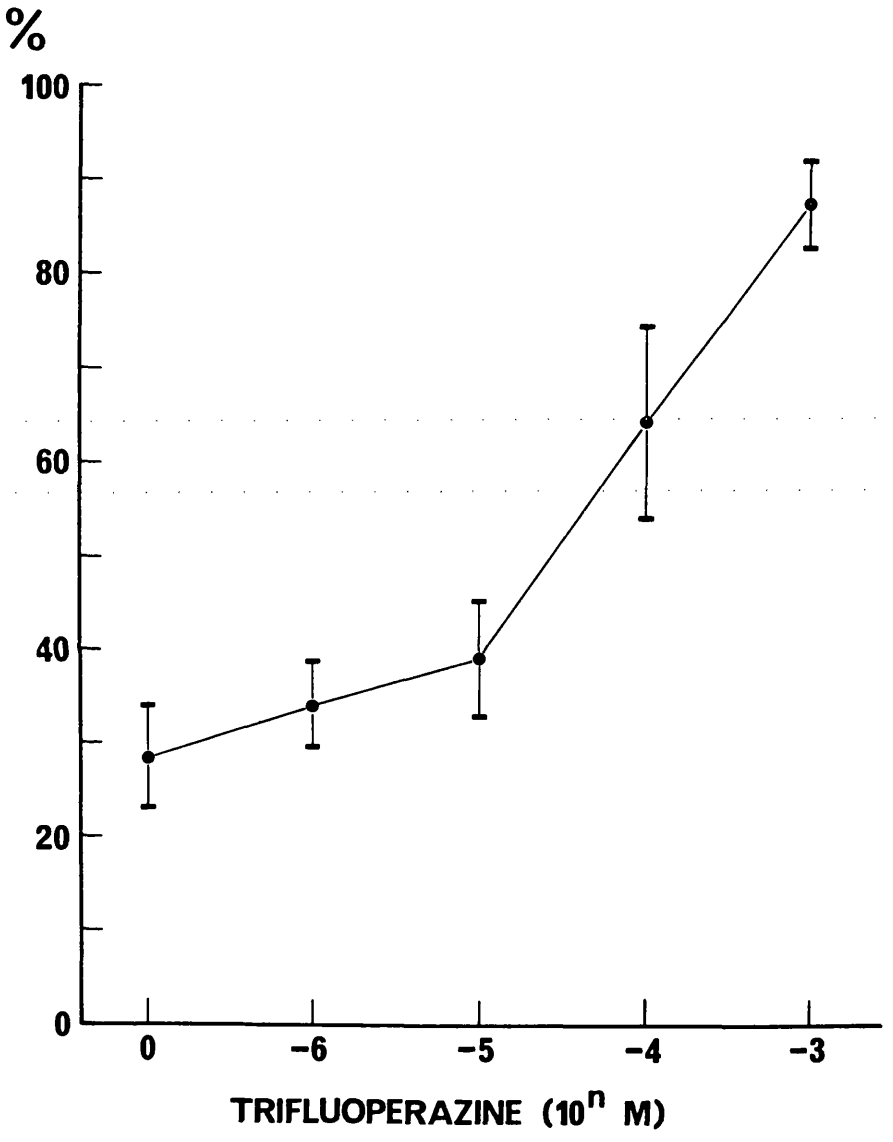


Fig.3.54. Graph showing the effect of 30 minutes pretreatment of rabbit Tenon's fibroblasts with trifluoperazine, followed by 5 mM ATP. 0 on the abscissa shows the contraction produced by 5 mM ATP without pretreatment with trifluoperazine. For each point 10 cells were measured. Bars are SEM. 10^{-4} and 10^{-3}M trifluoperazine significantly reduce the contractility of the cells ($p < 0.01$ and $p < 0.001$ respectively, Student t test).

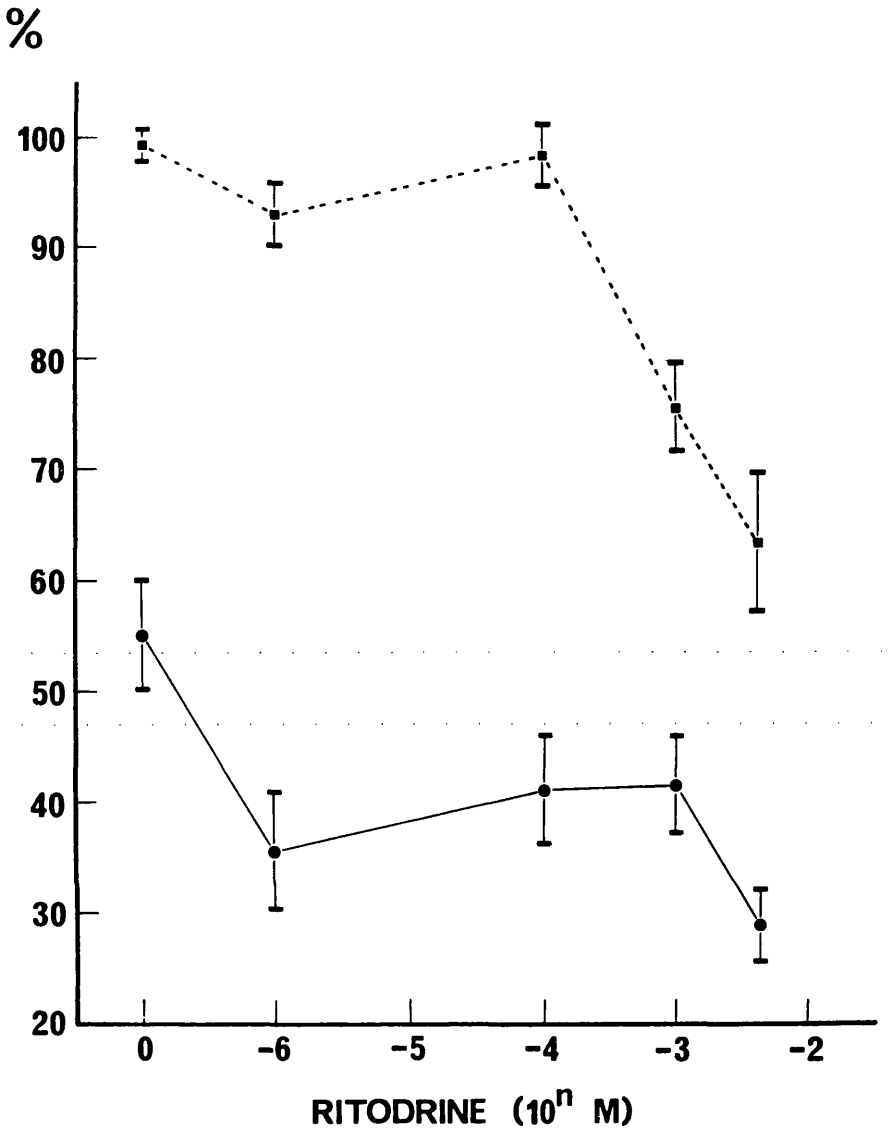


Fig.3.55. Graph showing the effect of treatment of rabbit Tenon's capsule fibroblasts with ritodrine HCl for 30 minutes followed by 5 mM ATP for 60 minutes. On the abscissa 0 represents cells exposed to F10 for 30 minutes followed by 5 mM ATP for 60 minutes.--■--after 30' exposure to ritodrine HCl; —●— after a subsequent 60' exposure to 5 mM ATP. 10^{-3} and 6.18×10^{-3}M Ritodrine HCl caused a significant reduction in the cell area after application for 30 minutes ($p < 0.001$ Student t test). After application of ATP to the cells, those cells pretreated with 10^{-3} and 6.18×10^{-3}M Ritodrine HCl underwent a significant further reduction in the cell area ($p < 0.05$ and $p < 0.001$ respectively, Student t test).

3.3.4. Error check

The intra-observer error for drawing around cells on the film viewer was 0.75%, while the inter-observer error was 5%. The intra-observer error for drawing around cells on the image analyser was 0.3% for one observer and 0.4% for the other, while the inter-observer error was about 0.25%.

3.4. CELL MORPHOLOGY

3.4.1. Drug effects

Normal fibroblasts were studied by a variety of techniques for comparison with drug treated cells. Trypan blue exclusion after trypsinising normal fibroblasts was 97%.

Drugs with activity against the cytoskeleton

After 4 hours exposure to 10^{-3}M colchicine, which optimally inhibited fibroblast migration and contraction, numerous cells were seen by phase contrast microscopy to be "trapped" in mitosis. Cells not in mitosis had partially lost their spindle shape and polarity (fig.3.56b.). By 24 hours these changes became more obvious. Scanning electron microscopy showed relatively good preservation of the normal cell morphology (fig.3.57b.). Immunofluorescent labelling of tubulin after 4 hours exposure to 10^{-3}M colchicine demonstrated destruction of the normal labelling pattern (fig.3.58d.). After exposure to this dose of colchicine, cell viability by trypan blue exclusion was 99%.

Within 30 minutes of exposure to 10^{-5} cytochalasin B, which maximally inhibited chemotaxis, marked morphological effects were demonstrated on the cells. There was substantial distortion of the normal architecture with rounding up of the cell body, leaving fine branching peripheral processes attaching the cells to the substrate. Phase contract and differential interference contrast microscopy clearly showed these morphological changes (fig.3.57d.). At 10^{-6}M, cell rounding up was far less evident. Scanning electron microscopy of cells treated with 10^{-5}M cytochalasin B showed marked rounding up of cells with blebbing of the plasmalemma (fig.3.57e.). By immunofluorescence, the normal filamentous actin staining pattern was destroyed within the rounded up cell body, and aggregates of actin were evident in some peripheral processes (fig.3.58b.). Trypan blue exclusion at four hours showed 98% viability.

Taxol at 10^{-6}M maximally inhibits chemotaxis. After exposure of a culture of fibroblasts to this dose of taxol for 4 hours, many cells were "trapped" in mitosis. There was a slight loss of the normal spindle shape and polarity of some cells (fig.3.56c.). By scanning electron microscopy, prominent membrane ruffles were evident. Microblebbing of the cell surface was prominent and some cells had lost their polarity (fig.3.57c.). Immunofluorescence confirmed the

loss of polarity of the cells, and showed a perinuclear accumulation of tubulin (fig.3.58e and f.). Viability by trypan blue exclusion after four hours exposure to 10^{-6}M taxol was 99%.

Fibroblasts were exposed for 24 hours to the three drugs at the optimally effective doses for inhibiting fibroblast migration. Following thorough washing and refeeding with F10 medium, the morphological effects of 10^{-5}M cytochalasin B were completely reversible, and the subsequent proliferation of the cells exactly parallelled that of the control cultures. The morphological effects of the 10^{-3}M colchicine persisted and subsequent cell multiplication ceased for the next 14 days. The morphological effects of taxol persisted for about six days in most cells, but cell replication was evident in nests throughout the cultures. After about 9 days cell numbers increased markedly and by 15 days, confluent cultures with about 3 million cells per flask were present (fig.3.59.).

Drugs which increase intracellular cyclic-AMP levels

At concentrations ranging from 10^{-12} to 10^{-3}M, dibutyryl-cAMP had no discernable morphological effects on the fibroblasts. Cell viability after four hours exposure to 10^{-3}M dibutyryl-cAMP was 95% which was similiar to untreated control cells. After 24 hours exposure to the same dose of the drug, cell proliferation was the same as untreated control cultures.

At concentrations varying from 10^{-11} to 10^{-3}M, Prostaglandin E_2 had similiarly no morphological effects on the cells. Trypan blue exclusion after 4 hours of exposure to 10^{-3}M PGE_2 was 95%.

Calmodulin antagonist

At the doses at which both cell contraction and cell migration were maximally inhibited, trifluoperazine had severe morphological effects on the cells. The drug appeared highly toxic and led to destruction of the cell membrane (fig.3.60.). Trypan blue exclusion after 4 hours exposure to 10^{-4} and 10^{-3}M trifluoperazine showed that all cells were dead. The apparent effects of inhibiting cell contraction and cell migration are thus largely attributable to toxicity of the drug.

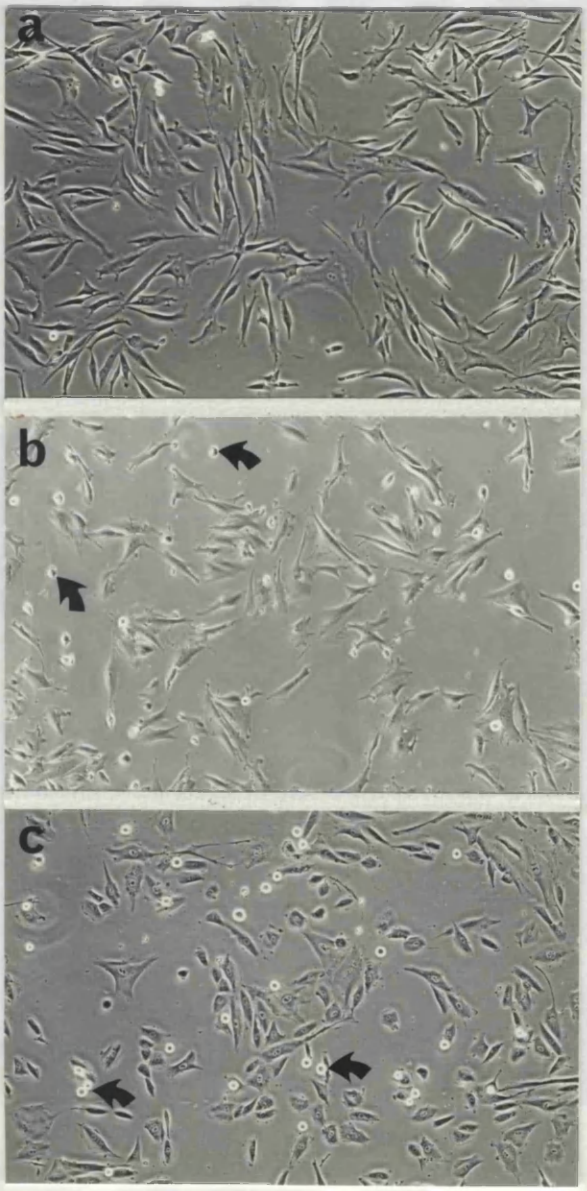


Fig.3.56. Phase contrast micrographs of 3 separate cultures of rabbit Tenon's fibroblasts. a.)Control cells. b.)After four hours exposure to colchicine 10^{-3}M. Numerous mitotic figures are evident but the remaining cells look little different to the control culture. c.)After four hours exposure to taxol 10^{-6}M. Again numerous mitotic figures are evident. The cells have partially lost their spindle shape and their polarity (Magnification X100).

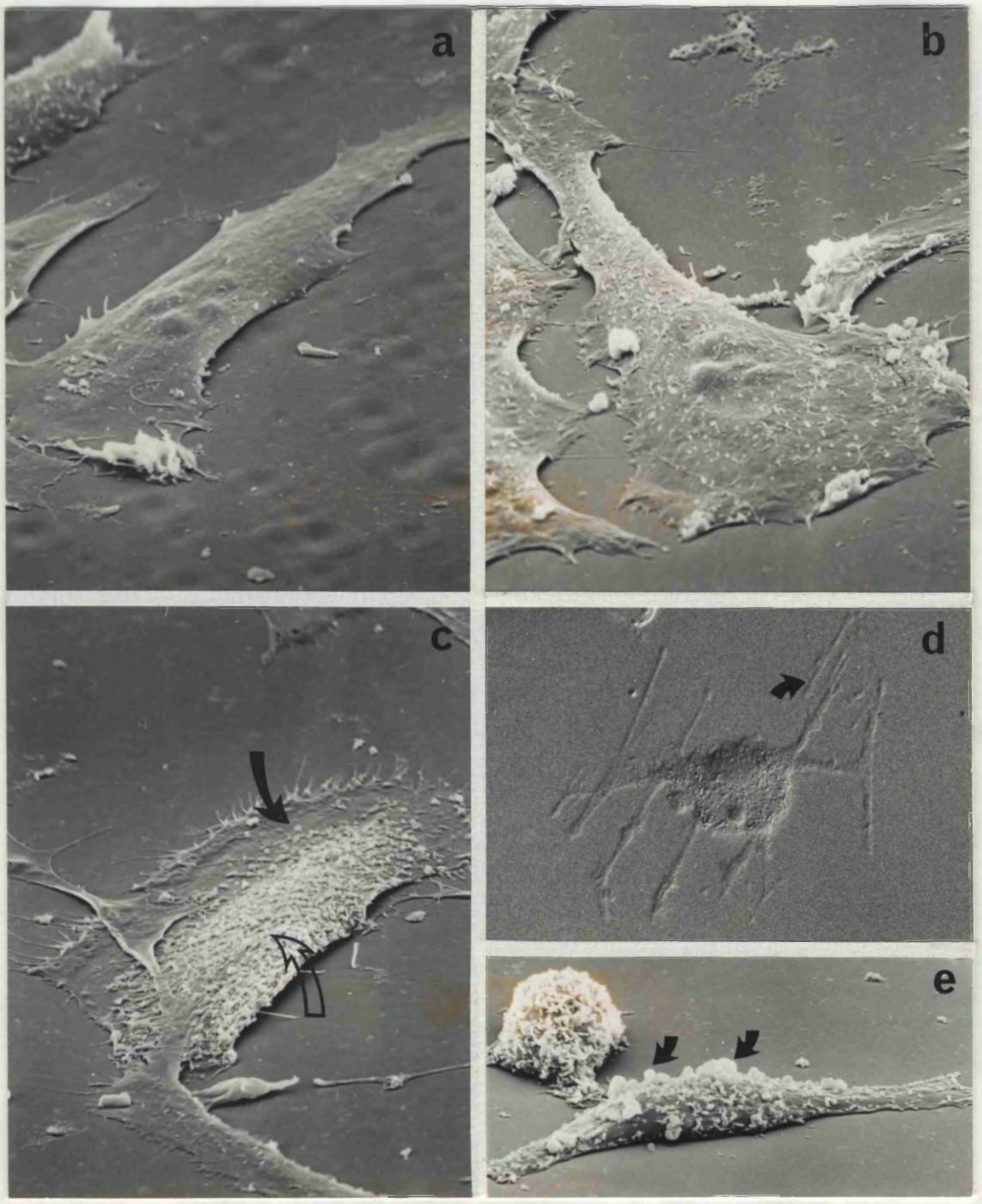


Fig.3.57. Scanning electron micrographs of rabbit Tenon's fibroblasts except for d.)which is a differential interference contrast micrograph. a.)Control cell (X2 000) b.)After treatment with colchicine 10^{-3}M. This cell looks little different to the control (X1 700). c.)After exposure to taxol 10^{-6}M. The cell has lost its polarity. There is a prominent fan and ruffle on the leading edge of the cell (solid arrow). Blebs of the cell membrane are evident (clear arrow) (X1 600) d.) and e.)After treatment with 10^{-5}M cytochalasin B (d.X540and e.X1 800). Cells have rounded up. Branching processes are evident in d. (arrow), while blebbing of the cell surface is shown in e.(arrows). Cells have been exposed to drugs for four hours.

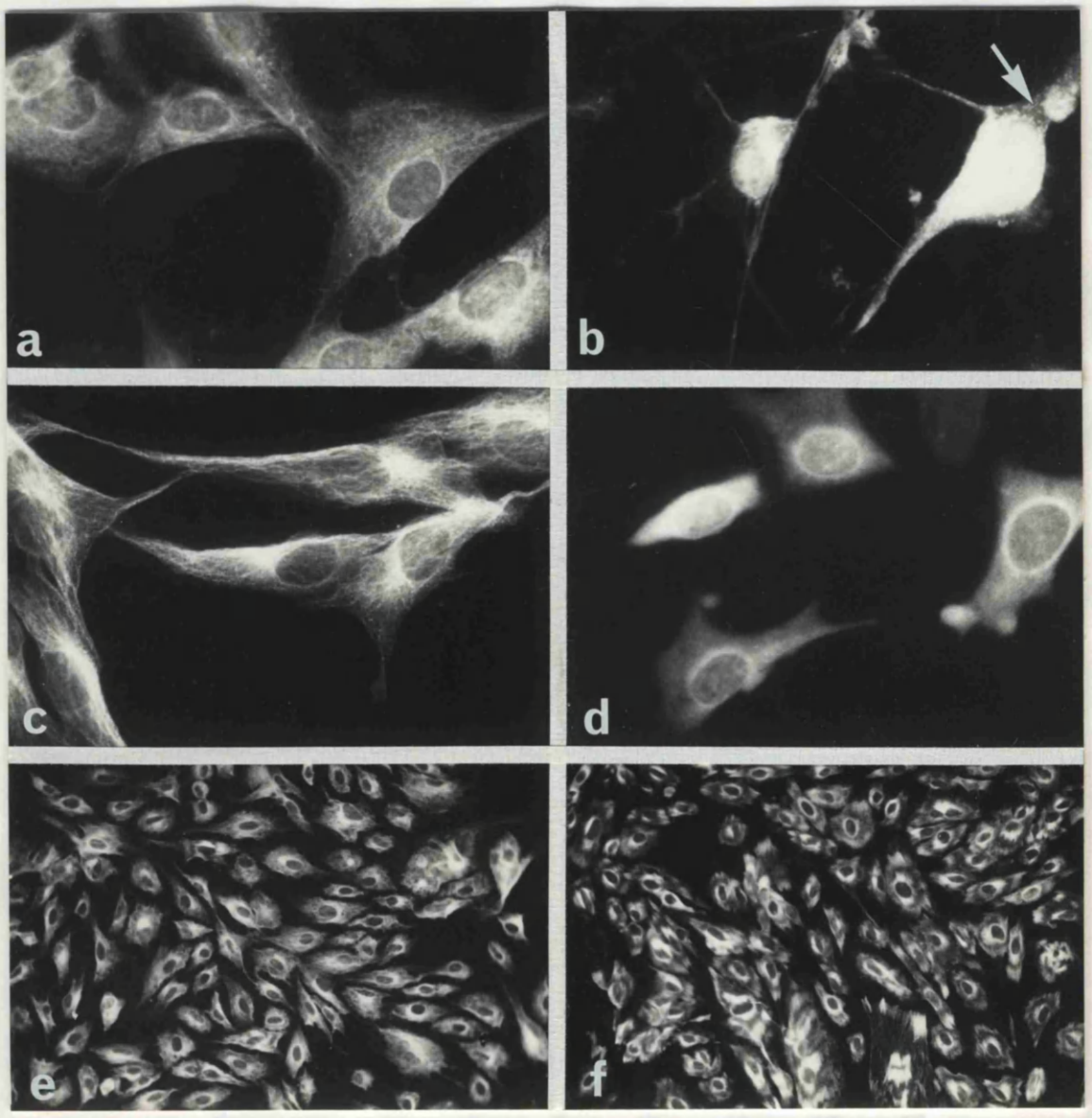


Fig.3.58. Rabbit Tenon's fibroblasts pretreated with drugs for four hours and then labelled with monoclonal anti-actin or anti-tubulin. a.)Control culture showing actin filaments. b.)Pretreatment with cytochalasin B 10^{-5}M. The cells are markedly distorted. The cell bodies have rounded up leaving fine peripheral processes. The filamentary actin pattern is no longer evident in the cell body and appears to be fragmented towards the periphery (arrow) c.)Control culture with labelled tubulin. d.)Cells pretreated with colchicine 10^{-5}M showing marked loss of tubulin. There is also a partial loss of the normal spindle shape of the cells. e.)Pretreatment with taxol 10^{-8}M and f.)Taxol 10^{-6}M. Prominent tubulin labelling is evident around the nuclei, particularly at 10^{-6}M. There is some loss of polarity of the cells at this dose (Magnification a. to d. X560; e. and f. X200)

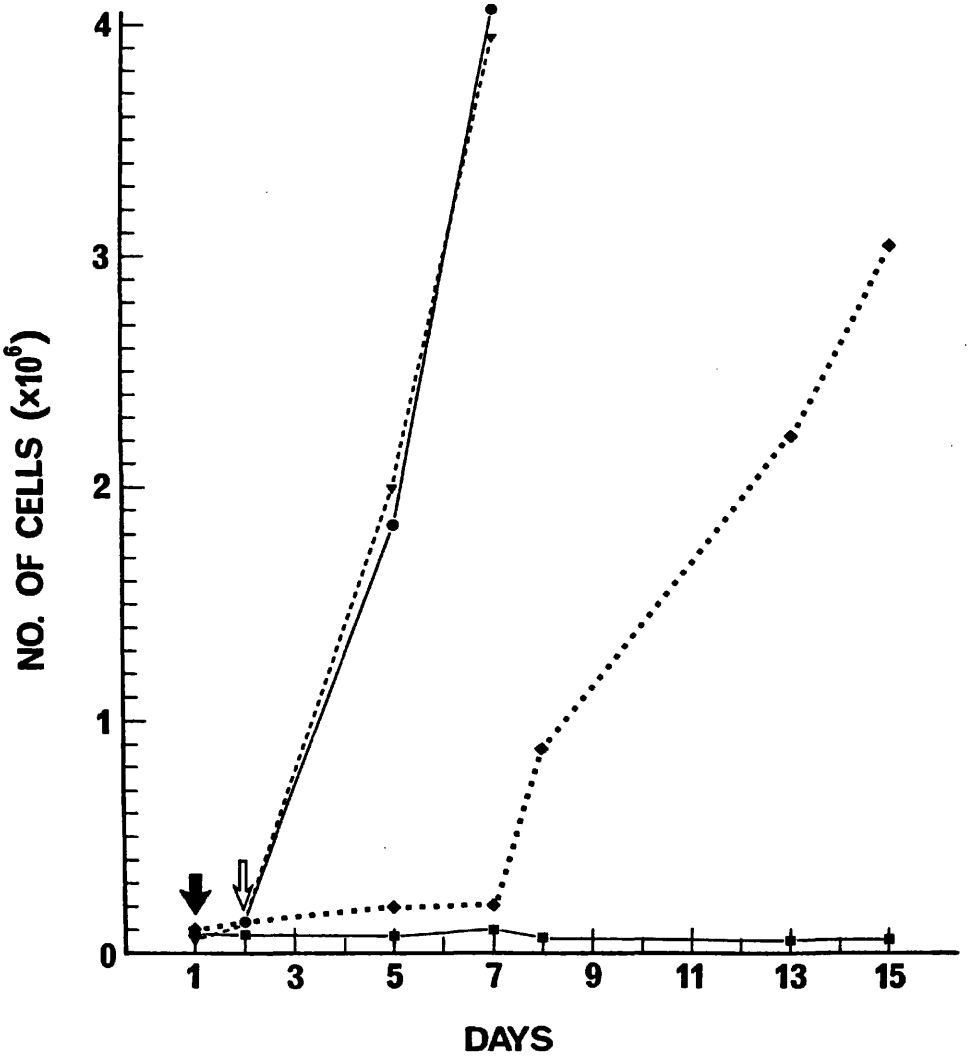


Fig.3.59. Rabbit Tenon's fibroblasts were passaged on day 0. On day 1 flasks were washed three times with serum free F10 medium and then fed with F10 medium with 10% new born calf serum containing either 10^{-3}M colchicine, 10^{-5}M cytochalasin B or 10^{-6}M taxol (filled arrow). After 24 hours the cells were washed three times with medium and refed (clear arrow). Points are the mean of three counts. ——●—— Control; --▼--10^{-5}M Cytochalasin B; ···◆··· 10^{-6}M Taxol; ——■—— 10^{-3}M Colchicine.

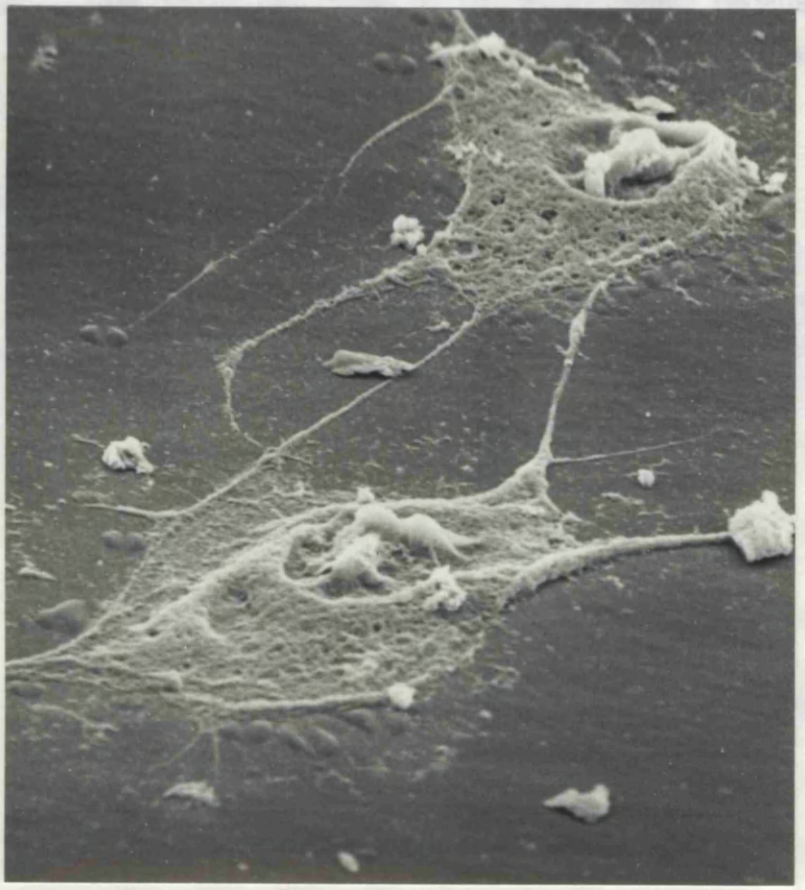


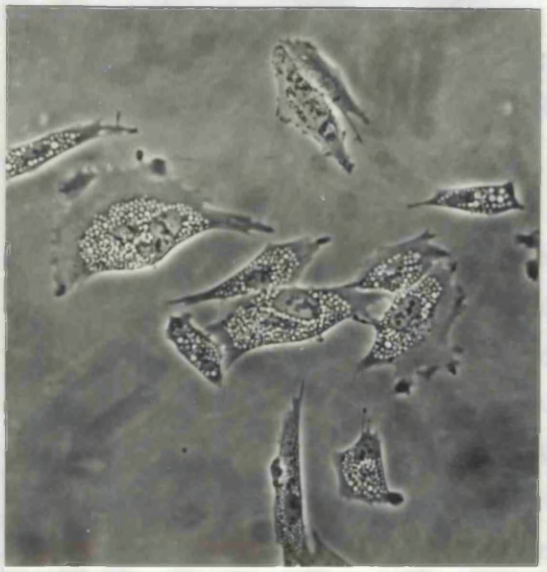
Fig.3.60. Scanning electron micrograph of fibroblasts exposed to 10^{-3}M trifluoperazine for four hours. There is marked destruction of the cell membranes (Xl 750).

Beta$_2$-adrenoceptor stimulant

Ritodrine hydrochloride led to marked vacuolation of the cytoplasm of the fibroblasts (fig.3.61.). Vacuolation was most evident at 6.18×10^{-3}M, the dose which also maximally inhibited cell migration. This peculiar concentration was used in the experiments because it was the maximum concentration attainable using the intravenous preparation of the drug.

After four hours exposure to 6.18×10^{-3}M ritodrine HCl, trypan blue dye exclusion showed cell viability of 96%. However, after 24 hours exposure to the drug, many cells had detached from the culture flask. After washout of the drug and replenishment with fresh F10 medium, virtually no proliferation of the few remaining cells was evident over the ensuing 10 days.

a



b

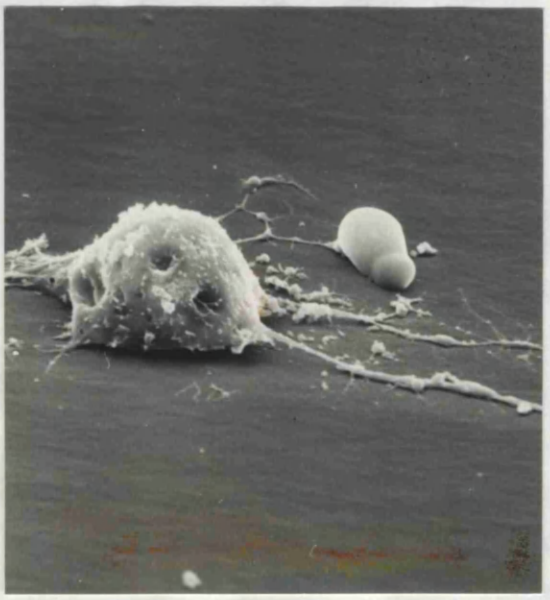


Fig.3.61. Fibroblasts have been exposed to 6.18×10^{-3}M ritodrine HCl for four hours. Marked vacuolation is evident and a number of cells have rounded up. a.)Phase contrast micrograph (X230), b.)Scanning electron micrograph (X1 600).

Chapter 4 DISCUSSION

4.1. CHEMOTAXIS

In this thesis it has been shown that fibronectin is chemotactic for both rabbit and human ocular fibroblasts. Fibroblast conditioned medium has also been shown to be chemoattractive for rabbit Tenon's capsule fibroblasts. Rather surprisingly, normal rabbit aqueous humour was found to be strongly chemotactic for the same cells. These observations on rabbit aqueous humour were confirmed when human aqueous humour was evaluated in chemoattraction assays. Control specimens of aqueous humour taken from patients undergoing cataract extraction all elicited a massive chemoattractant response on both rabbit and human ocular fibroblasts. This was demonstrated by chequer-board analysis to be primarily a chemotactic effect with a moderate chemokinetic response, which was reduced at higher doses of aqueous humour. All of the samples of aqueous humour taken from patients with glaucoma proved to be powerfully chemoattractant to both rabbit and human ocular fibroblasts. The response was again confirmed to be primarily chemotactic with a moderate chemokinetic effect at low concentrations, which was diminished at higher concentrations. Aqueous samples derived from patients who had previously undergone glaucoma surgery that had failed, consistently demonstrated greater chemoattractant activity than the control specimens derived from patients with cataracts, whether rabbit or human ocular fibroblasts were used as the indicator cells.

The demonstration in this thesis that fibronectin is chemotactic for ocular fibroblasts confirms the observations of other authors who have shown that fibronectin is chemotactic to fibroblasts of diverse origins (Postlethwaite, et al., 1981; Mensing, et al., 1983). It is important, however, to have substantiated this fact using cells appropriate to wound healing at the site of a trabeculectomy, since it is well described that fibroblasts derived from different organs do not necessarily behave in an identical manner (Freundlich, Bomalaski, Neilson, et al., 1986).

Fibronectin occurs in healing wounds (Grinnell, Billingham and Burgess, 1981) and is responsible for the adherence of cells to the connective tissue matrix (Hynes and Yamada, 1982). It has been shown

to be widely distributed within the eye, particularly within human trabecular drainage channels (Floyd, Cleveland and Worthen, 1985). Fibronectin occurs in bovine aqueous humour (Reid, Kenney and Waring, 1982) and is likely to be present in human aqueous humour, although it has not yet been measured. It is probable that fibronectin is one of numerous factors responsible for the migration of fibroblasts towards a healing trabeculectomy.

The optimally effective dose of fibronectin in this thesis for eliciting fibroblast migration was found to be 20 ug/ml when rabbit cells were employed in the assay, compared to 30 ug/ml when human cells were investigated. The literature mentions various optimal doses of fibronectin ranging from 1.2 ug/ml to 100 ug/ml (Postlethwaite, et al., 1981; Seppä, et al., 1981; Mensing et al., 1983; Albini, Richter and Pontz, 1983). The disparity probably relates to differences in the sources of fibroblasts used in the various assays.

Optimally effective concentrations of fibronectin were used as positive controls in all chemotaxis experiments in the current study. However, it must be emphasized that the choice of this substance as a control was a compromise. Fibronectin is relatively cheap and is readily available. It has been widely investigated and has been employed as a control in many other studies. However, it suffers from two major disadvantages. The first is that it is only moderately chemotactic. Therefore the number of the fibroblasts migrating to aqueous humour was many times greater than the number migrating to the control of fibronectin. As an ideal control a substance should elicit a near maximal effect. Unfortunately, a single substance which elicits such a dramatic reaction has not been identified, and therefore fibronectin was resorted to. The second major disadvantage of fibronectin as a control is that there was considerable day-to-day variability in the response of the cells to this substance, for reasons that have not been adequately explained. The sources of error in multiwell chamber chemotaxis assays have been investigated (Minkin, Bannon, Pokress, et al., 1985) indicating that this day-to-day variability is not unique to the experiments described in this thesis. As a result valid comparisons are only possible between samples run simultaneously in the same multiwell assay, if necessary incorporating more than one 48-well micro-chemotaxis chamber. Even the inclusion of controls does not allow the direct quantitative comparison of results

obtained on different days. Nevertheless, qualitative comparisons are still feasible.

The fact that I found fibroblast conditioned medium to be chemoattractive to rabbit Tenon's capsule fibroblasts is interesting as it helps to explain the self perpetuating nature of scarring. Once a few fibroblasts have been attracted to a healing trabeculectomy, they are able to call up reinforcements and the process continues. Some of the factors known to be synthesised by fibroblasts are collagen, fibronectin and proteoglycans. Collagen is an additional chemoattractant for fibroblasts (Postlethwaite, Seyer and Kang, 1978). Mensing, et al., (1983) have proposed that there are other as yet unidentified substances in fibroblast conditioned medium which are chemoattractive to fibroblasts. Although fibroblast conditioned medium is very cheap and easy to manufacture and is a powerful chemo-attractant, it is unsuitable as a control as its activity declines when stored at -20^{o}C. An explanation for the decay of activity may ultimately give some clues as to the nature of the contained chemoattractants.

I did not anticipate that rabbit primary aqueous humour would be chemoattractive for ocular fibroblasts. The initial observation was made by chance on aqueous taken pre-operatively on a rabbit about to undergo experimental glaucoma filtration surgery. While I thought it likely that the secondary aqueous would enhance the accumulation of fibroblasts at the operation site, I thought that the primary aqueous would be inert (see section 1.8.4). The unexpected observation that rabbit primary aqueous humour is chemoattractive for ocular fibroblasts was subsequently confirmed and all specimens obtained, without exception, exhibited a similiar response. Furthermore, the migratory response demonstrated by the test cells was one primarily of chemotaxis with a smaller chemokinetic effect, which was evidence of preservation of the blood-aqueous barrier during sampling of the aqueous. Since proteins such as albumin exert a chemokinetic effect, one would anticipate a far greater chemokinetic effect, increasing with higher concentrations of aqueous humour, had the blood-aqueous barrier been disrupted by the paracentesis (Rosenbaum, et al., 1984). The chemotactic effect of normal rabbit aqueous humour for fibroblasts is in contrast to the chemokinetic effect for monocytes demonstrated by Rosenbaum and Raymond, (1985).

Further evidence of the integrity of the blood-aqueous barrier was provided by the inability to influence the chemoattractive activity of rabbit aqueous humour by the administration of a variety of anti-inflammatory drugs up to one hour before collection of the specimens. Rabbit eyes are well known to have an extremely fragile blood-aqueous barrier (Unger, et al., 1975). The fact that the technique of aqueous sampling used in this thesis preserved the blood-aqueous barrier in rabbits, indicated that a similiar technique employed in human beings with a much more resistant blood-aqueous barrier, would be unlikely to cause artefacts in the aqueous humour.

Rabbit eyes are known to exhibit a marked inflammatory response and to heal up a filtration wound within 17 days (Miller, et al., 1985). A major part of the healing response may be attributable to the intrinsic chemotactic activity of normal aqueous humour, for fibroblasts, as demonstrated in the current study. The absence of a fibroblast infiltrate in normal rabbit aqueous humour and indeed in human aqueous humour, despite the presence of fibroblast chemo-attractants does not imply that this finding is an in vitro artefact since other factors that increase vascular permeability or expose connective tissue directly to the aqueous humour might be required for cellular migration in vivo (Rosenbaum and Raymond, 1985). An analagous situation of potential inflammatory activity exists in the vascular system. Blood consists of a complicated mixture of inflammatory agents and chemotactic substances, eg. the fibronectin level in human plasma is about 300 ug/ml, which is chemotactic for fibroblasts (Hynes and Yamada, 1982). The chemotactic activity of blood is also controlled by inhibitory circulating substances (Ochs, Postlethwaite and Kang, 1987). Blood is normally prevented from direct contact with the tissues. Once damage has occurred and blood leaks out of the vessels, an enormous inflammatory reaction commences which elicits healing of the wound.

Aqueous humour samples were taken from patients undergoing cataract extraction and were employed as the "normal" control specimens. Ethically it is not possible to get fresh specimens of aqueous humour from living patients with perfectly normal eyes. It is therefore conceivable that the control samples in this study were not normal since either a primary change in the aqueous humour may induce a cataract or alternatively the presence of a cataract may cause a

secondary change in the aqueous humour accounting for its chemotactic activity. Although it is impossible to rule out this possibility entirely, the fact that all the human aqueous samples, including those from patients with glaucoma and no cataracts, were qualitatively similiar, makes this extremely unlikely.

The chemoattractant activity of the control aqueous humour specimens appeared to be independent of the surgical approach to cataract extraction and the surgeon supplying the sample (providing care was taken not to make contact with any intra-ocular structures while the sample was being collected). The activity of the samples was also not influenced by the pre-operative administration of topical indomethacin, which has been shown to reduce inflammation and reduce breakdown of the blood-aqueous humour barrier (Sanders and Kraff, 1984). Protein estimation of randomly selected specimens of aqueous humour taken from patients with cataracts fell within the normal range, serving as further evidence that the chemotactic activity exhibited by the cataractous aqueous humour was not artefactually produced by sampling. pH measurement of the wells of the chemotaxis chamber demonstrated in addition that the observed cellular migration was not artefactual due to pH gradients across the membrane.

Paracentesis can lead to breakdown of the blood-aqueous barrier, and manipulation of the eye at the start of surgery, particularly when it has previously undergone multiple surgical procedures, may damage the barrier. Likewise, the preoperative administration of anti-glaucoma drugs such as pilocarpine (Novack and Leopold, 1988) and adrenaline (Miyaki, Miyaki and Kuratomi, 1987) may damage the blood-aqueous barrier. However, the preponderance of the chemotactic response over the chemokinetic effect, seen with glaucomatous aqueous humour substantiated the preservation of the blood-aqueous barrier when these samples were taken. Chemotactic activity was also observed in the aqueous samples taken from people undergoing primary trabeculectomies who had not applied any anti-glaucoma medications to their eyes, thus drug damage to the blood-aqueous barrier could be largely discounted as the cause of the chemotactic effect observed.

Partial characterization of the fibroblast chemotactic constituents of human aqueous humour indicated that there were at least two components, one heat labile and the other heat stable. The chemotactic

activity was resistant to high pH but was deactivated by low pH, and resided in substances with molecular weights over 30,000. Fractionation of small samples using for example, high performance liquid chromatography may enable the molecular weights of the chemotactic components of aqueous humour to be determined in future studies.

What this thesis has not demonstrated is whether the increased chemotactic activity seen in the aqueous of the "complex" patients who had sustained failed glaucoma drainage surgery predated the unsuccessful surgery or was caused by it. A prospective study of patients undergoing a first trabeculectomy would be needed to clarify the point. A study of this nature would need to incorporate patients at high risk of surgical failure, eg. young people and Blacks. However, because the average failure rate of trabeculectomy is about 15% (Watson and Grierson, 1981), large numbers of patients would have to be included in the investigation, which would be extremely difficult using the 48-well micro-chemotaxis apparatus that has been described here. Even if the increased chemotactic activity were produced by the previously unsuccessful surgery it would help to explain why repeat trabeculectomies have a lower chance of success than first trabeculectomies.

No attempt has been made in the current study to investigate the fibroblast chemoattractants produced by the inflammatory processes resulting from the surgical trauma of trabeculectomy. Without doubt, the acute inflammatory response will influence the formation of fibrous tissue at the operation site. However, study of these factors would necessitate taking specimens such as aqueous humour, post-operatively from patients who had undergone glaucoma surgery, which would not be ethically acceptable. Alternatively, animal models of failing glaucoma surgery (Miller, et al., 1985) could be used to clarify some of these factors. In this thesis it has been shown that rabbit ocular fibroblasts behave very similiarly to human cells, which indicates that data from such animal studies is likely to be relevant to the human situation.

The aqueous components responsible for the chemotactic effect are at present unknown. However, there are numerous substances which have been shown to be chemotactic for non-ocular fibroblasts. These include collagen (Postlethwaite, et al., 1978), activated complement

(Postlethwaite, Snyderman and Kang, 1979), lymphokines (Wahl and Wahl, 1981), fibronectin (Postlethwaite, et al., 1981), fibronectin peptides (Postlethwaite, et al., 1981), platelet derived growth factor (Seppä, Grotendorst, Seppä, et al., 1982), tropoelastin (Senior, Griffin and Mecham, 1982), platelet factor 4 and platelet derived beta-thromboglobulin (Senior, Griffin, Huang, et al., 1983), ATP (Okada, Yada, Ueda, et al., 1983), elastin (Senior, Griffin, Mecham, et al., 1984), leukotriene B_4 (Mensing and Czarnetzki, 1984), coagulation products (Senior, Skogen, Griffin, et al., 1986), and transforming growth factor beta (Postlethwaite, Keski-Oja, Moses, et al., 1987). In addition both macrophage- (Diegelmann, Schuller-Levis, Cohen, et al., 1986) and lymphocyte-derived chemotactic factors for fibroblasts (Postlethwaite, et al., 1976) have been identified. The list of fibroblast chemotactic substances is constantly being added to, however, none of them has been positively identified in normal human aqueous humour. Recently, however, a substance similiar to the powerful fibroblast mitogen and chemotactic agent, fibroblast growth factor has been identified in cataractous human aqueous humour (Tripathi, et al., 1988 and 1989).

As mentioned previously, fibronectin has been measured in pooled post-mortem bovine aqueous humour at 2.46 ug/ml (Reid, et al., 1982). It is likely to be present in human aqueous humour since it is synthesised by human meshwork cells in tissue culture (Worthen and Cleveland, 1982; Polanski, Wood, Maglio, et al., 1984), and by corneal endothelial cells (Zetter, Martin, Birdwell, et al., 1978). However if the level is similiar to that in bovine aqueous, it will contribute little to the chemoattractant activity of human aqueous humour. Many of the above substances are likely to occur in "inflammatory" aqueous humour post-operatively. In addition, a platelet derived growth factor-like substance has recently been identified in the vitreous in patients with proliferative vitreoretinopathy and this could conceivably leak into the aqueous, accounting for part of its chemotactic activity in some patients (Campochiaro, Jerdan, Glaser, et al., 1985). Platelet derived growth factor has a molecular weight of 30,000 and retains its chemoattractant activity after boiling (Seppä, et al., 1982), which are features of the aqueous chemoattractants identified in this thesis. Clearly, further biochemical investigations are indicated to positively identify the fibroblast chemoattractants in aqueous humour.

In the normal eye aqueous humour is not directly exposed to fibroblasts. Thus although the aqueous has potential activity to enhance the migration of fibroblasts, this is not realised until the integrity of the eye is breached. Where a trabeculectomy has been created, there will be a number of factors that determine whether the filtering channels remain patent. The main factor in favour of keeping the channels open will be the flow of aqueous humour. In opposition will be healing forces, which attempt to close the wound in the body. The pre-existing state of the aqueous humour and particularly its post-operative state, with regard to the ability to recruit fibroblasts to the wound site by the process of chemotaxis, may be critical in determining the success or failure of the operation.

4.2. FIBROBLAST CONTRACTION

In this thesis Triton X-100 cytoskeletons of rabbit ocular fibroblasts achieved a modest, slow contraction on the application of ATP. The optimally effective dose was 0.1 mM ATP which resulted in a reduction in area of the cells to about 65% of the initial area within 60 minutes, corresponding well with responses published by other authors (Masuda, et al., 1983; Hitchins, De Yong, Day, et al., 1988; Das, Frank, Weber, et al., 1988).

It was felt, however, that this technique of cell contraction was unacceptable for a number of reasons. Many cells were detached from the substrate during the course of preparation of the cytoskeletons. The procedure is entirely unphysiological because the cells are killed by permeabilisation. The cytoskeletons are placed in a solution which approximates the internal environment of the cell, which is necessary because the barrier function of the cell membrane has been removed. Although the contractile apparatus is "dissected out" for experimentation, the cell remnants are in a totally unphysiological environment. Since the cell membrane was extensively damaged by permeabilisation it proved difficult to define the cell margin accurately on the image analyser, making it difficult to quantify changes in the cell area. Lastly, it was felt that the absence of the cell membrane together with its receptors would make it extremely difficult to interfere with cytoskeleton contraction pharmacologically.

In addition, Masuda, et al., (1983) found that up to 70-80% of the total protein in whole cells is solubilised by treatment with 0.05% Triton X-100. The ability to reverse and repeat the contractile process is severely limited. Grierson, et al., (1986) have shown partial relaxation of cytoskeleton preparations with the smooth muscle relaxant, papaverine, but further cycles of contraction and relaxation were not undertaken by these authors. Other workers appear not to have reported relaxation of contracted cytoskeletons.

The single cell contractile assay developed in this thesis has demonstrated that whole rabbit Tenon's capsule fibroblasts undergo a pronounced, rapid, and reversible contraction, measured as a reduction in surface area in two dimensions, in response to exogenous ATP.

Conjunctival, scleral and skin fibroblasts behave in a virtually identical fashion. The reduction in area of the intact cells caused by ATP is about four times greater than that of the Triton X-100 cytoskeleton preparations. The intact cell response also occurs far faster than the cytoskeleton preparation with a 50% reduction in area achieved within about 90 seconds. Unlike the cytoskeleton preparation where the optimally effective dose of ATP was 0.1 mM, 15 mM exerted the optimal contractile effect on intact fibroblasts.

The mode of action of ATP in bringing about a contraction of intact cells is different to its effect on cytoskeletons, where it acts merely as an energy source. This is because ATP does not penetrate intact cells to any large degree (Gordon, 1986). Trams, (1974) has demonstrated that ATP acts on the surface of numerous cell types, including fibroblasts, and produces a biphasic change in membrane ion permeability, particularly involving an efflux of K^+. He noted that different cell types had different dose responses to ATP. Yatani, Tsuda, Akaike, et al., (1982) have shown that extracellular ATP activates membrane calcium channels in neurons, although the maximum concentration of ATP evaluated was 1 mM. Exogenous ATP also induces electrical membrane responses in fibroblasts, via a receptor system thought to be of the P_2-purinoceptor type (Okada, Yada, Ohno-Shosaku, et al., 1984). In Okada's study concentrations of ATP ranging from 0.2 mM to 100 mM were evaluated, and activity was shown within this range. Purinergic receptors have been extensively reviewed by Burnstock (1976; 1978). Oiki, Ueda and Okada, (1985) have further demonstrated that exogenous ATP induces a twofold rise in intracellular calcium concentration in fibroblasts, due to calcium influx, thereby inducing electrical membrane responses by activation of calcium-dependent potassium channels.

In the current study adenosine has been shown to be a partial agonist of fibroblast contraction. This appears further to substantiate the purinergic receptor hypothesis of the mode of action of exogenous ATP. Burnstock, (1978) has described a hierarchy of activity of adenosine, adenine nucleotides, and nucleosides at these receptors. Receptors responding principally to adenosine are designated P_1-purinoceptors, while those activated principally by ATP are P_2-purinoceptors, ie. the type of receptors that appear to be present on the fibroblasts studied in this thesis. At least 2 subtypes of P_2-purinoceptors have been

defined on the basis of the potency order of various agonists, selective antagonism by drugs, and on the principle effect of receptor stimulation, ie. excitation or relaxation. A photoaffinity analogue of ATP known as Arylazido aminopropionyl adenosine triphosphate or $ANAPP_3$, has been found to be a specific P_{2X}-purinoreceptor antagonist (Hogaboom, et al., 1980). However, when tested in this thesis on the intact single cell contraction system, $ANAPP_3$ failed to inhibit the contraction, and in fact enhanced the response of the cells to exogenous ATP. Likewise, Reactive Blue 2 has been found to selectively inhibit responses mediated via the P_{2Y}-purinoceptor (Burnstock and Warland, 1987). However, Reactive Blue 2 functioned as a partial agonist in eliciting contraction when applied to intact fibroblasts in the experiments conducted here.

Therefore, although exogenous ATP probably elicits the contraction of intact fibroblasts via P_2-purinergic receptors, the process is not antagonised by the currently known specific antagonists of this class of receptor. Nevertheless, it now appears that further subclasses of P_2-purinoceptors are emerging. These have been designated as P_{2T}- and P_{2Z}-purinoceptors (Gordon, 1986), and additional subtypes are likely to be found. The field is rapidly growing in complexity and purinoceptors have been identified on a wide variety of cells and tissues, being responsible for a diverse range of biological functions.

The maximally effective concentration of ATP (15 mM) which elicited fibroblast contraction, as described in this thesis, would appear at first sight to be supra-physiological. Certainly this dose is at least an order of magnitude higher than that described as optimal at various purinoceptors (Burnstock and Warland, 1987). Nevertheless, doses of ATP upto 100 mM induced electrical membrane responses in fibroblasts (Okada, et al., 1984), and 50 mM ATP induced a maximum contraction of isolated guinea-pig bladder strips (Sjögren and Andersson, 1979). ATP and ADP are stored in dense granules in blood platelets. ATP is also ubiquitous in all cell types, with a cytoplasmic concentration greater than 5 mM (Gordon, 1986). The "platelet release reaction" involving degranulation occurs at injury sites (Mills, Robb and Roberts, 1968), usually where platelets aggregate, and thus the granule contents are released from platelets that are clustered together, rather than suspended uniformly in the blood. The rate of accumulation of

platelets during the initial formation of a haemostatic plug is considerable, and when these aggregated platelets degranulate the pericellular concentrations of ATP and ADP will be very high as their concentration within the storage granules is about 1 M (Gordon, 1986). ATP will also be released from other damaged cells in the wound. Consequently, high localised concentrations of ATP within a wound are possible, and these may impinge on fibroblasts in the immediate vicinity. Okada, et al., (1984), who found that exogenous ATP induced electrical membrane responses in fibroblasts, have also demonstrated that fibroblasts exhibit chemotactic activity to exogenous ATP (Okada, et al., 1983). They therefore hypothesized that ATP may be one of the regulatory mediators for the activation of fibroblast function. This thesis has demonstrated that in vitro, exogenous ATP is capable of eliciting fibroblast contraction. It remains to be shown whether this has any relevance in vivo.

In the single cell contractile assay described here it was shown that placing the fibroblasts in a calcium- and magnesium-free environment leads to their contraction. Replenishing either calcium or magnesium to the levels found in F10 medium or Dulbecco's phosphate-buffered saline leads to the stability of the cells. These observations are in keeping with the fact that a decrease in the extracellular calcium leads to an increase in the excitability of cell membranes. Conversely an increase in extracellular calcium stabilizes the membrane by decreasing excitability (Ganong, 1983). Magnesium, which like calcium, is a divalent cation, has a similiar effect on cell membranes. The contraction of cytoskeletons has similiarly been shown to be calcium sensitive (Masuda, et al., 1983). Moore and Pastan, (1979) have demonstrated that microsomal membranes isolated from cultured fibroblasts have an energy-dependant calcium uptake system, similiar to that of the sarcoplasmic reticulum of skeletal muscle. The system may function to sequester calcium within fibroblasts. Depolarization of the fibroblast cell membrane due to increased excitability in a calcium- and magnesium-free environment may release calcium from the endoplasmic reticulum, thus leading to excitation-contraction coupling. This indicates that inhibiting the movement of calcium across the cell membranes pharmacologically may be a useful way of inhibiting fibroblast contraction.

The single cell contractile assay suffers from none of the disadvantages of either cytoskeleton preparations or collagen gel contraction (See section 1.8.3.). Preparation of the cells is not necessary, other than washing them in phosphate-buffered saline. Thus no cells are lost, as occurs with cytoskeletons both in preparation and during the contraction, and there are no complications in terms of analysis of the results for missing cells. The fibroblasts are in a physiological environment since they are contracting in their normal tissue culture growth medium. Cells are surrounded by their plasmalemma and so they are susceptible to the effect of drugs which act via the cell membrane. Since the contraction is readily reversible and repeatable, the experimental set up is similiar to that of a tissue bath, and consequently allows for the testing of compounds that may inhibit the process. A disadvantage of the assay, as performed, is that analysis of the results is extremely labour intensive. This has limited to some extent the number of potential anticontractile compounds that were assessed in this thesis. The analysis could however be improved by the use of an automatic computerised image analyser for evaluating changes in cell area, rather than the semi-automatic system used in this thesis.

Intact human ocular fibroblasts were assessed in a few preliminary experiments and were noted to respond in a similiar manner to exogenous ATP to the rabbit fibroblasts described in this thesis. It was, however, decided to restrict the investigation to rabbit fibroblasts alone, both because of the extreme labour intensiveness of the analysis of the results produced by the contractile assay, and because any drugs shown to have anti-contractile activity would first be evaluated in rabbit models of glaucoma surgery before being assessed in human beings.

The response of cytoskeletons to ATP has been regarded as a contraction, and is thought to provide valuable information about the response of cells in vivo, despite the totally unphysiological nature of the experiments (Izzard and Izzard, 1975; Kreis and Birchmeier, 1980; Masuda, et al., 1983; Grierson, et al., 1986). Similiarly, the reduction in volume of cellular gels is regarded as evidence of cellular contraction (Bell, et al., 1979). However, it remains to be clarified how the directly visualised reduction in cell area, which has been called a contraction in this thesis, produced by the

exogenous application of ATP, resembles the behaviour of fibroblasts in vivo in bringing about the contraction of healing wounds. Drugs developed using this system, which are shown to inhibit fibroblast contraction in vivo, thus diminishing fibrosis, will ultimately vindicate the assay.

4.3. DRUG EFFECTS

4.3.1. Drugs with activity against the cytoskeleton

In this thesis taxol has been demonstrated to inhibit powerfully the migration of rabbit Tenon's capsule fibroblasts to both fibronectin and to aqueous humour with an optimal effect at 10^{-6}M. This drug also inhibited fibroblast contraction optimally at 10^{-5}M. Exposure to 10^{-6}M taxol for 24 hours markedly inhibited proliferation of the cells for the ensuing six days, with no evidence of toxicity, and with normal proliferation after this period. Taxol was found to "block" fibroblasts in mitosis and led to a slight loss of the normal spindle shape and polarity of some of the cells.

Taxol promotes microtubule assembly (Horwitz, et al., 1981) and induces the reorganization of the cytoskeleton into unusual microtubule arrays (Green and Goldman, 1983). However, it is unknown why this effect should compromise the contraction and the locomotion of fibroblasts. Taxol has been previously assessed in vitro, in relation to proliferative vitreoretinopathy (van Bockxmeer, et al., 1985; Verdoon, Renardel de Lavalette, Dalma-Weizhausz, et al., 1986). It was found to inhibit rabbit skin fibroblast migration to fibronectin, causing a maximal inhibition of 50% at $5x10^{-6}$M. It also inhibited contraction of collagen gels populated with skin fibroblasts, causing a maximal inhibition of 50% at 10^{-6}M. In chorioretinal fibroblast populated collagen gels, greater than 90% inhibition of contraction was observed with 10^{-6}M taxol (van Bockxmeer, et al., 1985). The results from the work in this thesis are broadly in agreement with the other studies, however, Tenon's capsule fibroblasts were used in the current investigation, which is appropriate to the study of filtration surgery. Since it is acknowledged that fibroblasts from different sites in the body may respond differently, (Freundlich, et al., 1986), caution must be exercised in extrapolating results from one cell type to another. The study by Verdoon, et al., (1986) has in addition only shown inhibition of fibroblast migration to fibronectin, which is at best only one of the chemoattractants active at the site of a trabeculectomy. Since aqueous humour is more powerfully chemoattractant to Tenon's capsule fibroblasts than fibronectin and may effect a different population of receptors, it is important to have demonstrated that taxol blocks cell migration to this more potent stimulus.

The current study also confirms the previously demonstrated powerful antiproliferative effects of taxol, but on cells appropriate to the study of filtration surgery. van Bockxmeer, et al., (1985) showed inhibition of rabbit chorioretinal fibroblast proliferation by 10^{-7}M taxol, while rabbit skin fibroblast proliferation was virtually totally inhibited at 10^{-6}M taxol (Verdoon, et al., 1986). In this thesis however, drug exposure was for 24 hours only, but the inhibition of cellular proliferation persisted for far longer than this. Taxol therefore has a "hit-and-run" effect (Laurence and Bennett, 1980).

Taxol has been identified as having antiproliferative effects, and a change in cell morphology has been seen in the current study. Cell morphology, cell proliferation, migration and attachment may all affect the contraction of collagen gels (Section 1.8.3.). It was therefore unclear from the previous studies where taxol was used to inhibit gel contraction, whether the observed inhibition of gel contraction was indeed due to an inhibition of cell contraction. The current study which employs an assay which appears to be more specific for cell contraction, confirms a direct anti-contractile effect of taxol.

van Bockxmeer, et al., (1985) have used taxol in vivo in a rabbit model of proliferative vitreoretinopathy. 2.3×10^{-5}M taxol was injected into the midvitreous in conjunction with an injection of heterologous chorioretinal fibroblasts. The drug significantly diminished the severity of retinal detachments. However, pale optic discs were observed in 25% of eyes receiving this dose of taxol, 2 months after administration. At a dose of 3×10^{-7}M taxol which appeared to be equally effective at inhibiting retinal detachment, no toxicity was observed.

Taxol is currently undergoing evaluation in the United States of America as a systemic agent for treating disseminated malignancies. When administered in a dose of 250 mg/m^2 to patients with metastatic malignant melanomas, partial tumour responses were seen in one third of patients treated (Wiernik, Schwartz, Einzig, et al., 1987). The main dose limiting side effect in some of the patients was neurotoxicity, although recoverable neutropenia occurred in all patients treated. The study recommended that taxol should be further

investigated in malignant melanoma at the above dosage. Doses of taxol that might be administered to the eye would be orders of magnitude less than 250 mg/m^2, so that systemic toxicity is unlikely to be a problem.

In the current study, while cytochalasin B strongly inhibited the migration of fibroblasts to both fibronectin and aqueous humour at an optimal dose of $10^{-5}M$, it did not inhibit cell contraction. The drug produced progressively more marked morphological effects on the fibroblasts at doses from 10^{-10} to $10^{-5}M$, leading to severe disruption of the normal cell architecture. Even at the higher doses of cytochalasin B when the cells had attained a highly arborised morphology, they were still capable of contracting when stimulated with exogenous ATP. The morphological changes produced by cytochalasin B were entirely reversible and cell multiplication after exposure to $10^{-5}M$ cytochalasin B for 24 hours was normal.

Cytochalasin B disrupts microfilaments (Britch and Allen, 1981), which appears to inhibit fibroblast locomotion. In spite of the fact that microfilaments consist principally of actin, which is believed to be essential for cell contraction, cytochalasin B treated fibroblasts are still capable of contracting when stimulated with ATP in the assay developed in this thesis. The implication is that either microfilaments are not needed for cell contraction, or that the assay is inappropriate and that the response of the cells to ATP is independent of the cytoskeletal contractile system. Croop and Holtzer, (1975) have also demonstrated a further contraction of fully "arborised" fibroblasts, initially treated with $10^{-5}M$ cytochalasin B and subsequently exposed to colcemid. The effects of cytochalasin B are extremely complicated and therefore the response seen in this thesis is not at variance with other reports in the literature.

The lack of an anti-contractile effect of cytochalasin B in vitro, as demonstrated in this thesis, substantiates the fact that in vivo the drug has been found to be inactive. Topical administration of $2.1x10^{-5}M$ cytochalasin B to skin wounds on the backs of New Zealand White rabbits failed to retard wound contraction or wound healing in two separate studies (Ehrlich, Grislis and Hunt, 1977; Rudolph, Hurn and Woodward, 1981).

The results in this thesis with regard to cytochalasin B are diametrically opposite to those reported by Verdoon, et al., (1986) who found no inhibition of rabbit skin fibroblast migration to fibronectin using cytochalasin B, but 50% inhibition of contraction of a fibroblast populated collagen gel by $8x10^{-7}M$ cytochalasin B. The difference in results may be explained by the fact that fibroblasts from different source tissues were used in the experiments, although it is hard to attribute the diametrically opposite results to this alone. Cytochalasin B has also been found to have marked antiproliferative effects (Verdoon, et al., 1986), which may partially account for an apparent "anticontractile" effect seen on collagen gels, particularly since the reduction in gel volume was measured after 48 hours of incubation, at which stage a marked effect on proliferation would be anticipated.

Further evidence in favour of an anticontractile effect of cytochalasin B was provided by Kirmani and Ryan, (1985), who evaluated in vitro, transvitreal membranes formed after penetrating ocular injury in rabbits. Cytochalasin B at $10^{-5}M$ was found to relax previously contracted membranes. However, it was not made clear whether this might not possibly be a non-specific toxic effect leading to the apparent relaxation of the membrane.

In the current investigation colchicine was found to inhibit fibroblast migration to both fibronectin and rabbit aqueous humour. Colchicine also inhibited fibroblast contraction. However, the optimal doses were higher than taxol or cytochalasin B, and the effect was less powerful. The highest dose tested was $10^{-3}M$. This did not appear to be toxic to the fibroblasts, and although after exposure to the cells for 24 hours effectively abolished multiplication, there was no reduction in the number of cells already present over a 14 day observation period.

Colchicine has been found in vitro to inhibit proliferation, migration and contraction of skin fibroblasts (Verdoon, et al., 1986), and to inhibit proliferation and contraction of chorioretinal fibroblasts (van Bockxmeer, Martin and Constable, 1985). Other in vitro studies have shown that it inhibits collagen secretion from cultured fibroblasts (Diegelmann and Peterkofsky, 1972). Colchicine has also been used in a variety of in vivo situations to reduce wound healing,

with varying success. An uncontrolled study using oral colchicine in conjunction with beta-aminoproprionitrile to inhibit skin keloid formation at reoperation, showed promising results (Peacock, 1981). With skin wounds in rats the dose of intramuscular colchicine necessary to inhibit wound healing was close to the dose inducing non-specific toxic effects (Chvapil, Peacock, Carlson, et al., 1980). In rabbit skin wounds colchicine did not inhibit wound contraction or healing, while in rats its apparent anti-contractile effects were thought to be due to toxic effects on local tissues (Rudolph, et al., 1981). As mentioned in section 1.7.6, colchicine has been used as a component of combination chemotherapy to control bleb fibrosis after implantation of Molteno tubes (Molteno, et al., 1976 and Molteno, 1980). However, unacceptable side effects from the combination therapy have limited its general application (Brown and Cairns, 1983). Nevertheless, oral colchicine at a dose of approximately 3.5 mg per day has been used in a model of proliferative vitreoretinopathy in rabbits and was found to reduce the incidence of tractional retinal detachment (Lemor, Yeo and Glaser, 1986). As a note of caution, however, as little as 1 ug of intravitreal colchicine has been reported to cause damage to photoreceptors and ganglion cells in Rhesus monkeys (Davidson, Green and Wong, 1983).

In summary, taxol, cytochalasin B and colchicine have been shown in this thesis to have activity in inhibiting aspects of fibroblast behaviour that are regarded as crucial to wound healing, ie. fibroblast proliferation, migration, and contraction. Taxol appears to have the greatest potency in this regard and when used intra-ocularly at doses shown in the current study to be effective, has a reasonable therapeutic index (van Bockxmeer, et al., 1985). Toxicity is likely to be even less of a problem if the drug is administered extra-ocularly in relation to glaucoma drainage surgery. Taxol is very poorly water soluble and single intravitreal doses have persisted for upto 2 months (van Bockxmeer, et al., 1985). A dose of taxol administered sub-conjunctivally is therefore likely to persist for a prolonged period at the site of drainage surgery. The drug appears to have a "hit and run" effect on cell proliferation, ie. a sustained response long after removal of the drug, which also indicates that frequent dosing is unlikely to be required.

Further investigation of colchicine, cytochalasin B and particularly of taxol, is indicated in animal models of glaucoma drainage surgery as they may be useful either singly or as part of combination chemotherapy, in improving the surgical success rate of trabeculectomies.

4.3.2. Drugs which increase intracellular cyclic-AMP levels

In the current investigation, attempts were made to increase the intracellular cAMP level with theophylline, dibutyryl cAMP and prostaglandin E_2. Theophylline was ineffective at inhibiting fibroblast migration, dibutyryl cAMP was moderately effective at inhibiting migration to fibronectin, but prostaglandin E_2 showed a dramatic response at 10^{-3}M. However, when aqueous humour was used as the chemoattractant, both dibutyryl cAMP and PGE_2, while still showing activity at inhibiting fibroblast migration, demonstrated reduced effectiveness. Nevertheless 10^{-3}M PGE_2 still reduced fibroblast migration by 50%. Being the most active of the three compounds, PGE_2 was tested in the contractile assay, but in spite of application to the cells for 24 hours before testing, failed to inhibit ATP-induced fibroblast contraction. Neither dibutyryl cAMP nor PGE_2 had discernable morphological effects on the cells, cell viability was not compromised, and cell proliferation after 24 hours exposure to the drugs was normal.

The lack of effectiveness of theophylline may be related to the relatively short duration of the migration assay, which lasts four hours. Since theophylline inhibits phosphodiesterase, the enzyme which breaks down cAMP, it may require longer than four hours for the intracellular cAMP level to build up to high enough levels to influence fibroblast behaviour. As has been mentioned previously, fibronectin is likely to be only one of the chemoattractants in aqueous humour, and different chemoattractants may act on different receptors on the cells. It is therefore not surprising that although dibutyryl cAMP and PGE_2 inhibited the migration of rabbit Tenon's fibroblasts to fibronectin, they were less effective when aqueous humour was used as the chemoattractant.

The lack of an anticontractile effect of increasing the intracellular cAMP level, as demonstrated in this thesis, is at variance with other studies in the literature which have employed PGE_2 or dibutyryl cAMP

to inhibit cell contraction in vitro. Ehrlich and Wyler, (1983) found that the addition of PGE_2 to fibroblast populated collagen lattices partially inhibited their contraction. The preincubation of normal human dermal fibroblasts for 24-30 hours with $2.8x10^{-5}M$ PGE_2 or 1 mM dibutyryl cAMP, before permeabilisation with glycerol, reduced ATP-induced contraction to less than 20% of control cytoskeletons (Ehrlich, Griswold and Rajaratnam, 1986).

Experimental in vivo studies using these compounds are very limited. The lack of an inhibitory effect on fibroblast contraction as demonstrated here, may help to explain why dibutyryl cAMP was ineffective in vivo. Ehrlich, et al., (1977), applied $10^{-3}M$ dibutyryl cAMP to skin wounds on the backs of New Zealand White rabbits. Unfortunately, no inhibition of wound contraction or wound healing was shown when compared to untreated controls. A similiar lack of effectiveness, using the same dose of dibutyryl cAMP, was demonstrated by Rudolph, et al., (1981) in the same experimental model. Although these studies are not encouraging, only a single dose of the drug has been evaluated in a single species. Further investigation in other models of wound healing may provide more optimistic results.

These drugs which increase intracellular cAMP, have properties, as demonstrated in this thesis and as documented in the literature, which appear to give them potential for reducing wound healing in vivo. It therefore seems worthwhile to evaluate dibutyryl cAMP and PGE_2 in animal models of glaucoma surgery in the hope of reducing wound fibrosis.

4.3.3. Calmodulin antagonist

Trifluoperazine proved to be a powerful inhibitor of both fibroblast migration to aqueous humour, and of fibroblast contraction. However, the drug was highly toxic and at doses shown to be "therapeutically" active both in this and in other studies, resulted in 100% cell death. Extensive damage to trifluoperazine treated cells was shown by scanning electron microscopy, which accords with one of the known functions of calmodulin, which is cell membrane phosphorylation. Cell death was clearly evident in the single cell contractile assay as developed in this thesis, which demonstrates the probable superiority of this assay which utilises intact cells, over cytoskeleton contractile assays. Since the cells are already dead in the latter

assays and have a large proportion of their cell membrane removed, the toxicity of drugs like trifluoperazine will be missed.

Masuda, Owaribe, Hayashi, et al., (1984), found that calmodulin antagonists would inhibit the ATP-induced contraction of human fibroblast cytoskeletons prepared by Triton X-100. This led them to conclude that contraction in non-muscle cells was regulated by a Ca^{2+}-calmodulin-dependant mechanism, similiar to that occurring in smooth muscle cells. Similiarly, Ehrlich, et al., (1986), found that trifluoperazine at 10^{-5}M inhibited the ATP-induced contraction of glycerinated human skin fibroblasts. van Bockxmeer, Martin and Constable, (1985), found that 10^{-4}M trifluoperazine completely abolished chorioretinal fibroblast proliferation, while the contraction of chorioretinal fibroblast populated collagen gels was totally inhibited at $3.3x10^{-5}$M trifluoperazine. Cell viability was not compromised by these doses of the drug. Consequently, in an effort to inhibit proliferative vitreoretinopathy, trifluoperazine at doses ranging from 10^{-4} to 10^{-3}M was administered intravitreally to rabbit eyes simultaneously with an injection of chorioretinal fibroblasts. Trifluoperazine resulted in cataract formation in all eyes injected (van Bockxmeer, et al., 1985). It seems surprising that in vitro toxicity of trifluoperazine was not demonstrated in van Bockxmeer's study, particularly in view of the severe toxicity shown in vivo.

In summary, while trifluoperazine may have activity as an inhibitor of cell migration, cell proliferation and cell contraction, the diverse functions of calmodulin make interfering with its actions using trifluoperazine impossible at the present time without totally unacceptable side effects.

4.3.4. Calcium antagonist

Unfortunately, when assessed in the migration assay described above, nifedipine failed to have any effect on fibroblast migration over the dose range 10^{-11} to 10^{-3}M. It was far easier to screen compounds over a wide dose range in the migration assay than in the single cell contractile assay. Due to the lack of activity in inhibiting fibroblast migration, nifedipine was therefore not assessed in the contractile assay.

As has previously been mentioned, cell migration depends on contraction of the cytoskeletal actin-myosin system of the cell. The process is calcium dependant. The lack of activity of a calcium antagonist in inhibiting fibroblast migration is therefore surprising, particularly in view of a number of findings in the literature. The application of nifedipine to fibroblasts was found to block the ATP-induced rise in intracellular calcium (Oiki, et al., 1985). Likewise, nifedipine reduced the contraction elicited by the application of exogenous ATP to isolated guinea-pig bladder strips in vitro and to intact guinea-pig bladders in vivo (Sjogren and Andersson, 1979). In addition, Kirmani and Ryan, (1985) found that diltiazem hydrochloride, which is another calcium antagonist, would relax previously contracted fibrocellular intravitreal membranes, in vitro.

New calcium antagonists, with predelictions for various sites of action, are continually being developed. In view of the fact that there are good theoretical reasons why these drugs should interfere with actin-myosin contractile systems in fibroblasts, further work assessing some of these in vitro is indicated.

4.3.5. Cholinergic antagonist

Ipratropium bromide was ineffective in this thesis at inhibiting fibroblast migration over the dose range 10^{-9} to 10^{-4}M. Further experimentation using ipratropium bromide as a contraction inhibitor was not undertaken.

This lack of response implies that cholinergic receptors are unlikely to be implicated in the processes involving activity of the actin-myosin contractile system of fibroblasts. The findings above support the observations of Kirmani and Ryan, (1985) who found that acetylcholine was ineffective at eliciting the contraction of intravitreal fibrocellular membranes in vitro.

4.3.6. $Beta_2$-adrenoreceptor stimulants

Both ritodrine hydrochloride and salbutamol inhibited the migration of rabbit Tenon's capsule fibroblasts to fibronectin. However, when aqueous humour was used as the chemoattractant, salbutamol was no longer effective at reducing fibroblast migration, whereas ritodrine still reduced fibroblast migration by 83% at the maximum dose tested of 6.18×10^{-3}M. At a slightly lower dose of ritodrine, however, the

inhibition was not maintained and fibroblast migration was reduced by an insignificant 8%.

Since ritodrine was the most effective of the two sympathomimetics at inhibiting fibroblast migration, it was assessed in the contractile assay. Contrary to expectation, ritodrine appeared to elicit a contraction of the cells in its own right, prior to the application of ATP. This contraction was itself dose dependant.

Cell viability after 4 hours exposure to the dose of ritodrine that optimally inhibited fibroblast migration, was not compromised. However, marked vacuolation of the cytoplasm was evident at this time, and after 24 hours of exposure to the drug many cells had detached from the culture flask, and proliferation of the few remaining cells virtually ceased. Thus ritodrine hydrochloride appears to be toxic to fibroblasts after prolonged exposure.

The contraction of fibroblasts elicited by ritodrine hydrochloride in this thesis, is in accordance with the findings of Kirmani and Ryan, (1985) on intravitreal fibrocellular membranes in vitro. Approximately 90% of 200 membranes assessed contracted in response to noradrenaline. The contraction could be blocked by phentolamine, which is an alpha-adrenoceptor blocker. This suggested to Kirmani and Ryan that there were adrenergic receptors on fibrocellular intravitreal membranes. Since fibroblasts are a component of the experimental intravitreal membranes in Kirmani and Ryan's study, it is likely that the adrenergic receptors are on these cells in view of the contractile effect of ritodrine hydrochloride on fibroblasts, as demonstrated above.

Thus, although ritodrine hydrochloride may usefully inhibit fibroblast migration and may provide interesting insights into fibroblast locomotion, further investigation of this drug in vitro is obviously indicated before its use can be contemplated in experimental animals undergoing glaucoma drainage surgery. In addition further investigation of adrenoceptor antagonists should be undertaken as these may inhibit fibroblast contraction.

4.3.7. Smooth muscle antagonist

When tested in the whole cell contractile assay described in this

thesis, trocinate did not inhibit ATP-induced fibroblast contraction, and in fact appeared to enhance the effect of ATP. Further testing of this drug in the migration assay was not performed as it was thought unlikely to be useful.

The lack of activity of trocinate, which is a directly acting smooth muscle antagonist, and was shown by Madden, et al., (1974) to inhibit the contraction of skin wounds in rabbits, calls into question the concept of the "myofibroblast". Ehrlich, (1988) felt that there was little evidence to support the role of the myofibroblast in contractile processes, and that instead contraction was generated by fibroblasts producing tractional forces, as described by Harris, et al., (1980). The exact mechanism of fibroblast contraction has not yet been resolved, but certainly in the context of the experiments conducted in this thesis, a drug which inhibits smooth muscle contraction did not inhibit fibroblast contraction. Clearly, further work on the basic physiology of fibroblast contraction and locomotion is required.

4.3.8. Retinoids

The effects of the retinoids evaluated in the migration assay were contrary to expectation. Retinol was found to enhance rather than to inhibit both the random background migration of rabbit Tenon's capsule fibroblasts, as well as the migration to fibronectin. Retinoic acid merely increased the background random migration of the cells. This work suggests that in spite of some evidence to the contrary in the literature, topical retinoids would facilitate healing at the site of a trabeculectomy, which is clearly opposite to the desired response. This may be in keeping with the fact that retinoic acid has been found to expedite the healing of experimental corneal wounds (Ubels, Edelhauser and Austin, 1983).

The interesting effect of retinoids, as demonstrated in this thesis, illustrates the importance of using cells in assays that are appropriate to the site at which wound healing is to be studied. Thus, it is not surprising that the foetal dermal fibroblasts used in the studies of Hein, et al., (1984), respond in a different way to the mature Tenon's capsule fibroblasts used in the current investigation.

4.4. IMPLICATIONS FOR GLAUCOMA SURGERY

There is a pressing need to improve the success rate of the trabeculectomy. It is now generally accepted that of the trabeculectomies that fail, most do so because of the development of fibrosis at the operation site. Since surgical modifications of the original technique developed by Cairns, (1968) have not achieved the objective of improving the surgical success rate, it is likely that only pharmacological interference with wound healing will diminish fibrosis, thereby hopefully improving the results of surgery.

The most popular anti-fibrotic treatment at the present time, employed in the context of trabeculectomies, is 5-fluorouracil. In spite of its increasing use in the United States of America, however, it is very seldom advocated in the United Kingdom. No conclusion as to its efficacy has yet emerged from controlled clinical trials. Nevertheless, what is clear is that there are problems with administration of the drug, and there are potentially serious side effects associated with its use. It is unlikely to be a panacea for all glaucoma patients facing surgery, and even if it is shown to be of undoubted efficacy, the risk-benefit ratio will mean that it can only be administered in cases where there is a strong chance of failure of orthodox surgery. Less toxic and more efficacious drug treatments are urgently needed.

In addition, there is no way at the present time of identifying a particular patient who is likely to encounter a failure of a conventional trabeculectomy and who might benefit from adjuvant anti-fibrotic treatment. Certainly, groups of patients at high risk of surgical failure have been identified, but even within these groups success will be achieved in a proportion of patients with conventional surgery alone. Treatment of such patients with a potentially toxic drug with a highly unpleasant mode of administration, eg. sub-conjunctival injection for 5-fluorouracil, is undesirable. Some sort of predictive test to identify particular high risk patients would be beneficial.

In this thesis it has been demonstrated that aqueous humour is chemotactic for ocular fibroblasts. Moreover, it has been shown that the chemotactic nature of a patient's aqueous humour may determine

their propensity to sustain a surgical failure. The information that differences in the aqueous humour of various patients with glaucoma may determine their response to treatment, joins the growing body of data which indicate that changes in the aqueous humour may have aetiological implications for primary open angle glaucoma.

Detecting increased chemotactic activity in a patient's aqueous humour may form the basis of a predictive test to detect patients at high risk of surgical failure who might consequently benefit from drug treatment. The chemotactic assay as described in this thesis could be used for this purpose. However, there are considerable problems in overcoming the day-to day variability of the assay, thus chemotactic activity cannot as yet be calibrated against a fixed standard. Further investigation may resolve this problem. Once the chemotactic substances in aqueous humour have been identified, biochemical measurement of their levels may allow a more standardised estimation of the risk of surgical failure to be accomplished. In addition, once these chemotactic substances have been positively identified, drugs to inactivate them could be investigated.

The chemoattractant and the single cell contraction assays developed here provide a means to test drugs with potential anti-fibrotic activity. These in vitro systems used for screening compounds have considerable advantages over in vivo screening of drugs. A large number of drugs with various modes of action have been assessed over a wide range of dose levels. There seemed good theoretical reasons why many of these compounds should interfere with fibroblast function. In practice, only the drugs which affect the cytoskeleton of cells viz. taxol, cytochalasin B and colchicine, and drugs which increase the intracellular cyclic-AMP levels, viz. dibutyryl cyclic-AMP and prostaglandin E_2, had potentially useful activity. Other classes of drugs have been identified where further investigation is indicated. In addition, other drugs that are clearly not useful have been defined. It would have taken a great many experimental animals, numerous glaucoma operations, and a huge sum of money to have obtained the same result in vivo.

However, it must be emphasised that these in vitro tests cannot replace in vivo assessment. Any drug with potential activity identified in these assays will still have to undergo extensive

testing in experimental animals before being applied to man. Nevertheless, the in vitro systems allow a far greater number of compounds to be assessed more cheaply and more humanely than could be done with in vivo systems.

A major objection of in vitro systems generally, and tissue culture specifically is to determine their relevance in vivo. For the two assay systems described it is not possible at the present stage to establish this. The validity of the in vitro work will only be verified retrospectively if drugs tested in these systems are ultimately shown to improve the success rate of trabeculectomies. In addition, only fibroblast migration and contractile assays were employed. It is possible that additional assays, eg. proliferation, may have shown a positive response for some of the drugs that were regarded as ineffective, however, this is unlikely based on the known mode of action of these drugs. A similiar criticism could be levelled against the use of rabbit cells as opposed to human cells in the drug assays described in this thesis. It was felt that it was justified to use rabbit cells because any compound with potentially useful activity would first need to be assessed in rabbit or some other cheap experimental animal model of glaucoma surgery before being used in humans. Some drugs may prove ineffective in experimental animals but be active in human beings. This is certainly a potential problem of all experiments where animals are involved, but there is no easy solution to this.

It must also be born in mind that wound healing is an exceedingly complicated process. Only a small aspect of the problem of fibrosis has been addressed in this thesis. It may well transpire that the inhibition of fibroblasts themselves is too late in the process to inhibit wound healing in any clinically significant way. It may be more logical to attempt to inhibit the numerous inflammatory mediators initiated by the original surgical injury. This will necessitate the elucidation of the mechanism of acute inflammation, which at present is incompletely understood. Once all the inflammatory mediators have been identified, attempts to inhibit them could be performed in tissue culture.

Taxol, cytochalasin B, colchicine, dibutyryl cyclic-AMP and prostaglandin E_2 have been demonstrated to have activity as

anti-fibroblast agents in the assays conducted in this thesis. All five of these drugs seem worth investigating further in animals. However, taxol appears to be the most promising compound and is currently being assessed in a rabbit model of glaucoma surgery and in dogs undergoing similiar procedures, as a direct consequence of the in vitro data presented here. If taxol is effective in improving bleb survival in rabbit or in dog eyes, with a reasonable therapeutic index, it may ultimately come to be used in man to prevent fibrosis at the site of trabeculectomies.

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