STUDIES ON WOUND HEALING OF THE CORNEA

AND ON VASCULARISATION IN THE EYE

by

Fergus William Campbell

Institute of Physiology, The University

of Glasgow.

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

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P R E F A C E

After a century of increasingly intensive research there is still much doubt and controversy about the defensive response of the cornea of the eye to injury. This thesis is an account of the work carried out by me, over the past three years, to try to add some few facts to the physiology and pathology of corneal repair and of new vessel formation in the eye.

Firm in the belief that 'Die Methode ist alles', I evolved a relatively simple technique of inflicting standard heat injuries to the cornea, and utilised a quick and reliable method of following the course of wound healing. This method has been applied to a number of different but closely related problems.

The approach to the work here presented has been basically experimental rather than histological. Although much material has been examined histologically further work will be necessary to extract its full value. I have therefore only included histological results when they form an essential step in the evolution of a subsequent discussion. I hope to extend these histological observations at a future date.

The/

The work in Part Three of this thesis, on the development of the retinal vessels, was the natural result of personal contact with the studies of Professor A.J. Ballantyne and Doctor I.C. Michaelson on the pathology and comparative anatomy of the retinal vascular system. I am indebted to them for many stimulating ideas.

In reviewing the relevant literature I have, where possible, stressed the more recent trends of research which are not included in current textbooks. Only in this way have I been able to keep a reasonable balance between the presentation of original work and of literary research.

PART ONE

CORNEAL WOUND HEALING

Chapter I.

A REVIEW of the ANATOMY and PHYSIOLOGY of the CORNEA

The first part of this chapter is devoted to a brief description of the structure of the cornea in man, rabbit and guinea-pig. The purpose of the review on the physiology of cornea is to form a background against which to view the work presented im this thesis. A more detailed discussion of the relevant references is undertaken in each chapter.

The STRUCTURE of CORNEA

The cornea might be defined as that portion of the fibrous tissue of the eye which is transparent in the normal adult. Although the cornea is only 0.5 mm. thick in the human eye (Maurice and Giardini, 1951), it presents a well defined structure of remarkable strength. On microscopic section five layers may be readily distinguished.

1. Epithelial Layer.

Developmentally this layer is regarded as a continuation of the conjunctiva forwards over the cornea. The epithelium may be classified as stratified, pavement type. It consists of 5 or 6 layers of cells which can be/

be considered under three groups:-

(a) The BASAL CELLS are arranged in a single layer on Bowman's membrane. Salzmann (1912) described them as having flat bases with rounded heads. They fit together like a palisade.

(b) The WING CELLS are polyhedral in shape, and are arranged in 3 or 4 layers. To allow a close fit, these cells all have a convex anterior surface and a concave posterior surface. Their oval nuclei lie parallel to the surface. The more superficial cells are flattened.

(c) The SURFACE CELLS are arranged in 1 - 3 layers and lie superficially. Unlike the skin they never lose their nuclei or show keratinisation.

2. Bowman's Membrane. (Anterior elastic membrane).

This is a transparent, clear cut membrane about 10 μ thick, lying between the epithelium and the substantia propria (Bowman, 1847). It is probably a modified portion of the stroma. Peripherally it ends abruptly at the limbus in a rounded border. It does not regenerate when once destroyed. This membrane is well-marked in the human and guinea-pig eye, but it is not present in the rabbit eye (Fig.1).

3. The Substantia Propria. (Corneal stroma).

This/

This layer constitutes over 90% of the entire cornea and is composed of modified connective tissue lamellae, and cells. These fibres run the entire length of the cornea parallel to the surface but criss-crossing with each other in alternate layers.

In addition to these fibres the nuclei of the CORNEAL CORPUSCLES and WANDERING CELLS may be clearly distinguished on histological examination. The corpuscles or fixed cells were first described by Toynbee (1841). They appear to be ordinary connective tissue cells, which are deformed by the lamellar structure of the corneal stroma. Each consists of a flattened cell with a flattened nucleus and each has branching processes which intercommunicate with neighbouring cells to form an intimate syncytium.

The wandering cells are deformed leucocytes (Recklinghausen, 1860), which probably enter the cornea from the limbal vascular plexus. In the rabbit and guinea-pig they often possess eosin staining granules. The wandering cells increase enormously in number after injury or infection of the cornea.

4. Descemet's Membrane. (Posterior elastic membrane)

Duddell/

Duddell (1729) first referred to this structure, and it was later described in more detail by Descemet in 1758.

It is about 6μ thick and composed of elastic tissue. It is remarkably strong and resistant to inflammatory processes. It is structureless, but clearly defined and readily separable from the corneal stroma and the endothelium. Unlike Bowman's membrane it can regenerate after destruction by injury or disease.

5. The Endothelium

The endothelium is a single layer of flattened hexagonal cells attached to Descemet's membrane. It forms a layer over the whole posterior surface of the cornea and it is continuous round the angle of the anterior chamber with the cells on front of the iris. This membrane may be seen with the slit-lamp as a yellowish mosaic of six-sided cells.

The Limbus Corneae (Corneosclerotic junction)

At the periphery of the cornea there is a 1 mm. wide transitional zone between the cornea on the one hand/

hand and conjunctiva, episcleral tissue and sclera on the other. Bowman's membrane stops abruptly at this point and Descemet's membrane tapers off to form part of the ligamentum pectinatum iridis.

The epithelial layer becomes many layers thick at the limbus. The basal layers of the epithelium may be heavily pigmented in the rabbit and guinea-pig.

The limbal region of the cornea is richly supplied with blood vessels - the SUPERFICIAL MARGINAL PLEXUS (Leber, 1903). These vessels lie in the superficial layer of the substantia propria at the limbus. Here the capillaries of the plexus form a series of arcades which project a minute distance into the cornea proper. The limbal plexus anastomoses with the conjunctival vessels, the plexus of the capsule of Tenon and the episcleral and intrascleral plexuses (Leber, 1903).

The PHYSIOLOGY of the CORNEA

The remarkable transparency and avascularity of the cornea has attracted the curiosity of numerous physiologists and biochemists. Many are the problems that/

that have to be solved. How do the cells of the cornea respire so far removed from the nearest blood vessel? What conditions are necessary to maintain this avascular structure in a healthy state? Why does the cornea vascularise so readily in response to trauma? Is there a fluid circulation within the cornea? These are but a few of the questions to which an answer has been sought. In spite of much active research many problems remain only incompletely answered.

The Metabolic Requirements of the Cornea.

As yet comparatively little is known about the respiratory exchange of the cornea and many of the statements published are contradictory. The first investigators believed that the cornea obtained its oxygen from the surrounding atmosphere via the epithelium. As early as 1899, Bullot and Lor transplanted freshly enucleated eyes of rabbits into the peritoneal cavity of the live animal. They noted that the cornea became opaque after a few hours. However, if the corneal epithelium was first removed the transplanted cornea remained clear. The opacity, which/

which occurred under any area with an intact epithelium, was due to oedema of the endothelium. Similar results were obtained if the cornea of an enucleated eye was placed in a moist chamber containing a low percentage of oxygen. This loss of transparency always occurred in an environment of hydrogen whether the epithelium was intact or not. These workers concluded that the endothelium requires oxygen and that the epithelium resists the passage of this gas to the deep layers of the cornea. Sufficient oxygen reaches the endothelium only if the eye is in contact with the comparatively high oxygen content of the atmosphere or of the aqueous humour.

Fischer (1930) continued these experiments and investigated directly the gas exchanged between the cornea and the atmosphere in the rabbit. A small glass chamber, which fitted snugly over the cornea, was filled with known concentrations of gas. Later, the gas mixture was removed for analysis. He confirmed in vivo the finding of Bullot and Lor (1899) that the presence of an intact epithelium decreased the/

the rate of oxygen transfer. If the atmosphere were replaced with 100% CO_2 endothelial changes with opacity formation resulted. No loss of transparency occurred with pure hydrogen. Fischer believed that these changes with 100% CO_2 were due to the reduced rate of diffusion of carbon dioxide from the endothelium. From these findings he argued that normally there was a passage of oxygen from the atmosphere inwards and a transfer of carbon dioxide from the cornea outwards into the atmosphere. Both Bullot (1904) and Fischer (1930) maintained that the endothelium was the important structure in the cornea and that the oxygen was mainly required for this tissue.

Fischer's results were confirmed by Redslob and Trembley (1933). These workers found that the carbon dioxide elimination by a rabbit's cornea was between 0.013 to 0.05 c.c. per day per cornea, and by the human cornea 0.053 c.c. per day.

Much of this work must be viewed with suspicion for the conditions of the experiments were far from physiological. These workers, for example, have ignored/

ignored the possible role of the blood vessels at the corneosclerotic junction. The chamber attached to the cornea in the subluxated eye would almost certainly interfere with the limbal circulation.

Bakker, (1947), who had previously found carbonic anhydrase in the cornea and believed it to be there to assist the removal of carbon dioxide (Bakker, 1941), has repeated Fischer's experiments under more satisfactory conditions. He placed rats in an air-tight chamber into which any desired gas mixture could be introduced. The animals were kept alive by means of artificial respiration through a canula inserted into the trachea from outside the box. The animals were under general anaesthesia and their lids were stitched open to allow contact between the eye and the gas mixture. He found that the complete absence of oxygen did not produce macroscopic or microscopic changes in the cornea even after 12 hours observation, and he concluded that the cornea did not obtain appreciable quantities of oxygen from the atmosphere. Moreover, he pointed out/

out that a person asleep did not experience corneal changes even although the cornea was cut off from atmospheric oxygen. It should not be forgotten, however, that there may well be a reasonably high oxygen content in the closed conjunctival sac due to diffusion from the very rich vascular bed of the palpebral conjunctiva. Bakker confirmed the finding of Fischer, (1930) that the cornea became opaque in an atmosphere of pure carbon dioxide, but he demonstrated that a mixture of 8% carbon dioxide and 92% nitrogen did not cause any visible change in the cornea. As 8% carbon dioxide is well above the normal level found in the tissues it is unlikely that any diffusion of carbon dioxide from the cornea to the environment would take place. It is quite possible that, in Fischer's experiments, the profound changes in transparency that occurred with the high (100%) concentration of CO_2 were due to excessive alterations in the pH of the corneal tissues. The pH of a saturated aqueous solution of CO_2 at N.T.P. is 3.8.

The application of the Barcroft-Warburg manometric technique has permitted much more accurate estimations/

estimations of the metabolic requirements of excised corneal tissue. Kohra (1935) was one of the first workers to apply this technique to the cornea. He found that in the rabbit's cornea the epithelium consumed almost as much oxygen as the endothelium, while the stroma had only a very small oxygen requirement. The fluid in which the excised cornea was placed influenced Kohra's results. The respiratory uptake of the whole cornea or of the separate layers was greater in aqueous humour than in Ringer's solution. The nature of the medium did not influence the results during anaerobic glycolysis.

Gundersem (1938, 1939) considered that the oxygen consumption of cornea was predominantly due to the active epithelium.

The metabolism of the rat's cornea was further investigated by Bessey (1939) using the Barcroft-Warburg technique, and he found that the oxygen consumption was about 4 cu.mm. of oxygen per mg. of tissue per hour.

Kohra's work was criticised by Orzalesi (1939), who measured the respiration of human, rabbit and rat corneae/

corneae. He maintained that estimation of the metabolsim of endothelium could only be very approximate because of the inevitable trauma caused while removing the endothelium. It is possible, however, to obtain fairly accurate estimations of endothelial metabolism by measuring the oxygen uptake after the epithelium has been removed. Most of this uptake is due to the endothelium alone, as the stroma has only a very small requirement.

Lee and Hart, (1944) found that the oxygen consumption per hour for the whole rat cornea was 2.24 cu.mm., for the epithelium 1.16 cu.mm., and for the stroma 0.44 cu.mm. The respiration of the endothelium was too small to be measured. They considered that the trauma involved in the removal of the epithelium from the cornea reduced its oxygen consumption and so accounted for the discrepancy between the consumption for the individual parts of the cornea compared with the total intact cornea.

These workers also found that the metabolic activity of vitamin A deficient cornea was higher than normal, and that this was due to an increase in consumption by the epithelium. This result could be due/

due to the increased respiration of the hyperplastic epithelium. Moreover, they found that the respiratory intake of oxygen for riboflavin deficient rats' corneae was the same as for normal controls. On closer examination, however, it seems that in the early stages of riboflavin deficiency the metabolism of the epithelium is markedly depressed, but the metabolism of the substantia propria is greater, possibly because of the cellular infiltration. Thus the nett respiratory uptake for the complete cornea is normal.

The results of Simoyama, (1941) also suggest that cellular infiltration of the cornea increases its rate of metabolism. He studied the corneal metabolism during the healing of perforating wounds in rabbits, using Warburg's glycolytic quotient. Initially it decreased, probably due to the immediate effect of injury, but thereafter it steadily increased to a maximum at a time when the mitotic figures and proliferation of keratoblasts were most abundant histologically. Thereafter the quotient decreased steadily towards a normal level.

Robbie/

Robbie, Leinfelder and Duane (1947) utilised the technique of cyanide inhibition (Robbie, 1946) to determine how much of the normal respiration may be dependent upon an iron containing enzyme complex. They found that the respiration of the cornea was almost completely inhibited (90%) by 10^{-3}M. HCN indicating that most of the oxygen consumption was mediated by a cytochrome-cytochromeoxidase system. A 10^{-4} M. cyanide produced about 50% inhibition. Recovery from the cyanide exposure was rapid. Most of the oxygen consumption of the intact cornea was due to the epithelial layer. The stroma had very low oxygen consumption indeed. They considered that about 7% of corneal metabolism may be associated with a riboflavin containing carrier.

The possible danger of mustard gas injuries to the eyes during the second World War stimulated a considerable amount of research both in Britain and in America on corneal metabolism and the experimental pathology of mustard gas injuries. Much of the American work was published in 1948 in the Bulletin of the Johns Hopkins Hospital. Herrmann and Hickman (1948)/

(1948) found that the uptake of oxygen for the excised whole beef cornea averaged 70 cu.mm. per hour. In corneae which had been denuded of epithelium, however, the oxygen uptake was reduced to about 10% of this value. Furthermore, if the endothelium was then removed, less than 3% of the original oxygen consumption remained. If this figure is corrected for the autooxidation found in boiled corneal stroma, then less than 2% of the respiratory uptake can be attributed to the stroma.

The stroma of the cornea contains approximately one third of the corneal cells. About 5% of the stromal cells are wandering cells, and Friedenwald and Buschke (1948) found that these wandering cells almost certainly have an aerobic metabolism. If the oxygen consumption per wandering cell is equal to that of the cells in the epithelium or endothelium, then the total oxygen uptake of the stroma could be accounted for. One could conclude that the keratocytes have an anaerobic metabolism.

Friedenwald (1948), in his summary of the results of the wartime investigations, states that the/

the carbohydrate metabolism of corneal epithelium is very similar to that found in other body tissues. Its oxygen uptake is cyanide sensitive and is thought to utilise cytochrome oxidase. During metabolic studies it was found that, providing an adequate supply of carbohydrate was available, the oxygen uptake equalled that required for complete combustion of the carbohydrate consumed. However, under anaerobic conditions lactate is produced proportionate to the loss of glucose and glycogen. If adequate supplies of carbohydrate are not available the oxygen uptake may still remain at a normal level or even rise. No increase in non-protein nitrogen is found, and it is suggested that the alternative metabolites are probably fats.

The stroma, however, presents a very different picture. It has a negligible oxygen uptake which is probably required only by the aerobic wandering cells. However, the stroma does utilise glucose at a rate per cell about twice that of the epithelium. This is metabolised to a lactate by the stroma and no further. In the isolated cornea, the/

the stromal lactate can be utilised by the epithelium and indeed, constitutes about 25% of the total carbohydrate supply to the epithelium.

The epithelial consumption of stromal lactate can be markedly inhibited by exposing the cornea to small concentrations of mustard gas. It is interesting to note that this inhibition occurs even when the epithelium is still able to consume its endogenous stores of lactate. It is deduced from this finding that the epithelium must possess a special mechanism for the utilisation and transfer of the stromal lactate. There must be a special hydrogen transport system for the transfer of the lactate which is independent of the mechanism for metabolising the endogenous lactate of the epithelium.

Further study of the boundary between the epithelium and stroma will be required before a complete understanding of this relationship can be obtained.

After reviewing the relevant literature, de Roetth (1950) concluded that no work so far completed definitely demonstrated that the excised cornea/

cornea utilises atmospheric oxygen. He therefore placed fresh excised rabbit, cat and beef corneae in Warburg flasks dry, i.e., without any fluid in the main compartment of the vessel, and with the epithelial surface of the corneae facing upwards exposed to the air in the flask. He found that the cornea can utilise atmospheric oxygen directly. The oxygen uptake for cat, rabbit and beef corneae was 0.34, 0.74 and 0.62$\mu l\, O_2$/mg. dry weight/hour respectively. The average R.Q. for 48 beef corneae including the tissue-bound CO_2 was 1.00. He also noted that the excised bovine corneae maintained a steady rate of respiratory activity in the Warburg flasks regardless of the pH of the suspending medium, the gas phase being either 100% oxygen or air.

Langham, (1951) has recently confirmed in vivo that the cornea of the rabbit may utilise oxygen directly from the air. His findings also support the belief that some oxygen reaches the cornea from the conjunctival sac even when the lids are closed (personal communication). This supply may come from the rich vascular bed of the palpebral conjunctiva.

It is of interest to note that recent investigators, Herrmann et al (1942), Lee et al (1944), Robbie et al (1947) and de Roetth (1950), agree fairly closely on the oxygen requirements of the intact cornea. On the other hand the earlier investigators reported widely differing results - Kohra (1935), Bessey (1939), Orzalesi (1939) and Fischer (1930). This is almost certainly due to imperfections in the earlier manometric technique.

Source of Nourishment

From a consideration of the anatomy of the cornea it is obvious that nourishment can only be obtained from three sources, firstly from the anterior chamber through the endothelium or secondly directly from the capillaries of the limbus, or finally through the epithelium from the lachrymal fluid.

As early as 1852, Coccius noted that the cornea remained healthy after the aqueous had been replaced by air. Furthermore, Gruber (1894) found that the cornea remained transparent after all the limbal/

limbal vessels had been occluded. It was therefore established early that the cornea had at least a dual source of nourishment either from the limbal capillaries or from the aqueous. Either of these sources was sufficient to maintain an apparently healthy cornea.

These findings were further substantiated by Laquer (1872) and Gruber (1894) who observed that 'rust spots' in the cornea became blue after potassium ferricyanide had been injected into the blood stream or directly into the anterior chamber. A detailed examination of the more recent literature however, discloses many contradictions, and it is difficult to trace a coherent picture of the fluid exchange. Leber (1903), for example, attributed the main role to the limbal vessels and suggested they were responsible for nourishing the periphery of the cornea, although they may leave the centre in a precarious position. He did not, however, supply any experimental evidence to support this view. Wessley (1905), on the other hand, thought that the chief source of nutrition came from the aqueous/

aqueous. Rollet (1936) placed the main emphasis on the tears. Terry (1939) admitted the three sources of nutrition.

It is undoubtedly true that all of these sources contain the substances required by the cornea, i.e., oxygen and glucose. Weekers (1940) considered that there was insufficient evidence to assess accurately the importance of each source in the normal metabolism of the cornea.

Since 1940 rapid advances have been made in the preparation of radioactive isotopes and in their use in research. Here is an ideal method for the study of the route and rate of penetration of physiological substances into the cornea of the intact animal.

Sallman, Evans and Dillon (1949) using ^{24}Na have studied the topographical relation of certain tracer atoms in the eye by means of radioautography. Their investigations did not provide, however, convincing evidence of the penetration of the ^{24}Na into the cornea because of the accumulation of the tracer in the aqueous humour (Sallman, 1950; personal communication).

Palm/

Palm (1948) made an extensive study of the phosphate exchange between the blood and the eye. He also published a number of radioautographs of the distribution of ^{32}P in the guinea-pig eye (Palm, 1949). In these records the cornea always appeared as a clearly defined arc. It was not possible, however, to detect any regular variation in darkening between the different parts of the cornea (Palm 1950; personal communication).

Potts and Johnson (1950) report interesting results in a preliminary study using ^{32}P, ^{24}Na, ^{131}I and ^{134}Cs. They measured the amount of these tracers in various regions of the cornea at regular intervals after administration. When the rate of entry into the cornea was expressed as a percent of the five-minute serum level, each of the four ions tested showed a different and characteristic pattern. When the rate of ion entry from the blood into concentric circular corneal regions was determined, the outermost region had a significantly higher concentration of the measured substance than the more central region. If the limbal vascular plexus was damaged with silver nitrate the passage of ions into the cornea was drastically/

drastically reduced. They concluded the limbal plexus plays a predominant role in supplying the ions investigated to the cornea.

In a careful and analytical study of the permeability of the rabbit's cornea to sodium ions, Maurice (1951) found that there was a balanced exchange of ^{24}Na with the blood stream at the periphery of the cornea, amounting to about one-fifth of the exchange across the endothelium. He did not consider that the results given by Potts and Johnson (1950) justified their conclusion that the limbus is the predominant supply route for the cornea, although it does undoubtedly play an important role.

It is clear that the use of radioactive tracer elements has already advanced our knowledge of the corneal fluid exchange, and we can look forward to a considerable clarification of this problem in the next few years.

SUMMARY

The anatomy and physiology of the cornea is briefly reviewed.

Chapter 11

A REVIEW of the PATHOLOGY of CORNEAL WOUND HEALING

In spite of the ease with which the cornea may be used for the investigations of wound healing, comparatively little work has been published on the problem of tissue regeneration in the cornea. The onset of the World War in 1939, with its imminent threat of eye injuries from vesicant gases, has however, stimulated interest in this subject in recent years. Much of the resulting research has assisted in improving the therapy of corneal diseases in addition to extending our knowledge of the basic pathology of the cornea.

Healing of Aseptic Superficial Corneal Injuries

Since the work of Peters (1885) and Oppel (1912) it has been clear that corneal wounds, which involve only the epithelium, heal primarily by the migration or sliding of adjacent epithelial cells into and over the denuded area. Cellular multiplication appears to play only a late and secondary role in healing.

Arey (1942) and Arey and Covode (1943) studied/

studied the healing of epithelial wounds in the rat in greater detail. In these experiments primary closure of the wound by sliding of the epithelial cells over the defect was accomplished in a few hours. In the wound area the number of mitoses occurring in the epithelial cells was reduced by 50% for some 72 hours. At 96 hours the normal rate was regained. At no time during the 6 days of observation did the frequency of cell division per unit area exceed the normal rate. Indeed, Friedenwald and Buschke (1944,a) found in the rat that very small wounds (pinpricks) may heal completely during the period of mitotic inhibition, and that even with large wounds there is no general net excess of mitotic activity during or following the healing process. It would thus appear that the deficit in the cells must be made up by a diminution in the rate of desquamation. It is to be noted, however, that Smelser and Ozanics (1945) did find a net excess of mitotic activity during the healing of small thermal burns of the corneal epithelium.

Buschke, Friedenwald and Fleischmann (1943) pointed/

pointed out that the mere counting of the number of mitotes present in the corneal epithelium at any moment of time is subject to misinterpretation, as the duration of the mitotic cycle must also be known. Fortunately, colchicine may be used to arrest the mitotic cycle early in metaphase without affecting the rate of entry into mitosis. In this way the duration of the whole mitotic cycle may be estimated. Mitotic activity in the epithelium is limited to the two basal cell layers. In the rat, the process of mitosis requires approximately 70 minutes, and the intermitotic period is approximately 200 hours.

Friedenwald and Buschke (1944,a,c), using the rat, found that movements of the corneal epithelium commenced about one hour after infliction of the injury. They found that the movements were inhibited by systemic administration of morphine, of large doses of adrenaline or of ephedrine, and by local application of cocaine hydrochloride or pontocaine hydrochloride. They noted that the cells covering the defect are larger and flatter than normal and initially form a layer which is only one cell deep. Removal of the superior cervical ganglion did/

did not interfere with the process of healing. In vitamin A deficient rats the rate and speed of the mitotic cycle was reduced by 30% although the ability of the cells to move over the surface of a small wound was not significantly delayed. Furthermore, Friedenwald and Buschke (1944,b) noted that ascorbic acid and riboflavin deficiency had no effect on corneal mitotic activity even although the growth of the animal as a whole had been severely stunted by the deficiency.

Gunderson and Liebman (1944) investigated the effect of various local anaesthetics on the regeneration of corneal epithelium. In practically every instance the local anaesthetic used had an inhibitory effect upon epithelial healing, although in many instances the practical importance of the delay was negligible. 1% phenocaine hydrochloride solution and a hypertonic 0.5% solution of tetracaine hydrochloride appear to be the least toxic to regenerating epithelium.

Mann (1944) has studied the sliding action of the epithelium in rabbits and in man. She chose eyes that possessed pigment cells in the epithelium/

epithelium at the limbus. Any movement of these limbal cells can be detected by noting the distribution of the pigment on the cornea. She confirmed the belief that initially corneal wounds are covered by the migration of epithelium from around the wounded area. Greater degrees of heat injury appear to destroy the substance which activates the sliding cells. This substance appears to be a product of autolysis of injured cells, neither tissue nor species specific.

Puchkovskaya, (1940) studied the rate of regeneration of epithelium in dead guinea-pig eyes. Mitosis appeared to be minimal, but amoeboid movement of cells over the defect took place. In this instance, however, the superficial epithelial cells played the most important part. As this work was carried out on dead guinea-pigs and also at a low temperature these conclusions may not reflect the true mode of healing in the normal guinea-pig. These experiments do, however, demonstrate the remarkable reparative powers of the cornea under adverse conditions.

Healing/

Healing of Aseptic Deep Corneal Injuries

Even in corneal injuries involving both the epithelium and the corneal stroma, the ability of the epithelial cells to slide over the defect is clearly evident. Indeed, within a few hours after an injury involving the anterior two-thirds of cornea, a thin layer of epithelium one or two cells deep begins to cover the floor of the defect. Pullinger and Mann, (1943) experimenting with chemical injuries have shown that this initial healing is by a process of sliding from the surrounding intact epithelium, and also from the epithelium at the limbus.

Once the healing area is covered with epithelium, multiplication of the cells continues and the defect is filled with many layers of epithelial cells. Replacement of the stromal connective tissue occurs more slowly, but it ultimately fills in and replaces some of the epithelial cells at the site of injury. The epithelium covering the area may finally become of normal thickness and appearance.

Pullinger/

Pullinger and Mann (1943)successfully used intravitam dyes to demonstrate the entry of wandering cells into injured corneae. During healing these cells increase greatly in number. Some appear to act as macrophages, others form keratoblasts and fibrocytes.

Peters (1946) points out that mitosis is rarely observed in the cells of the corneal stroma. He was able to observe it, however, in colchicine treated amphibian eyes.

The new connective tissue fibres formed in the cornea after injury do not lie regularly in relation to the other fibres in the stroma. As a result, irregular refraction and reflection of light occurs at this area. In the human adult clearing of corneal opacities occurs only very, very slowly, although in the child, rabbit and guinea-pig even a dense opacity can in time diminish to a surprising degree.

Bowman's membrane once destroyed never regenerates, although Descemet's membrane can undergo reparative changes.

Duke/

Duke-Elder (1938) has reviewed the literature on corneal wound healing previous to 1937.

SUMMARY

The recent work carried out on the healing of aseptic corneal wounds is reviewed.

Chapter III

The USE of SODIUM FLUORESCEIN in ASSESSING the RATE of HEALING of CORNEAL WOUNDS

For nearly 70 years a solution of sodium fluorescein has been used by ophthalmologists for initial detection of breaks in the continuity of the epithelium of the cornea (Pfluger, 1882; Straub, 1888). In contrast, sodium fluorescein has rarely been used to follow the course of healing in corneal lesions or to determine the time taken for restoration of an epithelial covering over a corneal injury. In view of the need for comparing and assessing the efficacy of an ever increasing number of chemotherapeutic drugs and of antibiotics I undertook the following investigation.

The Action of Fluorescein

A solution of sodium fluorescein does not readily penetrate the healthy epithelium of the cornea. If allowed to remain in contact with the normal epithelium for a sufficient length of time, however, fluorescein can penetrate slowly to the underlying substantia propria/

propria of the cornea (Fromm and Groenouw, 1891). Here the fluorescein during its slow passage to the anterior chamber fluoresces vividly on exposure to ultra-violet light. If the fluorescein solution remains in contact with the healthy epithelium of the cornea for no longer than one minute practically no penetration takes place in the healthy cornea and no fluorescence occurs.

When the epithelium is absent, devitalised or thinner than normal, application of a solution of sodium fluorescein for one minute now leads to penetration, and the underlying stroma fluoresces on exposure to ultra-violet light. As an ulcer heals the corneal epithelium spreads over the surface of the ulcer and increases in thickness. Simultaneously, the intensity of fluorescence, after application of fluorescein solution under standard conditions, decreases, and finally ceases.

This end point is, however, obviously dependent on the various arbitrary features of the test. Moreover, the end point, disappearance of fluorescence, is no guarantee that the reparative processes are all complete. This point is illustrated in Chapter 6.

Nevertheless/

Nevertheless, if a standard technique in the use of sodium fluorescein is rigidly adhered to, it should be possible to gauge with some accuracy the course of healing of corneal lesions. The "end point" where fluorescence ceases makes it possible to evaluate, statistically if necessary, the efficacy of various remedial measures.

To follow the regression in the intensity of fluorescence and consequently the course of healing, it is necessary to establish standards of reference which can be used to estimate the progress in healing at each examination.

The Preparation of Standards

The preparation of suitable standards of reference required an investigation into the relationship of the concentration of sodium fluorescein to its intensity of fluorescence.

Strips of Whatman No.I filter paper were impregnated with known quantities of sodium fluorescein, (B.D.H.). The sodium fluorescein was dissolved in 50% alcohol and a known volume of this solution was evenly distributed on a piece of filter/

filter paper of known area. This strength of alcohol was found to dry more rapidly than a watery solution and it allowed more even distribution of the stain. Higher concentrations of alcohol were found to be unsuitable because sodium fluorescein was incompletely dissolved. Rapid drying, which was necessary for even distribution, was ensured by holding the paper in a stream of hot air from a hair-dryer.

A number of strips were prepared with concentrations of sodium fluorescein ranging from 0.125 microg./sq.cm. to 128 microg./sq.cm. of filter paper. These strips were then exposed to ultra-violet light from a G.E.C. mercury vapour ultra-violet dark bulb lamp. The intensity of the fluorescence was then measured photometrically with a low resistance galvanometer and a barrier-layer photoelectric cell. To absorb ultra-violet light the photocell was screened with a green Wratten filter (61 M). The impregnated strip of filter paper was placed in direct contact with this filter. The relative intensities of the light emitted from the fluorescein-impregnated paper was then read directly on the galvanometer scale. This photometer gave a direct linear relationship between the/

the fluorescent light intensity and the galvanometer reading.

Figure 2 shows graphically the results obtained. For convenience a geometric scale was used in the abscissa. The graph shows clearly that the intensity of the fluorescence rises to a maximum with increasing concentration of fluorescein up to 8 microg./sq.cm. Beyond this point increase in concentration of fluorescein not only does not increase the fluorescent response on exposure to ultra-violet light, but actually diminishes the response. This phenomenon is well known to physicists and is called "quenching". The explanation is obscure (Pringsheim, 1949).

From inspection it may be seen that concentrations of fluorescein ranging from 0.5 microg./sq.cm. to 8 microg./sq.cm. give a spread of intensity most useful in the preparation of standards for experimental use. Over this range a geometric increase in the concentration of fluorescein gives an almost linear increase in intensity of fluorescence. Moreover, the hue of the fluorescent light emitted is very similar to that from corneal lesions. Greater concentrations are not suitable for use as standards because the deep orange colour of the concentrated fluorescein/

fluorescein interferes with the hue of the fluorescence.

For experimental use five standard filter paper strips were chosen with concentrations in the range of 0.5 microg. of sodium fluorescein/sq.cm. to 8 microg./sq.cm. From the graph it can be seen that the intensities of fluorescence emitted from these strips bear, for all practical purposes, a linear relationship to each other. For simplicity we chose to represent these intensities by the figures 1,2,3,4, and 5, (Table 1). These figures are approximately proportional to the absolute intensity of fluorescence. Since the absolute intensity of fluorescence depends also on such factors as distance and strength of the light source standardisation of working conditions is essential.

For convenience the five strips were mounted behind glass under a black paper mask which exposed an area of 1 in. by 0.5 in. of each strip. There has only been slight fading over a period of nine months.

Use of the Impregnated Strips to Assess the Course of Healing

The/

The experimental use of a method such as this must be carefully standardised. One drop of a 2% aqueous solution of sodium fluorescein is instilled into the conjunctival sac. After exactly one minute the eye is washed with a sufficiency of isotonic saline to remove excess fluorescein solution. The set of standard strips is then held beside the eye.

Comparison is made under illumination by a G.E.C. ultra-violet lamp placed at a convenient distance, say two feet. The intensity of fluorescence from the lesion is then matched against the strips.

Ideally the matching should be carried out in darkness but is also satisfactory if the surrounding illumination is constant and of moderate intensity.

D I S C U S S I O N

The method outlined in this chapter has been used successfully to follow the course of healing of heat injuries in the guinea-pig's cornea, and also to follow epithelial healing in the human cornea.

The technique is simple and close agreement on the intensity of staining is obtained with different observers/

observers. The introduction of the sodium fluorescein solution into the eye causes no discomfort, and does not appear to delay the healing process.

SUMMARY

1. A method is described for assessing the rate of healing of corneal injuries using sodium fluorescein solution.

2. The steps taken to standardise the technique are described.

Chapter IV

The FATE of SODIUM FLUORESCEIN STAIN in the CORNEA

If a corneal injury which has been stained with sodium fluorescein is observed for several hours the intensity of fluorescence is seen to diminish gradually and finally to cease. Where does the fluorescein go to? There are several possible answers to this question. Firstly, the fluorescein could gradually diffuse deeper and deeper into the corneal stroma and pass through Descemet's membrane and the endothelium into the anterior chamber, there to be removed with the aqueous. Secondly, the fluorescein could diffuse centrifugally through the corneal stroma to reach the limbus and be there removed via the limbal capillaries. Or, lastly, the dye could pass anteriorly into the conjunctival sac to drain away with the tear fluid.

The following experiments were designed to establish the usual route of drainage and to test if the sodium fluorescein technique could be used to ascertain the mode of corneal fluid circulation.

METHOD

Animals. Ten adult healthy rabbits were used.

Apparatus. An "Agla" Micrometer Syringe (Burroughs Wellcome & Co., London) fitted with a gauge 20 hypodermic needle was used to inject the sodium fluorescein solution into the cornea.

Anaesthesia.

General anaesthesia was induced by slowly injecting 1-3 c.c. of a 5% solution of Pentobarbital Sodium (Nembutal) into an ear vein. Sufficient was administered to produce light anaesthesia for 15-30 minutes. In addition local anaesthesia of the eye was produced by instilling a 2% Amethocaine Hydrochloride solution (B.P.) into the conjunctival sac five minutes before the operation.

Technique.

The upper lid was retracted manually and the exposed bulbar conjunctiva firmly grasped with a small pair of toothed forceps. The forceps were handed to an assistant who was thereafter responsible for fixation of the head and the eyeball. Firm fixation/

fixation of the eye is essential for success. The syringe, filled with a 0.1% solution of sodium fluorescein in normal saline was then held at 45' degrees to the corneal surface. With the bevel of the needle facing away from the cornea the point of the needle was inserted just under the epithelium. The syringe was then lowered so as to make a tangent with the cornea, and the needle pushed further into the corneal stroma. The micrometer screw of the syringe was then turned and about 0.1 $mm.^3$ of solution injected. In most of the experiments the injection was made into the centre of the cornea. A slight leakage of the injected fluid sometimes occurred when the needle was withdrawn. This leakage was negligible.

RESULTS

Experimental Series 1.

Four corneae were injected as outlined above and observed for 8 hours under the ultra-violet lamp and also by means of slit-lamp microscopy.

For the first 10 or 15 minutes the fluorescence/

fluorescence was limited to a small circular area of about 2 mm. in diameter in the anterior third of the cornea. At the end of one hour the area staining had increased only slightly, but the whole corneal thickness excluding the epithelium and endothelium was stained. After 3 hours the intensity of the stained area had decreased, and a faint fluorescence was observed in the aqueous of the anterior chamber. The edges of the stained area were diffuse and difficult to define but the stained area was not greater than 3 mm. in diameter. Eight hours after the experiment commenced only a very faint fluorescence remained at the site of the injection.

Experimental Series 2.

It was apparent from the results of Experiment 1 that most of the fluoresceim had passed through the endothelium into the anterior chamber. To exclude this source of drainage it was decided to replace the aqueous in the anterior chamber with air. This was carried out fairly easily by fixing the eye as outlined above and passing/

passing a gauge 16 hypodermic needly obliquely through the cornea close to the limbus, and withdrawing the aqueous by means of a 1 c.c. syringe. The syringe was then detached from the needle, emptied, and filled with air. The syringe was then re-attached to the needle and the anterior chamber filled with sufficient air to return the intraocular pressure to a normal level. The needle was then rapidly withdrawn. Four rabbit eyes were treated in this manner.

Fluorescein was injected intracorneally as outlined in Experiment 1.

The fluorescein penetrated as far as the endothelium in 10 or 15 minutes as in Experiment 1. However, no fluorescein appeared to pass into the anterior chamber, for even after 16 hours observation the area stained almost as intensely as at the beginning of the experiment. The area of fluorescein staining tended to diffuse slowly towards the limbus, so that the total area stained increased from 2 mm. in diameter to about 5 mm. The stain never reached the limbus. The intensity of staining gradually decreased, taking about 36 hours to disappear.

Experimental/

Experimental Series 3.

This experiment was carried out upon 4 freshly enucleated rabbit eyes. The excised eyes were treated exactly as in Experiment 2, and then each eye was placed in 5 c.c. of normal saline in a small beaker. The eye and the surrounding fluid were examined from time to time for traces of fluoresceim. The intensity of the fluorescence from the cornea decreased more slowly than in Experiment 2, and after 16 hours a faint trace of fluorescence could be detected in the saline bathing the eye. The cornea was still staining faintly after 48 hours. Observations were not continued for a longer period as the eyes had started to degenerate.

D I S C U S S I O N

From these experiments it is fairly clear that the main source of drainage for fluorescein administered intracorneally is by passage through the endothelium into the anterior chamber. There is also evidence of some of the fluorescein passing through/

through the epithelium into the conjunctival fluid. The amount lost in this way is, however, small compared with the former route. The loss through this anterior passage may have been greater due to the damage to the epithelium at the point where the needle was inserted.

Evidence was obtained from these experiments of a passage centrifugally towards the limbus. The rate of diffusion in this direction, however, appeared to be too slow to remove significant amounts of dye from the centre of the cornea. This route may be important if fluorescein was injected nearer to the limbus.

SUMMARY

1. The fate of sodium fluorescein after intracorneal injection was investigated in the rabbit.

2. It was concluded that the main source of drainage is by passage through the endothelium into the anterior chamber.

3. Slow diffusion centrifugally towards the limbus was also noted.

Chapter V

The ROLE of ASCORBIC ACID in CORNEAL WOUND HEALING in the GUINEA-PIG

It is generally accepted that the repair of collagenous tissue is dependent on an adequate supply of ascorbic acid (Højer, 1924; Penney and Balfour, 1949; Wolbach, 1926 and 1933). Now, as the cornea is largely composed of collagen, it is reasonable to suppose that ascorbic acid will influence the healing of corneal injuries which are sufficiently deep to injure the substantia propria. Furthermore, as the cornea is avascular, vitamin C may also act as a hydrogen acceptor, and thus play an unusually important part in the normal metabolism of this special tissue.

Several workers have found that the cornea contains a very high concentration of ascorbic acid compared with other body tissues. (Table 2). Schmid and Burkd (1943), using a histochemical method, (reduction of silver nitrate) found the greatest concentration in the superficial epithelial layers of the cornea. The substantia/

substantia propria had a lower content although there was a high concentration in the region of Bowman's and Descemet's membranes.

On the other hand, Henkes (1946) found no ascorbic acid in Bowman's membrane and only a little in the corneal epithelium, but a high concentration in the substantia propria, and in Descemet's membrane. The sub-epithelial portion of the substantia propria contained the highest concentration of vitamin C. Henkes' method was to extract and titrate the ascorbic acid from the various regions of the cornea obtained by histological methods. It is interesting to note that he found in experimental scurvy in guinea-pigs, the ascorbic acid disappeared from the cornea in 2-3 weeks, although the glutathione content remained unchanged.

Pirie (1946), using microtitration, found the concentration of ascorbic acid to be greatest in the corneal epithelium of rabbit and beef eyes. The concentration in the corneal stroma was about equal to that found in the aqueous humour. No attempt/

attempt was made to separate the corneal tissue further, and analysis was carried out only on epithelium scraped off from the cornea or on the stroma. She believed that the corneal endothelium is permeable to ascorbic acid in either direction.

Galloway, Garry and Hitchim (1948) studied the influence of ascorbic acid on wounds of the corneal epithelium produced by a dental burr. The wounds were confined to removal of a small circular area of epithelium, leaving Bowman's membrane intact. They found no significant delay in healing in the scorbutic group of cavies (Table 3). No other well controlled experiment on the healing of corneal wounds in scurvy has been published. Pirie (1950, personal communication) found no histological difference in the mode of healing of deep corneal injuries produced with a trephine in scorbutic guinea-pigs when compared with normal animals.

The experiments in this part of the thesis were designed to repeat the findings of Galloway et al and to find if ascorbic acid influenced the healing of/

of wounds of the cornea which involved not only the epithelium but also the collagenous substantia propria.

E X P E R I M E N T A L

Animals

Female non-pregnant guinea-pigs were used. The initial weights lay between 450 g. and 650 g. The cavies were weighed every second day. The guinea-pigs were kept in groups of 5 or 6 in wire and metal cages, 24" by 18" by 12". The cages were sterilized twice weekly. The animals were kept and examined in one room with a temperature between 65'F. and 75'F.

Diets

The basal diet was crushed rat cake cubes (Thomson 1936) well moistened with water. These cubes are free from ascorbic acid. Corneal wound healing is known to be influenced by vitamin A deficiency (Friedenwald, Buschke and Morris, 1945) so that to supplement the diet six drops of Cod Liver Oil (minimum content 500 I.U. Vitamin A and

50/

50 I.U. Vitamin D per gram.) were added daily to the diet of each animal. The mash was placed in low set troughs so that the cavies had easy access to the food. Food and water were given without stint.

Ascorbic Acid

Although guinea-pigs require only about 2 mg. of ascorbic acid per day to prevent microscopic and macroscopic signs of scurvy, Kellie and Zilva (1940) believe that 20 mg. by mouth per day are necessary to maintain complete saturation of the body tissues. Furthermore, Kuether, Telford and Roe (1944) maintain that 21 days are required to bring a guinea-pig to a steady state of saturation with ascorbic acid.

In order to ensure that the cavies had a uniform initial level of saturation with ascorbic acid 20 mg. ascorbic acid (Roche) were given orally in 1 ml. water once a day. The solution was made immediately before use, and given to the animals by pipette. This daily intake was given for 21 days to all the animals to obtain tissue equilibrium (Jones, Bartlett, Ryan and Drummey, 1943).

Controls/

Controls

Control animals were injured after 21 days of saturation with vitamin C and the daily intake of 20 mg. was continued thereafter.

Principals

After the preliminary 21 days of saturation the principals were given 0.5 mg. ascorbic acid in 1 ml. water every second day for a further 21 days. Only then were injuries made to the cornea, the dosage of 0.5 ml. ascorbic acid every second day being continued thereafter.

A P P A R A T U S

Applicator

Small metal cylinders having a flat end of 1 sq. mm. area were heated among lead shot in a hot air oven to 120'C. or 180'C. The oven was thermostatically controlled to ± 2'C. These applicators were used to produce superficial corneal burns.

Cautery

To produce deeper lesions a cautery was made from a loop of 32 S.W.G. platinum wire. A predetermined/

predetermined constant voltage was fed to the cautery through a Londex relay, Type LF/FS, connected to the one second contacts of a Palmer AC Time Clock. The relay was activated every second from the time clock. The current flowed through the cautery every alternate second when a hand switch was closed. By closing this switch at the appropriate moment a current of exactly one second's duration would flow through the cautery.

Compression Balance

The apparatus shown in Fig. 3 was used to find the weight required to rupture the excised guinea-pig eye. The eye is compressed betweem a fixed support A and a platform C. The force is applied by adding weights to the scale pan B. Both the support A and platform C, pivoted on the end of the lever, are hollowed out to hold the excised eye.

This compression balance was used in two ways. Firstly, by attaching a metal bar to the pivoted platform C, (Fig. 2 x). it was possible to estimate the strength of a corneal lesion. Secondly, without/

without the bar, the resistance of the sclera to compression could be found.

T E C H N I Q U E

Anaesthesia

The cornea was anaesthetised by instilling into the conjunctival sac 2 drops of a 2% amethocaine hydrochloride B.P. solution. Anaesthesia was complete in 2 minutes, and lasted for about 30 minutes. The animals were steadied during the operation by holding the head lightly. The operation did not cause discomfort since the corneal reflex was never elicited and the cavies remained quiet both during and after the operation. No signs of distress appeared after the operation and in no case did infection occur.

Superficial Corneal Heat Injuries

The applicator was removed from the bed of lead shot in the oven by means of a Spencer-Wells forcep, heat insulated by placing rubber tubing over the points of the forcep. Exactly two seconds/

seconds after removing the applicator it was applied firmly to the cornea 2 mm. from the limbus at 12 o'clock. Contact was maintained for exactly 5 seconds. If a delay of more than 2 seconds occurred after removing the applicator from the oven it was discarded and a fresh one utilised. Histological examination showed that such injuries affected only the epithelium over the cornea. Macroscopically and microscopically the injuries produced were remarkedly uniform.

Deep Corneal Injuries

The cold cautery was pressed firmly and vertically on the cornea 2 mm. from the limbus at 12 o'clock. Just enough pressure was used to dimple the cornea. The hand switch was then closed and the current allowed to flow for one second. The cautery was removed 1 second after the time switch and relay had cut off the current. Histological examination showed that the resulting lesion was 1 mm. in diameter and destroyed the corneal epithelium and the anterior two-thirds of the substantia propria.

All/

All thermal injuries were carried out by myself but at the time of the operation I was unaware whether control or principal animals were being injured. I later learned that the injuries were inflicted alternately on the eyes of principal and control animals.

Method of Examination

To assess the degree of healing two drops of 2% aqueous sodium fluorescein solution (B.D.H.) were instilled into the conjunctival sac and allowed to act for exactly one minute. Excess solution was removed first by mopping with filter paper and then by instilling 6 ml. Ringer solution. The eye was examined immediately thereafter in darkness under a G.E.C. Mercury Vapour Ultra-violet dark bulb lamp, 8" from the injured eye. The intensity of fluorescence from the injury was compared with the standard described in Chapter 3.

A fresh injury in all cases fluoresced brightly. As healing progressed the intensity fell off gradually and was evaluated against the other strips with smaller fluorescein content.

In/

In addition, the degree of corneal oedema, of vascularisation and of opacity were noted under intense focal illumination with the aid of a binocular loupe.

Observations were carried out at intervals of exactly 8 hours day and night until fluorescence was absent at three consecutive examinations. Thereafter each eye was examined at 24 hour intervals.

RESULTS

General

Control guinea-pigs remained healthy and gained weight continuously before and after the operation.

The principal animals showed a gain in weight during the initial period of vitamin C saturation, and this gain in weight continued for 10 days on the decreased intake of 0.5 mg. ascorbic acid every second day. Thereafter the reserve supply of ascorbic acid in the body became insufficient, and the cavies began to lose weight (Fig. 4). They became quiet and less active, and the coat became staring.

After the lesions had ceased to show fluorescence/

fluorescence the guinea-pigs were maintained on their respective diets, and were thereafter killed at varying intervals for histological examination. A principal and its corresponding control were examined together.

Post mortem examinations were carried out on all animals. There were no macroscopic signs of disease.

Superficial Corneal Heat Injuries

Using the applicator heated to 180'C. six control and eight principal eyes were injured. The time of healing as indicated by cessation of fluorescence is shown in Table 4. The difference of one hour between principals and controls is statistically not significant. The degree of significance was calculated by means of Student's "t"test ("Student", 1925).

An attempt was made to evaluate the mean fluorescence in both groups at injury, and subsequently at 8 hour intervals. The values attributed to the intensity of fluorescence from each lesion were summed for each group and divided by the number of eyes/

eyes in the group. It is obvious from Fig.5 that there is no difference between the fluorescent gradients of the two groups.

After cessation of fluorescence a faint corneal opacity (nebula) persisted in 4 out of the 14 eyes. Wolff (1948) states that a healed epithelial defect does not leave an opacity. It must therefore be assumed that the injury had altered slightly the structure of Bowman's membrane and possibly also the substantia propria. It was therefore decided to repeat the experiment using the applicator initially heated to only 120°C. Ten control and eight principal eyes were injured under the identical conditions of the previous experiment.

As will be seen from Table 5 there is again no statistical significant difference between the end point of healing as indicated by the disappearance of fluorescence. In this series all opacities disappeared within 96 hours from the time of injury. It is therefore likely that in this experiment only the epithelium was injured.

It/

It is interesting to note that the mean time for healing was markedly affected by the initial temperature of the applicator (Table 6). There is a highly significant difference in the mean time of healing of the 180°C. and 120°C. experiments. This result is probably due to the greater area and depth of epithelium involved with increase in the temperature of the cautery. It is thus important to control this temperature carefully.

Deep Corneal Heat Injuries

With this more severe type of injury which involved the substantia propria, there was a highly significant difference between the rate of healing in principals and in controls as judged by the disappearance of fluorescence (Table 7). In the guinea-pigs with ample ascorbic acid healing was complete in 94 hours, in the scorbutic cavies healing took 126 hours.

In this experiment an opacity remained at the site of injury in all animals and persisted unchanged until the guinea-pigs were killed.

The mean gradient of fluorescence intensity

was/

was assessed in this experiment as outlined above (Fig. 6).

After the first few hours the lesions in the control cavies gave on the average less intense fluorescence than did the lesions in the principal animals. It would thus appear that ascorbic acid exerts its influence on corneal healing as early as 8 hours after the time of injury.

The Strength of the Injured Cornea

In preliminary examinations the manipulations required in enucleation of eyes previously injured by the cautery, not infrequently led to perforation through the site of the lesion. This occurred although healing had so far progressed that all fluorescence had ceased. Moreover, this rupture took place even although the eyes were enucleated with considerable care and only small transient rises in intraocular pressure could have occurred. Nevertheless, this increased pressure appeared to be sufficiently great to rupture the eyes through the site of the lesion. It would thus appear that disappearance of fluorescence cannot be taken as an index/

index of complete return to normal, for there is here a suggestion that a residual weakness is present at the site of the lesion. Moreover, we received the impression, at this stage, that such ruptures were more frequent among principals than among controls.

It was therefore decided to subject the eyes to known degrees of compression, and thus to attempt to find if there was any difference between the principals and controls. The compression balance described on page 56 was therefore designed. The balance was constructed in time to subject to stress 28 control eyes and 26 principal eyes from the experiment with deep corneal lesions.

To estimate the degree of compression necessary to rupture the cornea through the site of the lesion, the excised eye was placed cornea downwards with the bar of the pivoted platform along the 3 - 9 o'clock meridian of the eye. (Fig.3). The indentation of the cornea in this way built up considerable pressure in the anterior chamber. If, however, the eye did not perforate by the time

1100 g. had been applied to the scale-pan the eye tended to slip off the bar undamaged.

In the controls 21 out of 28 eyes slipped off the bar in this way without perforation. This is indicated by a * after the highest value recorded before slipping off the bar (Table 8). This value was perforce used for statistical analysis. On the other hand, in the principal group 21 out of 26 eyes perforated. The "chi square" test (Fischer, 1946) shows that such a result could only occur by chance 1 in 1000 times (Table 9).

Further inspection of Table 8 shows that up to 552 hours following injury eyes from principals perforated at a lower pressure than did eyes from the controls. These results have been analysed by the "t" test and the results are shown in Table 10. The difference between the two groups is highly significant.

Analysis of the pressures required to rupture eyes injured more than 552 hours previously is hardly justified, since all the eyes from the controls/

controls and many from the principals slipped off the bar before perforation.

Inspection of Table 8 also shows that as healing progresses a greater weight is required to cause perforation in both groups.

The Strength of the Sclera.

It is possible, although not probable, that the sclera in the scorbutic group of animals may have diminished in strength throughout its entire structure, as the tissues of the sclera are predominantly collagenous. Fortunately, it was possible to measure scleral strength even after the cornea had ruptured, for in the guinea-pig eye the relatively large lens is displaced forward after the anterior chamber empties and prevents escape of the vitreous humour through the ruptured cornea. To measure scleral strength the bar was removed from Platform C (Fig. 3) and the eyes were placed, cornea downwards, to be compressed between the fixed support and the platform. Weights were added to the scale pan until the sclera ruptured. As both the fixed support and the platform were hollowed out to fit the eyes, they could not escape from/

from the apparatus and the weight necessary for rupture of the sclera was obtained in every case. The results from this part of the experiment are given in Table 11. The "t" test showed that there was no significant difference between the eyes from principals and controls.

D I S C U S S I O N

Superficial Heat Injuries of the Cornea

The repair of heat injuries probably confined largely to the epithelial cells is not influenced by ascorbic acid deficiency. This finding is in keeping with the results of Galloway, Garry and Hitchim (1948) who inflicted mechanical wounds on the corneal epithelium by means of a dental burr. It would thus appear that epithelial cells of the cornea can divide and proliferate quite independently of a supply of ascorbic acid. It is well known, however, that corneal epithelium can fill in a defect of moderate size without formation of new cells, by a process of sliding in from the periphery (Arey and Covode, 1943, Friedenwald, Buschke/

Buschke and Crowell, 1945). If this latter method of healing was predominant in these experiments then there is no reason to expect a deficiency of vitamin C to delay the healing of wounds confined to the epithelium.

Deep Heat Injuries of the Cornea

Repair of injuries which penetrate Bowman's membrane and destroy the collagenous tissue of the substantia propria require separate consideration.

As judged by the time required for cessation of fluorescence the healing of deep corneal heat injuries in scorbutic guinea-pigs is very definitely retarded. The cessation of fluorescence is closely correlated with the degree of epithelialisation (Chapter 3). It has just been shown above, however, that the process of simple replacement of epithelium is not impaired by a lack of ascorbic acid. It may be that the slower rate of epithelialisation in the deep ulcers, where no addition of ascorbic acid had been given, was due to the absence of a suitable substratum of/

of collagenous tissue. This is in keeping with the hypothesis of Hartwell (1929) who studied skin epithelialisation in cases of scurvy, and also with the suggestions of Galloway et al (1948). Pirie (1950) has pointed out that new formation of collagen in deep corneal injuries is very slight and that the healing is predominantly by epithelial proliferation.

It can only be concluded that some change takes place in the exposed collagen in the bed of the ulcer to permit more rapid overgrowth of the epithelium in the control animals with adequate vitamin C intake.

This hypothesis is further strengthened by the evidence from the experiment with the compression balance. There is no doubt that the vitamin C deficient animals, in which the healing process had progressed as far as complete epithelialisation, still had a residual structural weakness in the cornea at the site of the lesion. This weakness could readily be accounted for if new formation of collagen fibres was deficient.

It/

It is unlikely that the diminution in structural strength in the deficient guinea-pigs was due to a general weakening of the connective tissue coat of the eye since the force required to rupture the collagenous tissue of the sclera was similar both in principals and in controls.

SUMMARY

1. Standard superficial and deep heat injuries were made on the cornea of guinea-pigs receiving either a wholly adequate (20 mg. daily) or a deficient (0.5 mg. every second day) intake of ascorbic acid.

2. The progress of repair was estimated both by instillation of sodium fluorescein solution and by subjection of the excised eyeballs to compression in a special balance.

3. The healing of superficial lesions, confined to the corneal epithelium, was not impaired by a deficiency of ascorbic acid.

4. On the other hand, deeper lesions, involving the substantia propria, healed significantly more slowly in the deficient cavies.

5/

5. The healing deep lesions were weaker in the scorbutic animals up to 20 days after injury.

6. It was concluded that, although restoration of corneal epithelium as such may be independent of an adequate supply of ascorbic acid, yet the rate of epithelialisation of a wound of the cornea involving collagenous tissue does depend on the provision of a suitable fibrous tissue substratum, and in turn on an adequate intake of ascorbic acid.

Chapter VI

The ACTION of ASCORBIC ACID on the RATE of HEALING of CORNEAL ULCERS in MAN

After completing the work recounted in Chapter 5 of this thesis I had the opportunity of extending the investigations to human cases of corneal disease. This work was carried out in close co-operation with Dr T.A.S. Boyd who was responsible for many of the clinical observations made at the Glasgow Eye Infirmary.

If the results of the experiments on guinea-pigs also apply to human beings then ascorbic acid may be of pronounced value in the therapy of corneal disease. Lyle and McLean (1941) and Summers (1946) reported on the effect of massive doses of ascorbic acid on a variety of corneal diseases. All these observers believed that ascorbic acid was of definite therapeutic value. Unfortunately, these workers made no attempt to design their investigations so that statistical analysis could be carried out. No controls were used. The conclusions formed depended solely upon their clinical acumen and on their experience of/

of how similar cases would have progressed without ascorbic acid therapy.

We decided to restrict our studies to the healing of small corneal ulcers so as to simulate the type of injury produced in the animal experiments previously described. The choice of this type of case enabled sufficient numbers to be studied in a reasonable period of time, and permitted statistical analysis of the results.

Unfortunately for the purposes of our experiment, but fortunately for the community as a whole, scurvy is a comparatively rare condition in this country and so we were unable to observe a group of patients which was comparable to our group of deficient guinea-pigs. All our patients were eating a more or less normal diet, the intake of vitamin C probably reaching 50 mg. daily in some cases. Therefore, in order to form a group in sufficient contrast to those patients on normal diet, half the patients were given a very large dose of vitamin C.

METHOD

The/

The patients studied in this investigation were in attendance at the Out-Patient Department of the Glasgow Eye Infirmary between November 1949 and March 1950.

Approximately half the patients received 0.5 g. ascorbic acid (Roche Products Ltd.) three times daily, and the remaining cases received control tablets, containing no ascorbic acid, but of identical appearance and taste. Thus the patients in the principal group probably received 30to 50 times as much ascorbic acid as the patients in the control group who received only the ascorbic acid in their natural diet. Administration was continued until healing was complete.

Throughout the experiment the observers were unaware of the type of tablets administered to each patient. This was achieved by numbering the boxes containing the tablets in a random fashion, and recording (for future reference) the contents to which each batch number referred.

A detailed clinical history was obtained from each patient. This was followed by a careful/

careful examination of the lesion, and such details as its size and position on the cornea were noted.

One drop of a 2% aqueous solution of sodium fluorescein (British Drug House) was then instilled into the conjunctival sac. After one minute exactly, excess fluorescein was washed off with a stream of isotonic sodium chloride solution delivered from a pipette. The intensity of fluorescence of the ulcer was then assessed in a dark room by placing alongside the patient's eye a series of standard strips of filter paper impregnated with varying concentrations of sodium fluorescein. Fluorescence was induced by exposure to a mercury vapour lamp fitted with a Wood's glass screen, and situated about 2 feet from the patient. Details of the standard strips used will be found in Chapter 3.

As healing of a corneal ulcer progresses, the intensity of the fluorescence decreases and finally ceases when epithelialisation is complete. The time of healing was recorded as the interval between the first examination of the ulcer and the/

the cessation of fluorescence. Observations were carried out daily.

Before the patient's final discharge the cornea was examined by slit-lamp and biomicroscope. The depth of the lesion was assessed by measuring the fraction of the thickness of the cornea occupied by opacity.

RESULTS

Analysis of the time taken for the corneal ulcers to epithelialise showed that the mean healing time for the group of patients who had received ascorbic acid (22 cases) was 4.00 days, whereas for the control group (29 cases) the mean was 4.82 days. The difference of 0.82 days is not significant.

However, several factors which may affect these times of healing are at work simultaneously. The following factors which may influence resolution will now be considered.

1. Depth.

The two groups above were further divided into sub-groups of patients with "superficial " and/

and with "deep" ulcers. Ulcers were considered to be "superficial" if, after healing, the residual opacity occupied a quarter or less of the corneal thickness as judged by slit-lamp and biomicroscope. The remainder were considered to be "deep".

Under normal circumstances one expects superficial ulcers to heal more rapidly than deep ulcers. This was indeed the case in the control group where the difference in healing time between superficial and deep ulcers was highly significant (Table 12,B,D).

In the group of patients receiving 1.5 g. ascorbic acid daily, the superficial ulcers also healed more rapidly than the deep ulcers, but this difference was not significant (Table 12,A,C). In other words, there is a suggestion here that depth of lesion has less effect upon the healing time when there is an abundant intake of ascorbic acid.

2. Ascorbic Acid.

The difference between the mean healing times of the groups of patients with and without additional ascorbic acid is not significant in the case of superficial lesions (Table 12,A,B), but is significant in the case of deep lesions (Table 12,C,D)($P < 0.05$).

This/

This evidence means that the administration of large doses of ascorbic acid does not influence the rate of healing of superficial ulcers, but does accelerate the healing of deep ulcers.

The significant difference between the time necessary for epithelialisation of "deep" ulcers in the principal and control groups is further substantiated by an examination of the course of healing in these two groups. Information on the course of healing was derived from daily estimation of the degree of fluorescence from each ulcer. In Fig.7 the mean intensity of fluorescence on each day of observation has been plotted for the "deep" ulcers in the principal and control groups. Although by chance the group receiving ascorbic acid started with a higher degree of fluorescence, epithelial healing occurred rapidly, and so their "fluorescent gradient" was much steeper than that of the controls. In contrast, the "superficial" ulcers in principal and in control groups healed at similar rates (Fig.8).

It is thus clear that the course and time of healing were appreciably influenced by ascorbic acid only in deep ulcers; but can it be said that the deeper/

deeper the lesion, the more pronounced is the beneficial effect of ascorbic acid?

This question was investigated by a further subdivision of the deep and superficial groups. The deep group was broken down into a group in which the ulcers infiltrated one half of the corneal thickness and another group affecting one third. Similarly, the superficial group was divided into groups affecting one quarter and one fifth.

Figure 9 shows diagrammatically the mean healing time of principals and controls in each of the four groups. The influence of depth upon the control groups is clearly shown by the progressive lengthening pf healing time with increase of depth. There is a similar influence at work in the principal groups, but it is much less marked. It can then be said that the deeper the lesion, the more pronounced is the effect of ascorbic acid.

3. Site and Size.

A priori one might expect the area of the ulcers/

ulcers and their location on the cornea to have some effect on the rate of healing. Only a slight tendency was found for central ulcers to heal more slowly than marginal ones, but analysis indicated that this difference could easily have arisen by chance.

As one would expect, the larger ulcers healed more slowly than the smaller ones, but the difference between the healing times of large and small ulcers in the series examined was not significant. This was probably due to the comparatively small size of the ulcers (0.25 mm. to 2.5 mm. in diameter) we deliberately included in this survey.

4. Local Therapy.

All the cases received either gutt. sodium sulphacetamide 10%, or gutt. penicillin 1000 units per c.c., three times daily, and it was found that there was no significant difference between the healing times of sulphacetamide and penicillin treated cases. It is of interest, however, to note that, compared with sulphacetamide, penicillin significantly shortened the duration of conjunctival discharge/

discharge.

5. Pyorrhoea alveolaris.

During the routine examination of these patients it was noticed that a considerable number had pyorrhoea alveolaris, and so its incidence in our patients was investigated. Pyorrhoea was diagnosed only if the gums showed marked retraction with ulceration, or if pus could be expressed from the parodontal sulcus.

Pyorrhoea was present in these patients with corneal ulcers most frequently in the 20-40 year age group. Younger patients were usually free from infection, while many of the teeth of the older patients had been extracted. Since age appeared to affect the incidence of pyorrhoea, we collected a control group with the same age distribution from out-patients attending with non-infective ocular complaints and examined them for the presence of pyorrhoea.

Pyorrhoea was more common in the group with corneal ulceration (Table 13). The X^2 test showed that this difference in incidence was significant (P/

($P < 0.01$).

DISCUSSION

Superficial Lesions of the Cornea

The absence of a significant effect of large doses of ascorbic acid on the healing times of superficial ulcers is in keeping with the results of Galloway et al 1948, who inflicted mechanical wounds on the corneal epithelium, and also with the results given in Chapter 5 of this thesis. The ulcers classified as superficial in the present series were not of course confined solely to the epithelium, but did involve a certain amount of stroma. Not unexpectedly the effect of involvement of stroma on the healing time appears to be insignificant under the conditions of this human survey.

Deep Lesions of the Cornea

Large doses of ascorbic acid in man do promote epithelialisation of deep corneal ulcers as judged by the fluorescein test. It may again be concluded that the slower rate of epithelialisation/

isation where no additional ascorbic acid had been given was due to an absence of a suitable substratum of collagenous tissue.

It was naturally impossible to test the strength of the cornea after epithelial healing had occurred in this experiment, but there is nothing to refute the conclusion formed in Chapter 5 of this thesis that more rapid reparative changes had taken place in the exposed collagen of the bed of the ulcer of those patients given very large doses of ascorbic acid. This rapid repair then allowed speedy overgrowth by epithelium.

Action of Ascorbic Acid

The question now arises, why should massive doses of ascorbic acid accelerate the healing of corneal lesions in patients whose vitamin C intake was normal by usual standards? Even after careful questioning on dietary habits only a very few patients were found who did not eat potatoes and vegetables daily. Furthermore, as no patient showed signs of scurvy it must be concluded that this intake was sufficient for all normal purposes. The findings suggest, however, that the intake was not/

not optimal for the healing of deep corneal lesions. It would thus appear that ascorbic acid in such massive doses as 1.5 g. daily has a value in therapy apart from its normal role as a vitamin at accepted levels of intake.

The nature of this action remains obscure at present. It may well be that there is a local deficiency at the site of any collagenous tissue lesion. If this be so, then benefit would accrue from the increased rate of diffusion which would arise from a temporary massive increase in the blood level of ascorbic acid. In the case of the avascular cornea this accelerated diffusion will tend to be of greater value than in vascular granulation tissue surrounding lesions elsewhere.

There seems little doubt that an increase in serum ascorbic acid in the rabbit will cause an increase in the ascorbic acid content of the aqueous (Kinsey, 1947). Langham (1950) confirmed this and established that a ceiling level of ascorbic acid in the aqueous humour (50 mg/100 ml.) results from a raised concentration in the plasma. However, he found/

found that im the cat this mechanism was far less efficient. No work has been published using the human eye.

Furthermore, Pirie (1946) considered that the endothelium of the rabbit's cornea was permeable to ascorbic acid in either direction and found that the ascorbic acid content of the corneal stroma approximately paralleled that of the aqueous humour.

It is therefore likely that in man an increased intake of ascorbic acid will raise the serum level, and that this in turn may increase the concentration in the aqueous. If the permeability of the human corneal endothelium is similar to that in the rabbit, a higher ascorbic acid concentration should occur in the corneal stroma when massive doses are given.

The original object of these observations on clinical cases was to find if conclusions, based on the experiments with guinea-pigs, could be applied also to human beings under conditions of much less perfect control. This aim has been fulfilled to an unexpected degree. This present study was restricted to small ulcers of a type which/

which would be expected to heal with the usual treatment within a week or ten days. It would now be justifiable to extend the investigations to more serious corneal conditions which to a greater extent involve the collagenous tissue of the cornea. The results undoubtedly strengthen the clinical reports of Lyle and McLean (1941) and of Summers (1946), and it is hoped at a later date to verify the optimistic reports of these workers on other corneal conditions.

Pyorrhoea

From time to time the importance of focal spesis has been stressed as an aetiological factor in ocular disease. In recent years, however, the tendency has been to ignore this factor. This whole matter has been recently reviewed by Duke-Elder and Goldsmith (1951).

There are several possible explanations of the higher incidence of corneal ulceration in patients with pyorrhoea. The cornea is frequently subjected to damage, and is daily receiving minor epithelial abrasions. In a patient with pyorrhoea the/

the transference of organisms to the eye could occur very readily. A blood stream spread is less likely since the cornea is avascular. A more likely route would be by direct transference with the fingers from the mouth to the conjunctival sac.

Our results could also be explained by assuming that the general health of individuals with pyorrhoea is below normal, and that they will in turn be more liable to develop corneal infection. Although detailed clinical examination was not carried out in these patients we had a definite impression that pyorrhoea occurred both in those whose general health was poor and also in those who appeared to be quite fit.

SUMMARY

1. Fifty-one cases of small acute corneal ulcers in man were examined, and the healing time ascertained by instillation of sodium fluorescein under standard conditions.

2. Approximately half the patients received 1.5 g. of ascorbic acid daily; the remainder were given placebo tablets.

3/

3. Deep ulcers healed significantly more slowly than superficial ulcers.

4. The administration of the massive dose of ascorbic acid had no significant effect upon the healing time of superficial ulcers, but significantly accelerated the healing of deep ulcers.

5. It was concluded that there may be a localised area around the site of regenerating corneal collagen where the ascorbic acid level falls below the optimum for rapid healing. Raising the general ascorbic acid level with massive doses may increase the local rate of replacement.

6. Pyorrhoea was observed significantly more frequently in patients with corneal ulcers than in others with non-infective eye complaints.

Chapter Vll

The ACTION of the ADRENOCORTICAL HORMONES on CORNEAL WOUND HEALING

While it is clear from the experimental results outlined in Chapters 5 and 6 that ascorbic acid plays a vital role in corneal wound healing, it is impossible to establish from these results whether ascorbic acid acts directly at the site of healing or indirectly. As certain glucocorticoids, such as Compound E, are known to depress granulation tissue formation in the rabbit (Ragan, Howes, Plotz, Meyer and Blunt, 1949) it is possible that ascorbic acid acts indirectly by influencing glucocorticoid formation in the adrenal cortex. The experiments presented in this chapter were undertaken to test this hypothesis.

Ascorbic Acid and the Adrenal Cortex.

Many investigators have noted a close correlation between adrenocortical activity and the ascorbic acid content of the adrenal gland. An increased adrenocortical activity is associated with a reduction in the/

the concentration of adrenal ascorbic acid.

Administration of ACTH depletes the adrenal ascorbic acid in the rat and the guinea-pig. This action is surprisingly rapid and consistent in the rat (20 minutes) and has been used for assaying ACTH preparations (Sayers, Sayers and Woodbury, 1948). Furthermore, in a hypophysectomised rat, adrenal ascorbic acid is unaffected by stress although the same degree of stress in a normal animal would lower the ascorbic acid concentration (Sayers, Sayers, Liang, and Long, 1945). This finding suggests that physiological quantities of ACTH do influence the ascorbic acid content of the adrenal gland. Harris (1951) has recently reviewed the possible mechanisms of ACTH release from the anterior pituitary gland.

The role of ascorbic acid in the metabolism of the adrenal is unknown. Lowenstein and Zwemer (1946) have reported the isolation and identification of a water soluble conjugate of a cortical steroid and ascorbic acid, but their observations have not been confirmed. If these workers are correct than an increase in the activity of the adrenal gland should increase the concentration of ascorbic acid in the blood/

blood leaving the gland. However, Vogt (1948) was unable to detect a significant difference in ascorbic acid concentration between the blood entering and that leaving the gland under conditions of increased activity.

Several workers have noted the similarity between scurvy and the signs of adrenocortical insufficiency. Lockwood and Hartman (1933) found that an adrenocortical extract, free from ascorbic acid, retarded the onset of signs of scurvy and diminished the body weight loss. Similarly Ratsimamanga (1944) claimed that adrenocortical extract increased the survival time of scorbutic cavies. Kendall (1948) also noted the longer survival time in scorbutic animals, but found that the extract did not alter the pathological signs found at death. Clayton and Prunty (1951), on the other hand, found that there is an increase in the activity of the adrenal cortex during scurvy in guinea-pigs. They estimated the activity of the adrenal cortex by measuring the output of the urinary 17-ketosteroids and by examining the weight of the adrenal gland. In all animals the 17-ketosteroid excretion/

excretion showed a gradual but definite increase as the animals became scorbutic. Excretion reached a peak in the terminal stages. Furthermore, adrenal glands from scorbutic cavies showed an increase in weight over the control animals. Daily administration of ACTH and cortisone failed to influence the fall in body weight or time of death of the guinea-pigs.

Many workers have noted that the scorbutic animal is hypersensitive to stress and has a reduced natural resistance to infection. (Parrot and Richet, 1945). In man there is a marked increase in the rate of utilisation of ascorbic acid following stress or trauma such as fevers, burns or fractures (Andreae and Browne, 1946; Beattie, 1947).

On the other hand, there is considerable evidence to suggest that ascorbic acid does not play an essential role in the metabolism of the adrenal cortex. For example, administration of adrenocortical extracts does not alter the impairment of deposition of liver glycogen found in scorbutic animals (McKee, Cobbey and Geiman, 1947; Murray, 1948). Human cases of scurvy excrete corticoids at a normal rate (Daughaday, Jaffe and Williams, 1948).

The/

The ascorbic acid content of the chicken adrenal is not influenced by ACTH administration. Finally, ACTH produces a fall in adrenal cholesterol and a lymphopenia after the ascorbic acid in the adrenal has been reduced practically to zero by a scorbutic diet (Long, 1947).

Sayers (1950) states that "ascorbic acid does not appear to be an essential component of these metabolic processes of the adrenal concerned with the secretion of cortical hormone. It is possible that some other constituent of the gland, i.e. glutathione, can substitute for the vitamin".

It would appear that the concentration of ascorbic in the adrenal gland is a fairly reliable guide of adrenocortical activity in acute experiments in a well nourished and healthy animal. In chronic experiments, or work involving poorly nourished animals, other factors, such as the rate of synthesis of ascorbic acid, probably vitiate the value of the adrenal ascorbic acid as an index of adrenocortical activity.

Experimental/

Experimental Series 1

Although the exact role of ascorbic acid in the metabolism of the adrenal gland is still unknown there is definite evidence that it does enter into the metabolism of the adrenal cortex. In view of the findings outlined in chapter 5 I felt justified in investigating the effect of adrenocortical hormones on corneal wound healing under similar conditions.

Initially I was unable to obtain a supply of cortisone for this purpose, and the first experiment was carried out using sodium γ-resorcylate. This is a compound which has been recently introduced by Reid, Watson, Cochran and Spreull (1951). It has been used in the treatment of acute rheumatic fever, and has been found to be ten times more effective therapeutically than sodium salicylate in this condition. The clinical effects of this drug resemble in many ways those of ACTH and of cortisone. This similarity has been supported by investigations on experimental animals by Buttle (1951) and Prunty (1951).

I am indebted to Dr J. Reid of the M.R.C. Chemotherapeutic Research Unit in Glasgow for a generous supply of sodium γ-resorcylate and for advice on dosage/

dosage and administration.

M E T H O D

Animals

Ten young male and ten virgin female guinea-pigs were used. The 20 animals were paired for weight and sex, and divided into a control and principal group. All animals were kept in two large cages , the 5 male control animals being kept with the 5 male principal animals in one cage, while the femals were kept in the other cage.

Diet

All cavies received greens, rat cake and water without stint.

Drugs

The principal group of animals was given intraperitoneally 25 mg. of sodium γ-resorcylate in 0.5 ml. distilled water daily. The control group received only the water intraperitoneally.

Injuries

Three days after commencing this administration standard heat injuries were inflicted on both cornea/

cornea of all animals under local anaesthia. The technique was similar to that described on page 58.

Estimation of Healing

Healing was estimated 12 hourly by the method described in chapter 3.

Histology

Eleven days after inflicting the injuries the eyes were enucleated, placed in 10% neutral formalim and later sectioned, using the celloidine method. Sections were stained in the usual manner with haemalum and eosim.

R E S U L T S

Weight

It can be seen from Fig.10 that there is no appreciable difference in the rate of gain of weight between the control and principal groups. Sodium γ-resorcylate in the dose of 25 mg. daily has not significantly affected growth.

Rate of Epithelial Healing

The fluorescent gradients obtained in this experiment/

experiment are shown in Fig.11. It can be seen that there is no appreciable difference in the rates of healing between the two groups for the first $4\frac{1}{2}$ days. Thereafter, however, the mean intensity of fluorescence of the group receiving sodium γ-resorcylate began to increase slightly and to fluctuate around a low value. On the other hand, the mean fluorescent intensity of the control group remained fairly steady near the base line.

The reason for this delay in healing becomes clear if we examine the fluorescent gradients of individual corneae. The upper 4 records in Fig.12 are taken from 4 corneae in the control group. It will be noted that most of the corneae ceased to fluoresce fairly rapidly, but in some cases a faint fluorescence reappeared, thereafter to disappear permanently. In the group receiving sodium γ-resorcylate, however, the intensity of fluorescence increased quite markedly, and frequently after the initial rapid stage of healing.

In the control group of animals only six lesions showed this recrudescence of fluorescence after/

after reaching zero intensity, while in the principal group 16 lesions fluoresced after reaching zero intensity. This difference in the incidence between groups is significant (Table 14).

A possible explanation of this phenomenon is that the corneae of the cavies are constantly receiving mild traumata in the communal life of their cages, and that these abrasions are especially liable to cause attenuation of the delicate layer of epithelium which has just formed over the base of the lesions. It may well be that in the group of animals receiving sodium γ-resorcylate there is some delay in the formation of a suitable collagenous substratum and as a result the epithelium is unable to adhere adequately to the underlying stroma.

Histological Examination

After healing had progressed for 11 days the eyes were enucleated, fixed and sectioned. Representative sections from control and principal eyes are shown in Fig. 13. Special care was exercised to ensure that the sections were made through the centre of the lesion.

The/

The upper section is from a control animal, and the lower section is from a principal receiving sodium Y-resorcylate. The cellular infiltration and proliferation in the cornea from the animal receiving sodium Y-resorcylate is clearly less marked when compared with the control section. In the latter group the fibrous tissue reaction is so well marked that the cornea at the site of healing is thicker than the normal cornea. On the other hand, in most of the sections from the group receiving sodium Y-resorcylate the cornea is thinned at the site of healing.

This difference in reaction between the two groups appears to be quantitative only. No qualitative difference could be detected. Fibroblasts, fibrocytes and eosinophil cells were present, but there was no difference between the groups in the relative proportion of the various types of cell present.

COMMENT

From the histological results it is clear that sodium/

sodium γ-resorcylate has profoundly modified modified the mode of stromal repair. Although there is no obvious qualitative difference in the tissue reaction to the heat injury, there is a well marked quantitative difference. It seems likely that the action of the sodium γ-resorcylate is to retard the cellular reaction.

In contrast, the rate of epithelialisation as judged by the fluorescein method is only slightly affected. Indeed, there is no difference during the first 4½ days. This result differs from that obtained in the scurvy experiments where a delay in epithelialisation was noted as early as 16 hours after injury. Moreover, there was little tendency in the scurvy experiments for the epithelium covering the injured site to break down again after covering the bed of the lesion.

However, the results obtained with sodium γ-resorcylate were sufficiently encouraging to justify proceeding with the next experiment.

Experimental Series 2

After the completion of experiment 1, sufficient/

sufficient cortisone was given by the Medical Research Council to enable this experiment to be undertaken. The cortisone used in this work was provided from a generous gift made jointly to the Medical Research Council and to the Nuffield Foundation by Merck & Co. Inc.

METHOD

The method used was identical with that described on page 96 under Animals, Diet, Injuries and Histology.

Drugs

Each animal in the principal group was given intraperitoneally 12.5 mg. of 11-dehydro-17-hydroxycorticosterone-21-acetate daily. The cortisone was supplied as a saline suspension of strength 25 mg./c.c. It also contained unknown suspending agents and 1.5% benzyl alcohol as a preservative. Each animal in the control group received intraperitoneally 0.5 c.c. of Aqueous Vehicle No.1 daily. This preparation was a saline solution containing suspending agents and 1.5% benzyl/

benzyl alcohol, supplied also by Merck & Co. Inc. This administration was started 3 days before the corneal injuries were inflicted.

R E S U L T S

Weight

It may be seen from Fig.14 that there is no appreciable difference in the rate of gain of weight between the groups during the first four days. After this point the group receiving cortisone did not gain in weight as rapidly as the control group. The difference is small, however, and the administration of cortisone over this short period has not unduly affected the growth rate.

Rate of Epithelial Healing

The fluorescent gradients obtained in this experiment are shown in Fig.15. The lesions from the animals receiving cortisone tend to fluoresce more brightly than the control lesions, from as early as 24 hours after injury. This difference is maximal about the third day, thereafter the difference in mean intensity diminishes.

End/

End Point of Healing

Table 15 shows the mean time for epithelial healing. Healing was taken to be the time of reaching the first of two zero recordings of fluorescence intensity. It will be noted that the delay in the end point of healing in the principal group does not differ significantly from the control group.

Recrudescence of Fluorescence

As in the first experiment described in this chapter the principal corneae tended to refluoresce again after reaching zero intensity rather oftener than the control lesions. However, the difference in incidence between control and principal groups was not significant statistically.

Histological Examination

After healing had progressed for 9 days the eyes were examined histologically. The upper section in Fig.16 is from a control animal and the lower section is from a principal receiving cortisone. It is clear that the cellular reaction in the principal cornea/

cornea is less marked than in the control cornea. This difference in reaction between the two groups is quantitative and not qualitative.

Other sections examined showed closely similar results.

C O M M E N T

The results obtained from this experiment are very similar to those obtained with sodium Y-resorcylate. The cortisone, however, has delayed epithelialisation from an earlier stage when compared with fluorescent gradient obtained from the sodium Y-resorcylate treated group. The histological appearances of the sections from both experiments are very similar.

Certain differences were noted in the incidence of corneal vascularisation in these experiments, but this aspect will be treated for convenience in chapter 10.

Experimental Series 3

After completion of experiment 2 it was felt that it might be of interest to find the effect of cortisone/

cortisone on a more superficial wound of the cornea. An opportunity to study this point arose during the experiment on corneal vascularisation reported in Chapter 10.

METHOD

The method used was identical with that described on pages 96 and 97 under Animals, Diet, Drugs and Histology.

Injuries

The injuries were inflicted according to the method described on page 58. In this experiment, however, the temperature of the cautery was slightly lower, so that a superficial injury was produced involving only the anterior one-third of the corneal stroma.

RESULTS

Weight

It may be seen from Fig.17 that the administration of 12.5 mg. of cortisone acetate per day has only slightly diminished the rate of body growth when compared/

compared with the control group.

Rate of Epithelial Healing

The fluorescent gradients obtained in this experiment may be seen in Fig.18. There is clearly no difference in the rate of epithelial healing between groups.

The rate of decrease of fluorescence is, however, more rapid than in the previous experiment (Fig.15) presumably due to the more superficial nature of the injury.

There was no significant difference in the end points of healing between groups.

Recrudescence of Fluorescence

There was a slight tendency for a few injuries to refluoresce. The difference between groups was not significant. The number of corneae which showed this tendency was much smaller than in the first and second experiment in this chapter.

Histological Examination

All animals in this experiment were killed on the 18th day of healing. Examination showed/

showed little difference between control and cortisone treated groups. This finding is almost certainly due to the small amount of stroma involved and to the lateness of the examination (18th day).

D I S C U S S I O N

Superficial Heat Injuries

The administration of cortisone to a cornea with only a superficial injury does not seem to delay the rate of epithelialisation appreciably. As the rate of epithelialisation is so rapid it is probable that healing is by means of a sliding action and not by cellular proliferation of the epithelium. It would thus appear that cortisone does not delay the epithelial healing of small superficial heat injuries.

Deep Heat Injuries

A definite delay in the rate of epithelialisation was demonstrated when cortisone was administered to animals with a deep corneal injury involving the anterior two-thirds of the stroma. As simple epithelialisation does not seem to be retarded/

retarded (Experiment 3) it may be that the delay with deep injuries is due to the defective formation of a suitable collagenous substratum. Indeed, the histological examination of those deep injuries on the 9th day does suggest that there is a considerable delay in the fibrous tissue regeneration of these cortisone treated eyes. A similar hypothesis was advanced in Chapter 5 to account for the delay in epithelialisation which occurred in deep injuries in scorbutic animals.

Ascorbic Acid and Cortisone Acetate

If the mode of healing of the corneae in scurvy (Chapter 5) is compared with the observations made in this chapter, a remarkable degree of similarity will be noted.

A deficiency of ascorbic acid appears to affect wound healing in a way similar to an excess of glucocorticoid administration. This is surprising in view of the opinion of several workers (Lockwood et al,1933; Ratsimamanga,1944; Kendall, 1948) that the signs of scurvy resemble the/

the signs of adrenocortical insufficiency, and that the survival time of scorbutic cavies may be prolonged by administering adrenocortical extract. However, Clayton and Prunty (1951,a) have shown that there may be an increased output of adrenocortical hormones in scurvy. Could the delay in wound healing which occurs in scurvy be wholly or partly accounted for by this hyperactivity of the adrenal cortex? At the present time there is insufficient evidence to answer this question (Prunty, 1951, personal communication).

It may be that the systemic administration of cortisone as in my experiments has upset the balance, which Selye (1950) believes to exist, between the mineralocorticoids and the glucocorticoids. The administration of cortisone may have decreased the ACTH production from the anterior pituitary gland and thus in turn diminished the mineralocorticoid output of the adrenal cortex. It is unlikely that my results could be explained on this basis, however, for the/

the local application of cortisone acetate to the eye is known to delay corneal stroma regeneration (Newell and Dixon, 1951) in a similar manner.

It is clear that while the experiments here described have answered the immediate questions asked, the results have not assisted in defining the connection between ascorbic acid and the adrenal cortex. They do, however, add further weight to the belief that ascorbic acid and the hormones of the adrenal cortex exert, indirectly or directly, a profound influence on new fibrous tissue formation.

Sodium γ-Resorcylate

It is interesting to note that the administration of a substance with a structure as simple as sodium γ-resorcylate should produce a histological picture so similar to that found in the cortisone treated eyes. However, in view of the definite difference in the mode of epithelial healing (Figs.11 and 15) it would be unwise to conclude that their action on wound healing/

healing was identical.

Clayton and Prunty (1951) used the Sayers test (Sayers, Sayers and Woodbury, 1948) and failed to observe adrenocorticotrophic activity with resorcylate in hypophysectomised rats. It thus seems improbable that this substance acts directly on the adrenal cortex although the possibility remains that it acts through stimulation of the anterior pituitary.

Reid (1951), after weighing all the evidence obtained so far, considers it probable that sodium γ-resorcylate owes its action to chelate ring formation and thinks it conceivable that this also applies to cortisone. It may thus have a local action similar to cortisone. Further discussion on its mode of action would be fruitless until further experimental work has been completed.

SUMMARY

1. The role of ascorbic acid in the metabolism of the adrenal cortex is reviewed.

2./

2. Standard heat injuries were inflicted on the corneae of guinea-pigs receiving sodium γ-resorcylate, cortisone acetate or a control solution.

3. Sodium γ-resorcylate administration did not alter the rate of epithelialisation of deep injuries for the first four and a half days, but thereafter the healing lesions tended to break down and to fluoresce once more.

4. Histological examination on the eleventh day showed a marked diminution in the cellular reaction at the site of healing when compared with control sections.

5. Administration of cortisone acetate to cavies with deep corneal burns produced a delay in the rate of epithelialisation.

6. A well marked reduction in cellular proliferation at the site of the lesion was noted on the ninth day of healing.

7. Cortisone acetate did not influence the rate of epithelialisation or the histological appearance of the wound in superficial heat injuries.

8/

8. The results of these experiments are surprisingly similar to the results obtained from the scorbutic guinea-pig experiments outlined in Chapter 5. The interpretation of these findings is discussed.

PART TWO

CORNEAL VASCULARISATION

Chapter VIII

CORNEAL VASCULARISATION in the RABBIT

Although the cornea normally contains no blood vessels, under certain conditions new vessels freely enter the substance of the cornea from the limbal plexus. This neovascularisation occurs in a wide variety of conditions, and many attempts have been made to find a general theory which would adequately account for this vascular invasion under diverse conditions.

Several methods have been used experimentally to produce vascularisation in animals under control conditions. Ehlers (1927) made daily applications of ethyl alcohol to the cornea of rabbits resulting in a loss of transparency and an ingrowth of vessels from all round the limbus into the anterior two-thirds of the corneal stroma. He observed and beautifully illustrated the evolution of corneal vascularisation by this method.

Julianelle and Lamb (1934) and Julianelle and/

and Bishop (1936) studied the resulting new vessel formation that occurred after intracorneal injection of proteins in the sensitized rabbit and monkey.

Swimdle (1938) produced vascularisation by inducing corneal ulcers.

The interest in vesicant gasses during the recent World War led to many attempts to produce standard corneal injuries with these agents. Opinions differ widely as to the success of these attempts.

The method for producing standard heat injuries described in Chapter 5 seemed ideal for producing small discreet corneal injuries at any point on the cornea, and this technique was applied to study corneal vascularisation in the rabbit.

This investigation was stimulated by the clinical observation that a localised lesion of the human cornea is often accompanied by vascularisation if the lesion is sufficiently near to the limbus.

EXPERIMENTAL

Animals/

Animals

Healthy adult rabbits, both pigmented and albino, were used.

Diet

The animals were fed on bran and green vegetables. Six drops of cod liver oil were added to the diet daily.

Apparatus

The lesions were produced by means of the electric cautery described on page 55.

The vascularised area in the cornea was demarcated by injecting Indian ink into the carotid arteries. The injection apparatus is shown in Fig.19. It consisted of a 2 lb. carbon dioxide cylinder fitted with a reducing valve. The pressure of the gas flowing from the valve could be controlled by adjusting the spring of the valve. The pressure was measured with a simple mercury manometer. A 250 c.c. graduated measuring cylinder was fitted with a three hole rubber stopper. This cylinder was filled with Indian ink/

ink through a funnel. When the gas cylinder valve was opened the ink was forced out of the measuring cylinder along the exit tube. This tube was connected to a length of rubber tubing to which was attached a small cannula.

TECHNIQUE

Anaesthesia

The cornea was anaesthetised by instilling into the conjunctival sac two drops of a 2% amethocaine hydrochloride solution (B.P.). Anaesthesia was completed in 2 minutes. This degree of anaesthesia was found to be sufficient, for the animals did not elicit the corneal reflex during the operation, and they behaved quite normally afterwards.

Procedure

A standard lesion was produced by applying the cautery to the cornea and allowing the current to flow for one second. The burn to each cornea was repeated daily on the same site until adequate vascularisation resulted.

The/

The resulting lesion was about 1 mm. in diameter and involved the epithelium and the superficial two-thirds of the substantia propria (Fig.10). In two cases infection of the wound occurred, and these animals were excluded from the experiment.

For simplicity, all the injuries were placed along the 12 o'clock meridian of the cornea. Their distance from the limbus, however, was varied from 1.4 mm. to 5.3 mm. The radius of an adult rabbit's cornea is about 6 mm.

At the end of the serial cauterisation the rabbit was anaesthetised deeply with ether, the carotid arteries exposed and a cannula inserted, directed cranially into each artery. 5-10 c.c. of a 50% dilution of Indian ink (Reeves) in water was then run in under a pressure of 100-150 mm. Hg., until the vascularised area of the cornea was filled with ink particles.

The animal was then killed with chloroform. The eyes were enucleated and placed in 10% neutral formalin for 48 hours. For examination the/

the whole corneal thickness including the limbus was mounted in 50% glycerine.

The vascularised area was measured with a microscope eye piece micrometer, and with a Vernier moving stage.

RESULTS

Sequence of Events

Within a few hours of the first injury a congestion of the conjunctival blood vessels occurred in that quadrant of the limbus nearest to the lesion. This congestion usually passed off in 8-12 hours. There was then a latent period lasting from 24-48 hours, when no change could be observed in either the conjunctival or limbal vessels. The nearer the burn was to the limbus the shorter the latent period tended to be.

The first change observed in the limbal plexus on slit-lamp examination was an engorgement of the capillaries and venules nearest to the lesion. The venules increased to an enormous size compared with the arterioles.

During/

During the next 48 hours this engorgement continued and some of the distended venules showed saccular aneurysms on the side facing the lesion.

The next stage in the development of corneal vascularisation was difficult to observe due to its rapidity. The aneurysms suddenly ruptured, and the expelled blood forced its way into the tissues of the substantia propria and formed spicule-like hemorrhages radiating out from the site of the aneurysms (Fig. 21). After this event the venules returned to a more normal size.

I was unable to observe the rupture of an aneurysm but this event has been seen and described by Cogan (1949) - "One of the larger aneurysms erupted, and small columns of blood moved into the adjacent portions of the stroma, losing all connection with the blood vessel. The formation of these small hemorrhagic extravasates, as they were seen to be on histological examination, reminded one of groups of miniature freight cars being backed into a freight yard. The hemorrhages occurred/

occurred at intervals of several minutes, and the aneurysms slowly disappeared. Within the subsequent hour, several other aneurysms erupted similarly and disappeared, leaving a venule much reduced in caliber, and showing only a few small unerupted aneurysms on the side of the venule away from the lesion".

The shape of these spicule-like hemorrhages seemed to be determined by the structure of the corneal stroma. The hemorrhages opened into the venule from which it had formed, but there was no circulation of red cells in and out of it. The section shown in Fig. 22 was well injected with Indian ink and yet none of the ink penetrated into the spicule-like hemorrhages. The hemorrhages always pointed in the direction of the centre of the cornea and not towards the lesion.

This event was quickly followed by the formation of a maze of fine capillaries in the cornea which gradually replaced the hemorrhages. It was impossible to observe where these capillaries came from or what cells form the walls.

This/

This maze of capillaries was gradually replaced by a few larger vessels which form regular loops. The original fine capillaries regress and finally disappear. The larger capillaries extended by forming arches between the arterioles and venules. I have the impression that these arches form from the capillaries of the original capillary maze.

In actual experience the vessel formation was not so well ordered as I have outlined above, for as additional injuries were made to the cornea, fresh outbursts of hemorrhages might occur from some of the new formed vessels in the cornea itself. At times several stages were observed simultaneously in one cornea.

Several days after the last injury, the vessels would gradually become less congested, and the blood flow through them diminish. One by one the capillary loops closed down until all the new vessels had finally disappeared. The regression of these vessels may take as long as 3-6 weeks after the lesion has apparently healed. It is possible to observe the larger of the empty capillaries with the/

the slit-lamp as "ghost vessels" for many months thereafter.

We can now summarize the stages of new vessel formation in the cornea in response to a localised heat injury as follows:-

(1) A latent period of 24-48 hours.

(2) Congestion of the limbal plexus.

(3) Aneurysm formation of the venules.

(4) Rupture of the aneurysm with the formation of spicule-like hemorrhages.

(5) Formation of a fine plexus of capillaries.

(6) Formation of a definitive network of capillaries which probably extends by peripheral looping.

(7) Regression and final disappearance of these vessels.

Relation of Site of Lesion

It will be seen from Fig.23 that the vascularised network tends to have a triangular distribution, with the apex of the triangle towards the lesion. During preliminary studies it was noted that the distance of the lesion from the limbus/

limbus modified to some extent the size and shape of this triangular area of new vessel formation. It was therefore decided to measure the dimensions of this triangle with the lesion at different distances from the limbus. It is excessively difficult to make accurate measurements with a slit-lamp in the intact animal, due to small movements of the animal's head. The measurements were, therefore, made on corneae which had been injected with Indian ink and mounted in 50% glycerine.

Measurements were taken as shown in Fig. 24.

Experimental Series 1.

Focal points in the right corneae of five adult rabbits were cauterised daily for 13 days. In four of the corneae a typical triangular vascular area was produced. In these corneae, as in the others subsequently examined, the vascular triangle was isosceles. The results are shown in Table 16.

In rabbits A and B, with a lesion 1.7 mm. from the limbus, the length of "d" (Fig. 24) was the same - 3.3 mm. That is, vessels grew from all parts of/

of the limbus within 3.3 mm. of the centre of the lesion. Again, in rabbits C and D, with the lesion 2.1 mm. from the limbus, vessels grew from all parts of the limbus within 3.2 mm. of the lesion. On the other hand, when the lesion was placed 4.2 mm. from the limbus, as in rabbit E, the limbal vessels were not affected, and no vascularisation was produced.

The surprising constancy of the measurements for "d" in these four corneae made it imperative to repeat the experiment on a larger scale, to exclude the possibility of a chance finding.

Experimental Series 2

This series was carried out along similar lines, but both corneae were injured in each rabbit under local anaesthesia. In this group only 10 successive daily cauterisations were done. Moreover, the cautery point was larger, and the temperature slightly higher in this experiment, so that although the standard lesion was constant for this experiment, the results differ from these in Series 1.

The/

The results in the second series of eight rabbits involving 16 corneae, are shown in Table 17. Triangular areas of corneal vascularisation were obtained in all the eyes. The experiment on the left eye of rabbit G was spoiled by an error in the technique. In rabbit F the wounds became infected, as shown by a general redness of the conjunctiva, and the discharge from the eyes. These three corneae were therefore excluded from the experiment. All the other eyes were white and without discharge.

As will be seen from Table 17 in the remaining 13 corneae the distance from the centre of the standard lesion to the basal angle of the vascular triangle is fairly constant. The mean of the measurement of "d" in these 13 corneae is 4.2 mm. with a standard deviation of 0.21 mm. The range is from 3.8 to 4.4 mm.

In cornea H right and H left, the triangle of pigmentation was noted to occupy the same position as the vascular area (Fig.25). This pigmented triangle had formed from the limbal ring of/

of pigment by the spread and migration of the epithelial cells from the limbus. In the remaining rabbits limbal pigmentation was very slight or absent.

In all cases where new vessels appeared in the cornea, the vessels were confined to the anterior (superficial) two-thirds of the corneal stroma (Fig.26).

Experimental Series 3

Special care was taken to carry out these experiments under conditions identical with those of Series 2. Six corneae were cauterised at 4.5, 4.6, 4.8, 5, 5.1 and 5.3 mm. from the limbus. In no case was ingrowth of limbal vessels observed.

D I S C U S S I O N

These results suggest that if a lesion in the rabbit's cornea is sufficiently close to the limbus, it can produce an area of vascularisation which has the form of an isosceles triangle. If, however, the lesion is placed beyond a certain critical distance from the limbus then no vascular-

isation/

vascularisation results. The distance probably depends also upon the magnitude of the lesion.

The shape of the vascular triangle will depend upon the distance of the lesion from the limbus. As the sides "d" are always of the same length the base of the triangle must vary with distance. The shape of the resulting vascular area can be readily appreciated by studying Fig. 27. Each large black circle represents a standard lesion, and the distance between the concentric lines is 1 mm. It can be seen from Fig. 27 that the closer the lesion is to the limbus the broader is the base of the triangle.

I suggested in 1949 (Campbell and Michaelson, 1949) that these results could be explained by assuming that the injury liberated a factor which diffused to the limbus and stimulated new vessel formation. The experiments, however, shed no light on the possible nature of this factor. Furthermore, the results could be explained by other theories.

Cogan (1949) believes that vascularisation

of the cornea never occurs without oedema of the corneal stroma. This observation was also made by Julianelle et al (1934) and by Mann and Pullinger (1942) while studying mustard gas keratitis. The presence of oedema fluid in the cornea opens up the spaces between the fibres of the substantia propria and this in turn may allow ingrowth of the limbal capillaries.

While it is true that in my experiments corneal oedema did occur and did spread to the limbus, it is difficult to believe that this physical factor is the only one operative in inducing new vessel formation. It is quite common in clinical experience for corneal oedema to be present for many days without new vessel formation.

It has been suggested by Wise (1943); Bacsich and Riddell (1945); Bacsich and Wyburn (1947) that the cornea may be avascular, due to the presence of an inhibiting substance in the cornea. These workers base their theory on the fact that cartilage, Wharton's jelly, and cornea give a metachromatic/

metachromatic staining reaction with toluidine blue, and are also avascular. Meyer and Chaffee (1940) have identified the mucopolysaccharide of the cornea as a monosulphuric acid ester of hyaluronic acid. Woodin (1950 a,b) has thrown some doubt on this conclusion, however, and hopes to identify the substance at a later date (Personal communication). It is possible that a localised burn would destroy this substance and permit invasion of blood vessels into the cornea.

The results could also be explained by assuming that the trauma to the cornea increases the metabolic requirements above the normal level. This increase could be due to the increased number of polymorphonuclear leucocytes and wandering cells which are known to invade the rabbit's cornea in response to an injury. This increased oxygen demand might produce an area of relative anoxia around the lesion. This low oxygen tension may act as a stimulus to new vessel formation from the limbus if the lesion is sufficiently close to it.

It can thus be seen that the above experiments may/

may be equally well explained on several rather different hypotheses. Further work should be designed to establish which of these mechanisms is effective. In the next chapter of this thesis the role of ascorbic acid in corneal vascularisation will be considered, and the results discussed in the light of the theories of corneal vascularisation outlined here.

SUMMARY

1. Corneal vascularisation was induced in the rabbit by inflicting small discrete heat injuries daily.

2. The evolution of the resulting vascularisation is described stage by stage.

3. In another series of experiments it was found that if the lesion was placed farther than a certain critical distance from the limbus no vascular response occurred. At less than this distance vascularisation occurred and the vascular area had the form of an isosceles triangle/

triangle.

4. If a series of "standard lesions" be placed at different distances from the limbus, but within the critical distance for that lesion, the distances between the sites of the lesion and the basal angles of the triangular vascular areas are fairly constant.

5. The results suggest that new-vessel formation in the cornea, under the conditions of this experiment, involves a factor released by the lesion which diffuses to the limbus and induces vascularisation.

Chapter IX

The ROLE of ASCORBIC ACID in CORNEAL VASCULARISATION

During the experiment on the effect of scurvy in the healing of deep corneal injuries outlined in Chapter 5, the eyes were also examined for new vessel formation and corneal oedema. The results of these observations are presented in this chapter.

METHOD

The method used was identical with that described in Chapter 5 under Animals, Diet, Ascorbic Acid and Injury.

Measurement of Vascularisation and Oedema

The corneae were examined with intense focal illumination and a Zeiss binocular loupe. Observations were made 8 hourly.

Depending on the extent and density of new vessel/

vessel formation the vascularisation was recorded as 0, +, ++ or +++. This method of recording vascularisation, although approximate, allowed quick classification.

The notation was similar for the degree of corneal oedema around the lesion.

R E S U L T S

Effects on Vascularisation

During healing vascularisation of the cornea occurred in 9 eyes out of 32 controls and in 19 out of 32 deficient animals (Table 18). The results were analysed by the 'chi' square method and it was found that the greater incidence of vascularisation in the scorbutic animals was significant.

This higher incidence may be due to the greater time required for healing in the group of deficient animals (Table 19), for it is a common clinical observation in man that a corneal ulcer of long duration is more likely to vascularise than one of short duration. If this/

this explanation is true, then the eyes which have vascularised will be associated with the injuries which have taken longer to heal. Table 20 shows the mean time of healing for the vascularised and nonvascularised eyes in the scorbutic group. The difference of 4.5 hours between the two groups is not significant. A greater difference in the means exists in the control group (Table 21). The difference of 17.7 hours is, however, not significant. There is thus no conclusive evidence to suggest that under the conditions of this experiment the greater incidence of vascularisation in the scorbutic group is due to the longer time required for epithelial healing.

The onset of vascularisation, the time of its maximum extent, and the time of disappearance all tended to be delayed in the scorbutic group compared with the control group (Table 22).

Effects/

Effects on Corneal Oedema

The time of onset and the time of maximum oedema are shown in Table 23. The difference between the means of the times of onset is not significant. However, the scorbutic group did take significantly longer to reach the time of maximum oedema. The figures could not be analysed for the time of disappearance of the oedema as some of the animals were killed for histological examination before the oedema had disappeared.

D I S C U S S I O N

In experimental scurvy in guinea-pigs, ascorbic acid is known to disappear from the cornea in from 2 to 3 weeks (Henkes,1946). Nevertheless, the cornea shows no obvious change even in severe and prolonged scurvy in human beings or in guinea-pigs. It would thus appear that ascorbic acid is not essential for normal corneal metabolism. This is surprising in view of its high concentration in the cornea.

These negative findings do not apply, however, to the injured cornea. The results of the experiment above suggest that the injury unmasks a deficiency not otherwise apparent. Presumably new formation of collagen makes additional metabolic demands which cannot be adequately met in a state of scurvy. As a result there is a tendency for new vessel ingrowth.

It is interesting to note that a similar theory is being formulated to account for the vascularisation of the cornea which occurs in ariboflavinosis. Recent work suggests that spontaneous vascularisation does not occur in man even in severe riboflavine deficiency (Gordon and Vail, 1950), although vessel ingrowth may occur readily in this condition if the cornea is damaged (Landau and Stern, 1948). Stern (1950) has reviewed the problem of corneal vascularisation in ariboflavinosis and states:- "The appearance of corneal vascularisation may be/

be precipitated by conditioning factors such as chemical or mechanical trauma to the cornea in the presence of a subliminal riboflavin deficiency".

It may well be that avitaminosis C may also lead to vascularisation when there is an extra metabolic demand following on injury. There is no evidence so far that this conclusion, based on the guinea-pig cornea, also applies to man.

This experiment throws little light on the nature of the stimulus for vascularisation. Failure to satisfy fully a metabolic need, however caused, may lead to an accumulation of metabolites. These metabolites could act as a direct stimulus to new blood-vessel formation. On the other hand, the metabolites may lead to oedema and opening up of the lamellae of the substantia propria and thus permit invasion of blood vessels such as Cogan has suggested. Against this theory, however, is the finding in this experiment that the degree of oedema was

no/

no greater in the corneae that became vascularised. Again, the stimulus may be a local oxygen deficiency around the site of the trauma due to loss of vitamin C in its role as an H acceptor.

It is clear that final explanation of this experiment must wait until the metabolic role of ascorbic acid in the tissues is more completely understood.

SUMMARY

1. Standard heat injuries were inflicted on control and scorbutic guinea-pigs at a constant distance from the limbus.

2. New vessel formation occurred with significantly greater frequency in the scorbutic group than in the control group.

3. The causal factor in new vessel formation is discussed in the light of these findings. It is suggested that repair of an injury makes additional metabolic demands which can only be met, in a state of ascorbic acid deficiency, by the formation of new vessels.

Chapter X

The ACTION of the ADRENOCORTICAL HORMONES on CORNEAL VASCULARISATION

Although the cornea is normally an avascular structure, new blood vessels enter the stroma in response to a wide variety of stimuli. These new vessels can be readily examined in vivo and the effect of any agent on vascularisation can be accurately assessed.

Jones and Meyer (1950) have reported that subconjunctival injections of cortisone acetate in the rabbit markedly inhibit the vascularisation of the cornea which follows intracorneal injections of sodium hydroxide solution. Moreover, several other workers have noted that cortisone causes a diminution of vascularity in granulation tissue (Ragan et al, 1950; Spain, Molomut and Huber, 1950).

Towards the end of the experiment on the influence of cortisone on deep corneal lesions note was taken of the number of vascularised corneae/

corneae. The results are shown in Table 24. It can be seen that the incidence of vascularisation is significantly greater in the cortisone treated group. This observation conflicts with the finding of Jones et al (1950), and it was therefore decided to extend our experiments and to study the action of both the mineralo and glucocorticoids on corneal vascularisation. The results of this investigation are presented in this chapter.

EXPERIMENTAL

Animals

Fifteen young male albino and fifteen virgin female albino guinea-pigs were used. The 30 animals were balanced for weight and sex, and divided into three similar grpups. All animals were kept in two large cages, the sexes being separated.

Diet.

All cavies received greens, rat cake and water without stint.

Drugs/

Drugs

Each animal in the control group was given intraperitoneally o.5 ml. of Aqueous Vehicle No.1 (Merck & Co.) daily. This preparation contains the suspending agents, and 1.5% benzyl alcohol used in the dispensing of the cortisone acetate.

All the animals in the cortisone group were given intraperitoneally 12.5 mg. of cortisone acetate daily (Merck Suspension 25 mg./ml.).

Each animal in the third group received intraperitoneally 10 mg. of deoxycorticosterone glucoside (Ciba Laboratories). This preparation is readily soluble in water and is absorbed from the site of injection more rapidly than the usual oily suspension of DOCA.

Technique of Injury

The apparatus and technique is described in Chapter 5. The cautery was adjusted so as to injure only the anterior half of the corneal thickness/

thickness.

Assessment of Vascularisation

Each eye was examined every 12 hours for the first 6 days of the experiment and thereafter every 24 hours. The corneae were examined with a Zeiss binocular loupe (magnification 2.5) with illumination from a hand slit-lamp. An arbitrary scheme of notation was adopted whereby the length of the new vessels could be assessed on a numerical basis (Table 25).

R E S U L T S

Results of Cortisone Experiment

Weight

Figure 17 shows that cortisone did not significantly affect the rate of gain of weight of the guinea-pigs.

Vascularisation

Three eyes in the control series developed an infective keratitis, and these eyes/

eyes have been excluded from the results.

The mean degree of corneal vascularisation in arbitrary units in the control and cortisone group is shown in Fig.28. It is apparent that in both groups vascularisation occurred in two phases.

There was an initial stage of vascularisation which reached a peak at the end of the second day of healing. Thereafter it declined, and by the fourth day vascularisation was only moderate in extent.

After this initial stage, vascularisation increased once more, and by the sixth day reached a peak which was higher than the initial peak recorded on the second day. A gradual decline in the degree of vascularisation then occurred, and at the termination of the experiment on the 18th day there remained only slight residual vascularisation in a few eyes.

It can be seen that although the general form of vascularisation in the two groups is similar/

similar, there are certain minor differences. The cortisone treated group showed slightly less vascularisation than the control group up to the 7th day of healing, but thereafter the degree of vascularisation was slightly greater.

Results of Deoxycorticosterone Experiment

Weight

It is apparent from Fig. 29 that the administration of deoxycorticosterone has not altered the normal growth curve.

Vascularisation

Fig. 30 shows the mean degree of corneal vascularisation in the group receiving deoxycorticosterone glucoside as compared with the control eyes. This experiment was stopped on the 13th postoperative day.

In this group the two phases of vascularisation were also observed. It is interesting to note that both the first and second peaks of/

of vascularisation occurred slightly earlier than in the control group, but the degree of vascularisation was less than in the control group. The vascularisation decreased fairly rapidly after the 6th day.

Note:- It can be seen from Figs. 28 and 30 that the mean vascularisation of each of the three groups in this experiment showed two separate phases. I should like to stress that this phasing was representative of the course of events in the individual corneae and was not due solely to adopting a mean figure. Indeed, 45 eyes out of the total of 57 clearly showed this double phase of vascularisation.

DISCUSSION

The infliction of small discrete corneal heat injuries close to the limbus and involving about two-thirds of the substantia propria evoked a definite vascular response in all the eyes of these experiments.

It/

It was found that this vascular response occurred in two well defined phases. The initial phase took place within the first 3 days. During this period a local oedema of the cornea was apparent around the lesion extending to the limbus. The oedema became less obvious after the second day of healing, and its subsidence coincided with the transient regression of the vessels which occurred about the third day.

It is possible as has been mentioned already, that corneal oedema facilitates the ingrowth of new vessels from the limbal plexus, perhaps by opening up the collagenous tissues. Similarly, subsidence of the oedema may produce occlusion of some of these newly formed vascular channels. This correlation between corneal oedema and new vessel formation has been noted by Mann and Pullinger (1942) and by Cogan (1949).

In this initial phase of vascularisation there was no marked difference between the control/

control and cortisone treated groups. The peaks occurred at the same time, although the degree of vascularisation was somewhat less in the cortisone treated group. Also, in this latter group, the regression of vessels was more rapid and reached a somewhat lower level. It may be that this difference is significant. Cortisone diminishes tissue response to injury and it may, therefore, cause some reduction in the localised corneal oedema following injury. This in turn may lessen the degree of corneal vascularisation.

The second phase of vascularisation started on the fourth day and reached a maximum about the sixth day. Some of the vessels seen at the beginning of this stage probably represented the reopening of vascular channels formed in the initial phase of vascularisation. The second phase, however, was more intense, and it must include the formation of further new vessels. Oedema was not a feature of this second/

second phase, and it appears unlikely that oedema can be the mechanism accounting for this second production of new vessels. This is in conflict with Cogan's theory that oedema plays a dominant role in vascularisation.

The mechanism underlying the second phase of vascularisation is obscure, and several theories could be advanced to account for this further vascularisation.

Meyer and Chaffee (1940) have suggested that the mucopolysaccharide which is normally present in corneal tissue might account for its avascularity. This suggestion is based upon the observation that corneae, Wharton's jelly, vitreous and cartilage all contain some mucopolysaccharide and are all avascular. Meyer and Chaffee believed that this polysaccharide is hyaluronosulphate although this is doubted by Woodin (1950 a,b). Destruction of this substance by hyaluronidase may stimulate new vessel ingrowth (Meyer 1948). Cortisone is known to act as an anti-hyaluronidase agent (Seifter/

(Seifter et al, 1949), and if the theory of Meyer and Chaffee is substantially correct cortisone should produce a decrease in the degree of vascularisation. This might explain the slight decrease in vascularisation found in the cortisone group of this experiment during the early stages of healing, but would not account for the results in the later stages.

In Chapter 9 it was suggested that an unknown factor might be liberated at the site of a corneal injury. After diffusing to the limbus this substance may stimulate new vessel formation. If cortisone blocked or prevented the formation of this substance a greater diminution in the degree of vascularisation would be expected.

On the other hand, it is possible that the cellular reaction within the healing area increases the metabolic requirements of the cornea and that this in turn induces a vascular response. Indeed, Simoyama (1941) noted that in perforating wounds of the cornea there was initially/

initially a decrease in metabolism, but subsequently it steadily increased to a maximum at the time when the mitotic figures and proliferation of keratoblasts were most abundant. It was demonstrated in Chapter 7 that cortisone diminished the cellular reaction in this type of heat injury. A priori one would expect cortisone to diminish the ingrowth of vessels. It must, however, be borne in mind that cortisone also delays the progress of healing and that in the later stages of repair this delay may cause a prolongation of the vascularisation phase.

This latter theory accounts for most of the findings in this experiment. The diminished degree of vascularisation with cortisone in the early stages of healing may be due to the fewer aerobic cells present in the stroma. The prolongation of the second phase of vascularisation in the cortisone group may be indirectly due to a prolongation of the cellular reaction to injury. The establishment of this theory would require/

require the demonstration of a direct correlation between the degree of vascularisation and the number and type of cell present in and around the site of injury. Unfortunately, insufficient histological material was available during this investigation to substantiate this hypothesis.

Like cortisone, deoxycorticosterone in my hands has had no dramatic effect on the course of vascularisation. There was, however, a slight tendency for the peaks of vascularisation to occur somewhat earlier and for a more rapid recession of the vessels towards the end of the experiment. These results could also be explained on the theory that the accumulation of aerobic cells in the stroma encourages corneal vascularisation. Deoxycorticosterone is known to increase the formation of and rate of healing by granulation tissue (Pirani, Stepto and Sutherland, 1951). It is therefore probable, that the corneae in the deoxycorticosterone group responded to trauma more rapidly and healed earlier. This would in turn/

turn account for the earlier occurrence of the peaks of vascularisation and its more rapid recession in the later stages of healing.

It would thus appear that these two adrenocortical hormones act on corneal vascularisation not directly but indirectly, probably through their effect on tissue regeneration.

The results of this experiment conflict with those of Jones and Meyer (1950) who found that subconjunctival injection of cortisone caused a marked inhibition of new vessel formation within the cornea of the rabbit following intracorneal injection of alkali. There were, however, certain differences in technique between the work of these workers and my own. Jones and Meyer used rabbits, and their standard injury was produced with sodium hydroxide solution. There may well be a species difference between the rabbit and the guinea-pig in their vascular response to corneal injury, or to adrenocorticohormone administration. Further work will be required to elucidate this point. Moreover, these/

these workers administered the cortisone acetate subconjunctivally. (1.25 mg./eye every 3 or 4 hours). Although this dose appears small an effective concentration in the ciliary body, aqueous and cornea occurs with this administration both in the rabbit and man (Woods, 1950). The intraperitoneal dose of 12.5 mg./day used in the guinea-pigs was the maximum amount which could be given with safety to cavies of this weight. An effective level of cortisone would almost certainly be reached in the cornea for this dose in a previous experiment caused a marked delay in healing.

The results obtained in this investigation are in part supported by Leopold, Purnell, Cannon, Steinmetz and McDonald (1951). These workers found that the topical application of cortisone acetate suspension, used hourly for 10 hours for seven days, and daily subconjunctival injections of 1.25 mg. failed to influence the degree of opacification and vascularisation following either application of 0.01 c.c. of concentrated/

concentrated sodium hydroxide solution to the corneal centre, or intracorneal injection. Four treated and control rabbit eyes were used for each type of therapy and type of application of alkali. They suggest that the negative finding in these experiments was due to the severity of the alkali burns used, and that any slight effect would be masked with such toxic lesions.

SUMMARY

1. Standard heat injuries were inflicted on the corneae of guinea-pigs receiving cortisone acetate, deoxycorticosterone glucoside or control solution.

2. The resulting vascularisation occurred in two phases. The initial phase reached a maximum on the second day and lasted to the fourth day of healing. The second phase reached a peak on the sixth day and thereafter declined slowly to the eighteenth day.

3. Administration of cortisone acetate slightly decreased/

decreased the degree of vascularisation in the initial stage but increased the degree in the later stages of healing.

4. Deoxycorticosterone glucoside caused the peaks of vascularisation to occur slightly earlier than in the control group. The degree of vascularisation was also less and tended to diminish more rapidly in the later stages of healing.

5. These results could best be explained by assuming that these agents influence corneal vascularisation indirectly through their action on the cellular response at the site of healing. The presence of aerobic cells in and around the healing area may be responsible for the second phase of vascularisation.

PART THREE

RETINAL VASCULARISATION

DURING DEVELOPMENT

Chapter XI

The INFLUENCE of a LOW ATMOSPHERIC PRESSURE on the DEVELOPMENT of the RETINAL VESSELS in the RAT

The retina is in many ways an ideal site for the study of the growth of the peripheral vascular system. The retinal vessels all grow from a central point, the optic disc, and spread out on the retina in a two dimensional manner. Furthermore, an artery can be clearly distinguished from a vein from an early stage of development.

Michaelson (1948 a,b) has described and illustrated the retinal vascular development in man and in the cat. The vascular system was found to develop as an extension of the hyaloid vessels at the optic nerve head. A system of arterio-venous loops, or arcades, emerges from the hyaloid vessels and gradually extends towards the periphery of the retina. The capillaries develop from the venous limb of this arcade.

The/

The morphogenesis of the vascular system in the rat is similar to that in man. The state of the retinal vessels in a rat 150 hours old can be seen in Fig.31. The vessel marked A is an artery and the vessels on either side are veins (V). The capillaries have extended across from the veins towards the artery only for a certain distance, leaving finally a well marked capillary-free space around the artery and its branches. The capillary-free zone is traversed, of course, at a few points by arteriae afferentes to supply the capillary bed with arterial blood.

The presence in the retina of a perivascular zone free from capillaries was noted long ago by His (1865) and by Schwalbe (1872). They considered it to be part of the retinal lymphatic system. Gifford (1886 and 1892) however, who studied the action of phagocytic cells after intraocular Indian ink injections, made no reference to these perivascular spaces. Moreover, Weed (1914) and Wegehurth (1914) were unable to demonstrate/

demonstrate definite lymphatic channels in this region. After extensive research Krückmann (1918) maintained that an accessory pial sheath surrounds the endothelium of the retinal capillaries, and that between this sheath and the endothelium lay the perivascular space. Surrounding all is another layer, the glial mantle or limiting membrane.

The capillary free space on the other hand was demonstrated by Leber (1903) in injected specimens. This space probably corresponds to the pale zone on either side of an artery, occasionally observed in ophthalmoscopic examination (De Schweinitz, 1921). This capillary free space is certainly much bigger than the perivascular space so far described and Parsons (1904) is probably very near the truth when he states - "there is an area on each side of the larger vessels (arteries) which is free from capillaries. This is occupied in part by the perivascular lymph-sheath".

Clearly/

Clearly the retima, in this capillary free zone, must obtain its oxygen supply by diffusion either from the nearby artery or from the neighbouring capillaries, or from both. Evans (1938) has proved,by means of angioscotometry, that this area of the retina does function, although its sensitivity is readily depressed by factors such as mild anoxia.

The presence of this capillary free zone led me to postulate that the higher oxygen tension which might exist around a retinal artery may inhibit further extension of capillaries during development, and so account for this clearly demarcated capillary free zone.

This hypothesis was tested on the rat in which, fortunately, the retinal vessels are just commencing to develop from the hyaloid vessels at birth. It is thus possible to follow the entire process of vascularisation during extrauterine life.

METHOD

Animals/

Animals

Twenty young virgin female rats (Wister Strain) were mated with two young male rats. Each alternate litter born was placed in a low-pressure chamber 24 to 30 hours after birth. The mother usually refused to nurse her young if placed in the strange interior of the pressure chamber immediately after delivery. The remaining litters were used as controls.

Diet

The animals were fed on rat cake cube (Thomson, 1936) and water without restriction. Three drops of cod liver oil daily per animal were added to the diet.

The Low Pressure Chamber

The pressure chamber measured 3 feet in diameter and 8 feet in height. Entry was gained through an airtight manhole. A 6 inch diameter thick glass window allowed full observation of the interior. The chamber was warmed with a

250/

250 watt heater which was controlled by a thermostat.

The pressure within the chamber was regulated by withdrawing air from one side of the chamber by means of an electric vacuum pump, and at the same time allowing a controlled flow or air to enter from the other side through a screw valve. In this way a continuous air change occurred within the chamber. An R.A.F. altimeter was placed within the chamber close to the observation window. Very small changes of pressure within the chamber were detected by the altimeter and permitted accurate adjustment of the air leak through the screw valve. The altimeter was calibrated against a mercury manometer.

Procedure

The litter was carefully watched for 48 hours after birth, and if the mother was found to be nursing the young satisfactorily, the mother and litter were transferred in the same cage to the low pressure chamber. The pressure within/

within the chamber was then slowly lowered over a period of one hour until the pressure was equivalent to an altitude of 17,500 feet. This altitude is equivalent to a partial oxygen tension of 76 mm. Hg. in the air of the chamber. At this reduced pressure the respiratory rate of the mother was increased although no signs of distress occurred. Both mother and litter tended to be less active and to sleep for longer periods than at normal atmospheric pressure. The pressure was raised at least once each day to gain entry to the chamber. Food and water were then replenished, and one of the litter was selected for examination. Occasionally a mother did not tend her litter when placed in the chamber. She and her litter were discarded from the experiment.

To examine the vascular system of the retina one of the litter was selected at random. The age and weight of the selected animal were noted. It was then deeply anaesthetised with ether and its heart exposed. A hypodermic needle was inserted/

inserted into the left ventricle and a 50% dilution in saline of Reeves Indian Ink was injected under a pressure of 100 mm.Hg. until the skin of the head and neck became jet black in colour. The heart usually stopped beating a few minutes after the injection. The eyes were then enucleated and placed in a 10% formalin for 24-48 hours. Thereafter, they were washed in water, and the cornea, iris and lens removed. The retina, after separation from the choroid, was placed on a microscope slide with a few drops of 50% glycerine and covered with a cover slip. No attempt was made to remove the hyaloid blood vessels from the optic disc as this invariably resulted in damage to the retina. The slide could be examined a few hours later when clearing had occurred.

The dimensions of the capillary network were determined with a microscope fitted with a graticule eye-piece and a Vernier moving stage. Four measurements at right angles to one another were made on each retina. These were made from the/

the centre of the optic disc to the outer limit of capillary formation. The mean of the eight readings, four from each eye, then gave an estimate of the extent of capillary growth in that rat.

In addition, the breadth of the capillary-free zone around the arteries was measured with the graticule eye-piece, the distance between the artery and the nearest capillary being measured at regular intervals along the vessel. It should be noted that these estimations of the capillary-free zone inevitably included the thickness of the arterial and of the capillary wall. The Indian ink filled only the lumen of the vessels, and did not render visible the structure of the walls. The breadth of the lumen of the vessel was not included in the estimation as the measurements were made from the outer surface of the lumen as defined by the Indian ink.

RESULTS

A/

A decrease in the calibre of the retinal arteries, veins and capillaries was found in all young rats that had been subjected to the low pressure conditions for 64 hours or longer. Fig.32 shows that state of the retinal vessels in an animal 220 hours old which had been kept in the low pressure environment for 195 hours. The veins, arteries and capillaries are thin. The capillary bed is less dense than in a normal rat and resembles the definitive vascular bed found in a more mature animal. The capillary-free zone around the arteries (Fig.32,a) is less well defined and is narrower than in a control animal of the same age.

Extent of Capillary-free Zone

Table 26 summarises the results of 250 measurements of the breadth of the capillary-free zone in control and in principal rats of comparable age. The capillary-free zone is 14μ smaller than in the rats exposed to low pressure. This difference is highly significant.

Rate/

Rate of Growth of the Retinal Vessels

The radial extent of retinal vascularisation from the optic disc to the periphery of the vascularised area was plotted against the age of the rat at the time of investigation (Fig.33). The upper regression line was derived from the data of the control group and the lower regression line from the data of the low pressure group. The mean rate of vascular growth in the control eyes was 10.2μ/hr. and in the low pressure group 8.3μ/hr. This difference in the regression coefficients is significant. Thus the low pressure conditions have decreased the rate with which the vessels grow from the optic disc to the periphery.

However, the low pressure conditions in addition to decreasing the rate of retinal capillary growth also retarded the rate of general body growth as judged by body weight. It can be seen from Fig.34 that the rate of body growth in the low pressure group is less. It is possible that the reduced rate of retinal vascularisation is due to this decrease in the rate of body growth as a whole and is not a direct/

direct effect of the low environmental oxygen tension on the blood vessels.

The extent of vascularisation was therefore plotted against body weight at the time of examination (Fig.35). It will be noted that in terms of body weight the rate of vascularisation in the two groups is almost identical. That is, the regression lines are almost parallel. The low pressure group is on the average 1.9 gm. lighter.

Age, body weight and extent of retinal vascularisation are clearly closely correlated. The results were therefore analysed by means of multiple regression. In this way the influence of age on vascularisation could be studied when the body weight was held constant. Using this method of analysis it was found that the rate of vessel growth had decreased from 8.8μ/hr. of age in the control group to 7.2μ/hr. in the low pressure group. It would thus appear that the effect of the low pressure environment has really been to decrease the rate at which the retinal vessels develop.

D I S C U S S I O N/

D I S C U S S I O N

The low pressure environmental conditions used in this experiment have resulted in three dominant changes in the mode of retinal vessel development in the rat. The capillaries first formed develop towards the definitive type found in the adult rat more rapidly. Secondly, the capillary-free zone normally found around the arteries is narrower and finally, the rate of vascularisation of the retina is diminished.

These results could be explained by postulating that the oxygen tension in the tissues around the developing vascular bed controls to some extent the mode of formation of the developing vessels.

The presence of a capillary-free zone could be accounted for if the possibility of oxygen diffusion through an artery wall is allowed. When the developing capillaries reach an area of retina with a relatively high oxygen tension further extension ceases. This occurred in the control/

control rats at a mean distance of 63.2 μ from the lumen of the artery. In the low pressure group of animals, however, the capillaries grew nearer to the arteries (49.1 μ), presumably due to the lower oxygen tension in the arterial blood stream diminishing the diameter of the surrounding zone adequately supplied with oxygen.

The reduction in the rate of vascularisation and in body growth may be due to a general reduction in the rate of tissue anabolism. Unfortunately, little is known about the optimum oxygen tensions required for maximum growth of young animals, and further work would be required before a definite conclusion could be formed on this point.

The results of this investigation suggest that the oxygen tension in the retina is one of the factors influencing the morphogenesis of the retinal vessels. If it is assumed that the retinal blood vessels grow towards an area of low oxygen tension and cease to extend when an area of higher oxygen tension is reached, how many of the facts

of retinal vessel development could be explained?

Firstly, it has now been established that in man, and in all animals so far studied with a similar retinal vascular system, the retinal vessels only grow over the retina at a comparatively late stage of eye development. Presumably during a great part of the life of the developing retina adequate nutrition for growth is obtained from the choroid and by diffusion from the hyaloid vessels in the vitreous. However, in the rat and in man, once the development of the lens, iris ans ciliary body is advanced, the hyaloid system of vessels degenerates and finally disappears. At this time, moreover, ganglion and bipolar cells of the retina are undergoing rapid development, and presumably increasing the oxygen demand from the choroid. This oxygen requirement will become increasingly difficult to satisfy on account of the greater distance through which oxygen diffusion must occur. It is quite reasonable therefore to suggest that the retinal vessels/

vessels develop at this late stage because the developing retina has an increased need for oxygen which can no longer be supplied by the choroidal vessels alone.

Once this extension of vessels from the optic disc over the surface of the retina commences in the rat, it extends fairly evenly in all directions. Michaelson (1948,a) found in the human eye, however, that the extension of vessels started earlier on the temporal side of the disc than on the nasal side. This finding may be explained by the greater temporal area of retina present in the human eye as a result of the medial insertion of the optic nerve. It is quite possible that an oxygen debt occurs sooner on the temporal side and so stimulates an earlier invasion of vessels.

If we next consider the mode of capillary formation, we find that the capillaries arise from the venous loop of the arterio-venous arcades. This may be due to the inherently greater powers of proliferation of the vein endothelium/

endothelium, or due to the lower oxygen tissue tension which one would expect to find around a venous channel, when compared with an arterial vessel. This assumes, of course, that there is some gaseous exchange between the surrounding tissue and the blood in the lumen of these retinal vessels, but it should be borne in mind that at this stage of development the retinal vessels are in reality fine arterioles and venules. Indeed, many reach capillary size after only three or four bifurcations.

The tendency for capillaries to grow towards an area of low tension may also account for the interesting ophthalmoscopic observation that although arterio-venous crossings are very common, an artery has never been seen to cross an artery, or a vein to cross a vein, except in fundus drawings by rather unobservant artists. This may be explained by assuming that during the development of the capillary network the tendency of the sprouting capillaries will always be to grow through an area already supplied with capillaries to reach

an/

an avascular area beyond. The attendant artery and vein supplying one area will therefore never have need to cross the vessels serving another area.

Finally it should be recalled that the retinal capillaries only penetrate as far as the inner nuclear layer in the human retina. Could the cessation of growth in this plane also be influenced by the gradient of oxygen tension arising from the choroid?

SUMMARY

1. Litters of Wister strain rats were placed in a low pressure environment 24–30 hours after birth.
2. They were removed at varying intervals and the development of the retinal vascular system was studied after Indian ink injection, and compared with rats allowed to develop normally.
3. The retinal vessels formed by an extension of the hyaloid vessels at the disc. The capillaries developed by a process of budding from the veins.
4. In the low pressure group the arteries, veins and/

and capillaries were of smaller calibre, and resembled the definitive network of the more mature animals.

5. The capillary-free zone in the low pressure group of animals was significantly smaller than the zone in the control group.

6. The rate of progress of the vascular system from the optic disc to the periphery of the retina was diminished under the conditions of the low pressure chamber.

7. An important factor in the formation of the retinal vascular system appears to be the oxygen tension of the inner layers of the retina.

APPENDIX

Statistical Treatment

The equations for the regression of vessel growth on age (Fig.32) are:-

Control Group $y = 573.3 + 10.2x$
Principal Group $y = 479.9 + 8.31 x$

Where y = extent of vessel growth in microns and x = age of rat in hours.

The difference of the regression coefficients was shown to be significant by the 't' test ($P < 0.05 > 0.02$).

The equations for the regression of body weight on age (Fig.33) are:-

Control Group $y = 5.149 + 0.03185 x$
Principal Group $y = 3.355 + 0.02096 x$

Where y = body weight in grams and x = age in hours.

The regression coefficients differ significantly ($P < 0.01$).

The equations for the regression of vessel growth on body weight (Fig.34) are:-

Control Group $y = -821.0 + 289.0 x$
Principal Group $y = -202.6 + 287.3 x$

Where y = extent of vessel growth in microns and x = body weight in grams.

There is no significant difference in the regression coefficients ($P > 0.8$).

The partial regression equations are:-

Control Group $y = 338 + 41.95 x_w + 8.753 x_a$
Principal Group $y = 307 + 51.76 x_w + 7.222 x_a$

Where/

Where y = extent of vessel growth in microns
x_w = weight of the rat expressed in grams
x_a = age of the rat expressed in hours.

The significance of each regression coefficient was tested by analysis of variance. Each coefficient had a significant effect on y and between groups the coefficients differed significantly (P = 0.05 or less).

ACKNOWLEDGEMENTS

I would like to express my gratitude to Professor R.C. Garry for his constant interest and constructive criticism of the work presented in this thesis. Dr J.B. de V. Weir has kindly instructed me in the theory and practice of statistics and assisted in the design of the compression balance described in Chapter 5.

During many of the experiments I have been fortunate in having the assistance and collaboration of other workers then in Glasgow, and I would particularly like to express my indebtedness to Doctors T.A.S. Boyd, I.D. Ferguson, I.C. Michaelson and K.C. Wybar for active assistance and keen interest at various stages in this work.

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RÉSUMÉ

The structure and physiology of the cornea is briefly reviewed, and an outline, from personal experience, is given of the mode of healing of superficial and deep aseptic corneal wounds.

A technique was devised for the infliction of standard reproducible heat injuries to the cornea in guinea-pigs and rabbits.

The value of a sodium fluorescein solution in determining the rate of epithelial healing in the cornea was carefully tested and a detailed account is then given of the standard conditions under which it ought to be used. Experiments were carried out to determine the fate of sodium fluorescein after entering the cornea of the rabbit and it was concluded that the main source of drainage was by passage through the endothelium into the anterior chamber.

The fluorescein technique was then used to assess the rate with which standard corneal heat injuries healed in scorbutic and in normal guinea-pigs. It/

It was found that the healing of superficial injuries confined to the epithelium was not impaired in scurvy. On the other hand, deeper lesions healed significantly more slowly in the ascorbic acid deficient guinea-pigs. Moreover, the healing deep injuries were structurally weaker in the scorbutic cavies up to 20 days after injury.

Attention was then turned to the clinical use of massive daily doses of ascorbic acid in the treatment of acute corneal ulcers in man. It was found that administration of ascorbic acid did not accelerate the healing of superficial ulcers but did shorten the healing time of deep corneal ulcers. It was concluded that, although restoration of corneal epithelium as such may be independent of an adequate supply of ascorbic acid, yet the rate of epithelialisation of a wound of the cornea involving collagenous tissue does depend on the provision of a suitable fibrous tissue substratum, and in turn on an adequate intake of ascorbic acid.

The role of ascorbic acid in the metabolism of the adrenal cortex is then reviewed, and experiments are described to determine the action of cortisone acetate/

acetate on corneal wound healing. Administration of cortisone to guinea-pigs with standard deep corneal burns produced a delay in the rate of epithelialisation and a reduction of cellular proliferation at the site of the lesion as determined histologically. These findings are discussed in the light of the similarity noted between the mode of corneal healing when ascorbic acid was deficient and when cortisone was present in excess.

Corneal vascularisation frequently follows corneal trauma and Part 2 of this thesis describes the new vessel formation which results from the infliction of heat injuries to the cornea. The mode of new vessel formation in the rabbit is described in detail. It was found that vascularisation only occurred if the heat injury was within a certain critical distance of the limbus. The significance of this finding is discussed.

The role of ascorbic acid in corneal vascularisation in the guinea-pig was also determined. New vessel formation occurred with significantly greater frequency in the scorbutic cavies. This finding/

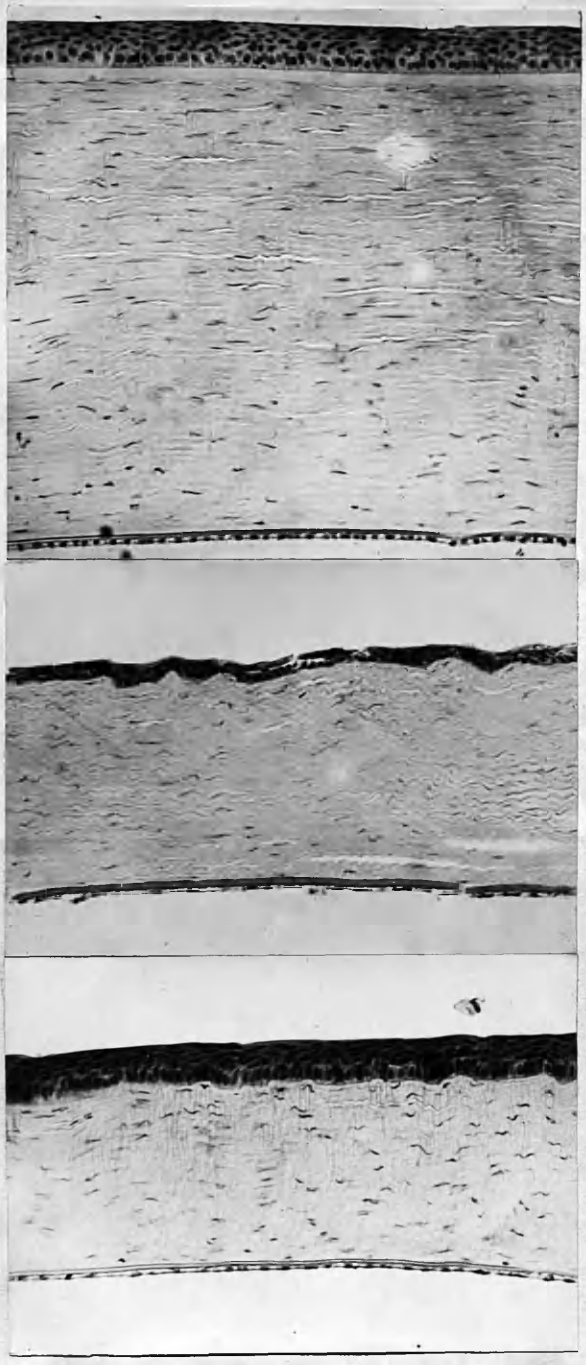
finding suggests that repair of an injury makes additional metabolic demands which can only be met, in a state of ascorbic acid deficiency, by the formation of new vessels.

Corneal vascularisation following heat injury was then studied in greater detail in the guinea-pig and it was found that the resulting vascularisation occurred in two phases. The initial phase reached a maximum on the second day and lasted to the fourth day of healing. The second phase reached a peak on the sixth day and thereafter declined slowly to the eighteenth day. Administration of cortisone acetate intraperitoneally slightly decreased the degree of vascularisation in the initial stage but increased the degree in the later stages of healing.

Deoxycorticosterone glucoside administration in the guinea-pig caused the peaks of vascularisation to occur slightly earlier than in the control group. The degree of vascularisation was also less and tended to diminish more rapidly in the later stages of healing. These results can best be explained by assuming that these adrenocortical hormones influence corneal/

corneal vascularisation indirectly through their action on the cellular response at the site of healing. The presence of aerobic cells in and around the healing area may be responsible for the second phase of vascularisation.

In Part 3 of this thesis the influence of a low atmospheric pressure on the development of the retinal vessels in the rat is described. It was found that in rats exposed to the low pressure the arteries, veins and capillaries were of smaller calibre and resembled the definitive network of the more mature animal. Furthermore, the capillary-free zone in the low pressure group of animals was significantly smaller than the zone in the control group. However, the rate of progress of the vascular system from the optic disc to the periphery of the retina was diminished under the conditions of the low pressure chamber. It is concluded that the oxygen tension in the inner layers of the retina is an important factor in the formation of the retinal vascular system during development.



HUMAN

RABBIT

GUINEA-PIG

Figure 1.

Sections of human, rabbit and guinea-pig cornea stained with H. & E. (Magnification, 70). Note the absence of Bowman's membrane in the rabbit cornea.

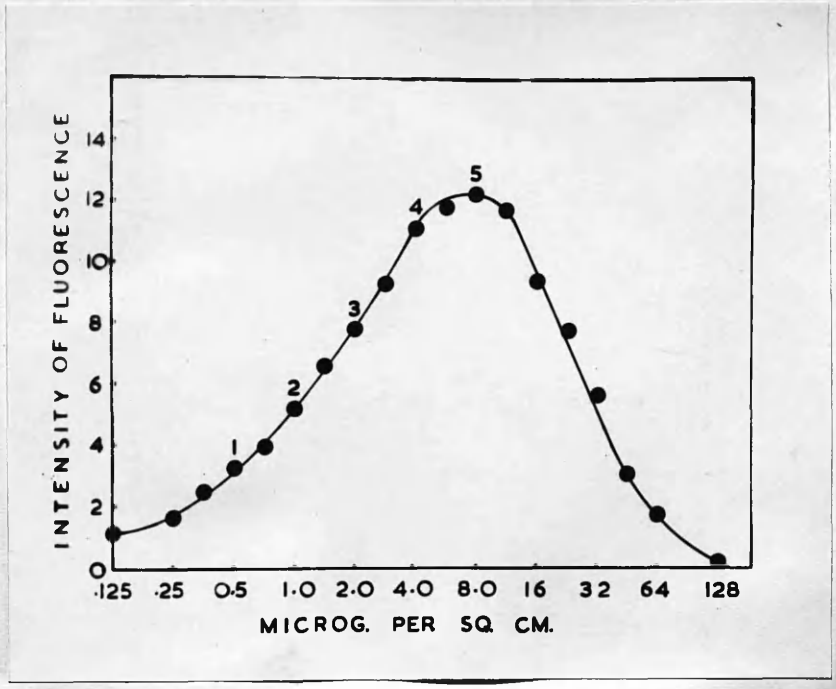


Figure 2.

Relation of intensity of fluorescence (galvanometer readings) to concentration of fluorescein in microg./ sq. cm. of filter paper (logarithmic scale). The points 1 to 5 on the graph correspond to the concentrations chosen for clinical use.

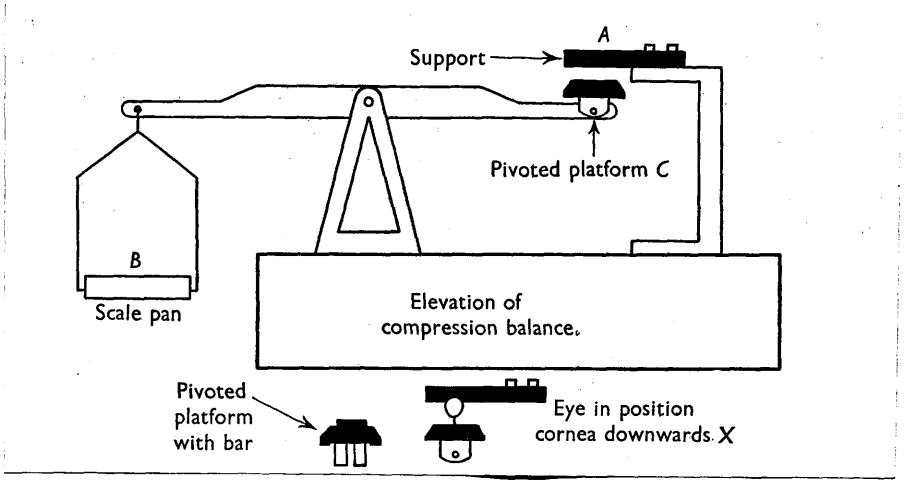


Figure 3.

Compression balance for estimating strength of cornea and of sclera. X shows position of bar and eye during estimations of corneal strength. The eye is compressed between a fixed support A and a platform C. The force is applied by adding weights to the scale-pan B. Both the support A and platform C, pivoted on the end of the lever, are hollowed out to hold the excised eye.

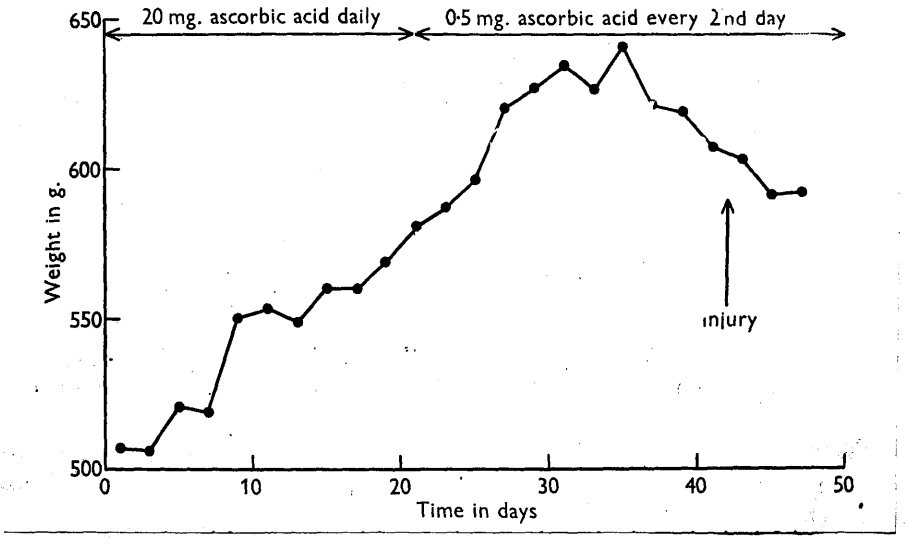


Figure 4.

Curve plotted from mean weights on alternate days of guinea-pigs in deficient group.

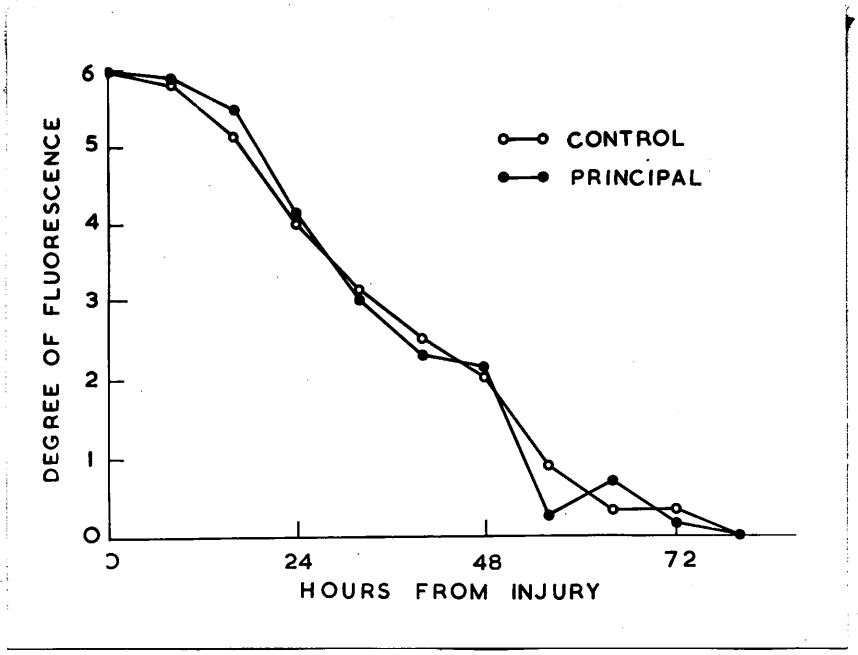


Figure 5.

Graph showing decline of mean intensity of fluorescence from corneal injuries in guinea-pigs made by applicator at 180°. The figures on the ordinate correspond to the strip intensities described in chapter 3.

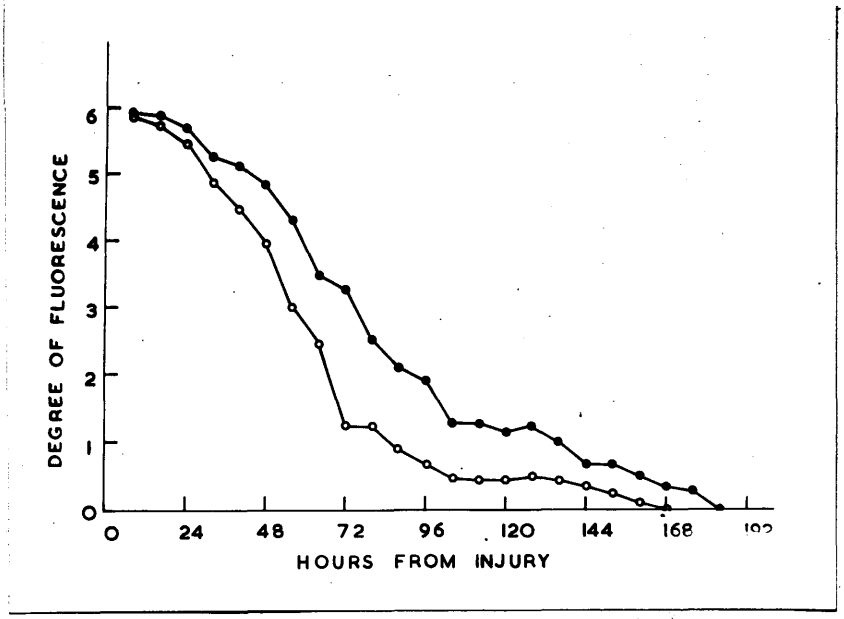


Figure 6.

Intensity of Fluorescence following deep injuries to the cornea of guinea-pigs. Control group o---o. Scorbutic group ●---●.

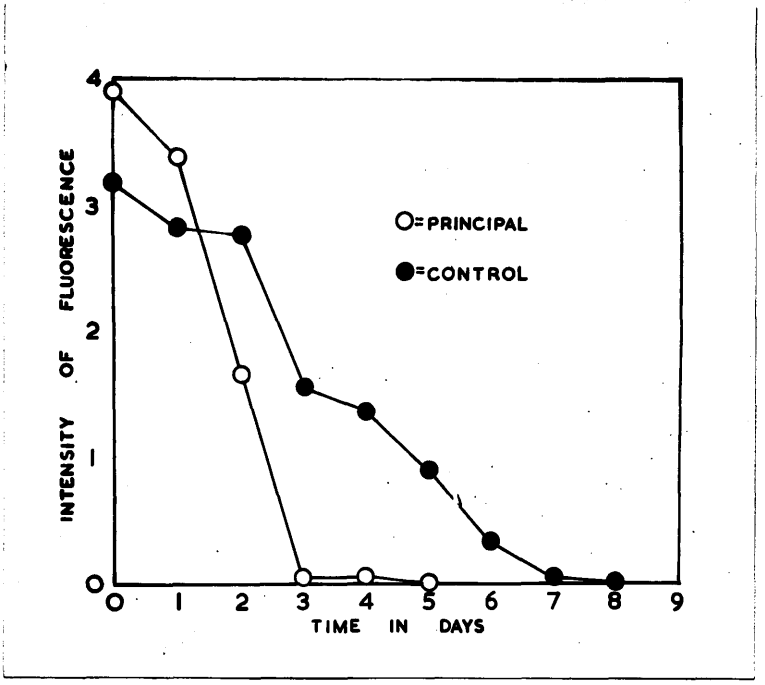


Figure 7.

Mean intensity of fluorescence of deep ulcers in man treated with (principals) and without ascorbic acid (controls) plotted for each day during healing. The figures in the ordinate correspond to the strip intensities described in Chapter 3.

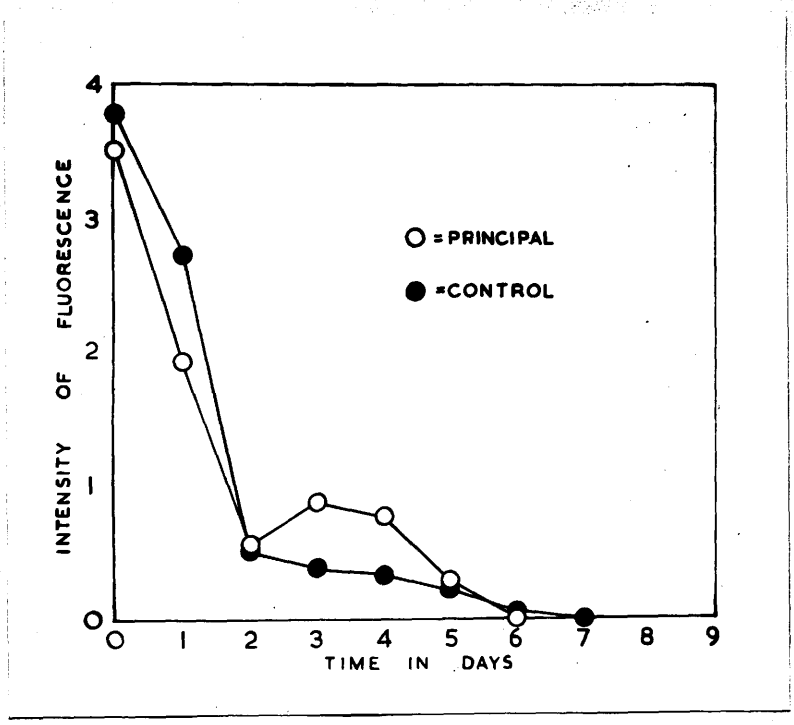


Figure 8.

Mean intensity of fluorescence of superficial ulcers in man treated with (principals) and without ascorbic acid (controls) plotted for each day during healing. The figures on the ordinate correspond to the strip intensities described in chapter 3.

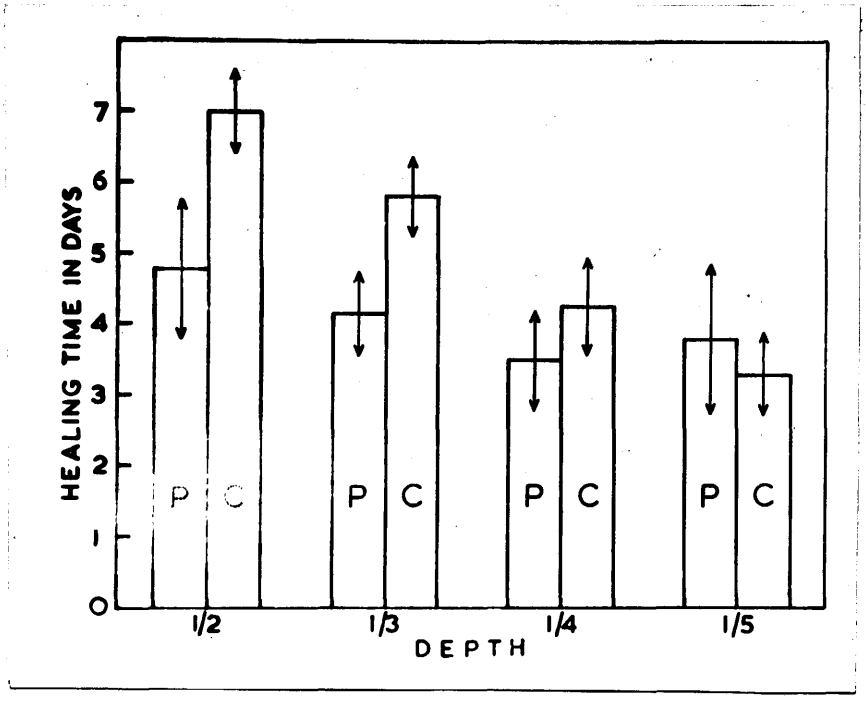


Figure 9.

Mean healing time related to depth of infiltration in corneal ulcers in man treated with (P) and without ascorbic acid (C).

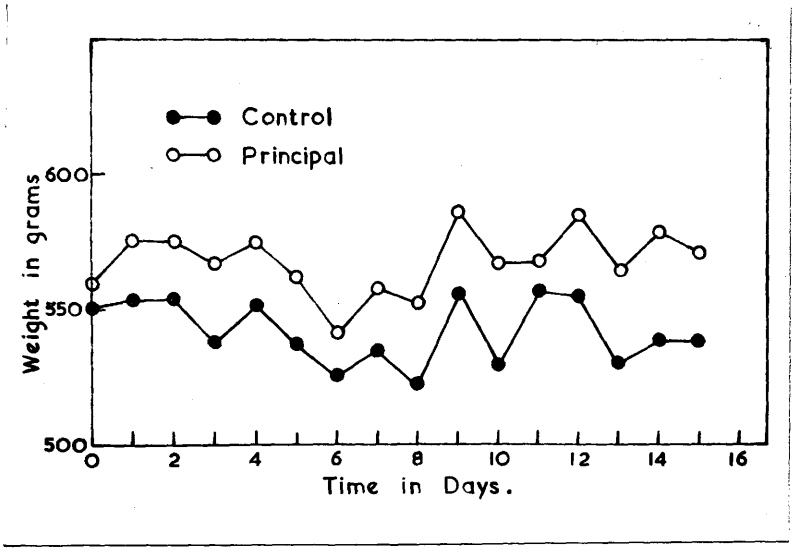


Figure 10.

The growth curves of the control and sodium γ-resorcylate treated group (principal) of guinea-pigs.

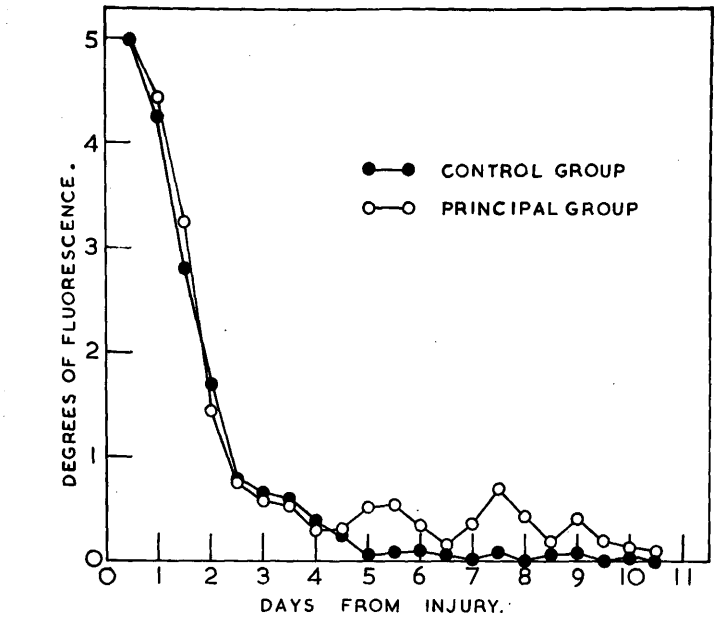


Figure 11.

The mean fluorescent gradients of the control and sodium γ-resorcylate treated group of guinea-pigs.

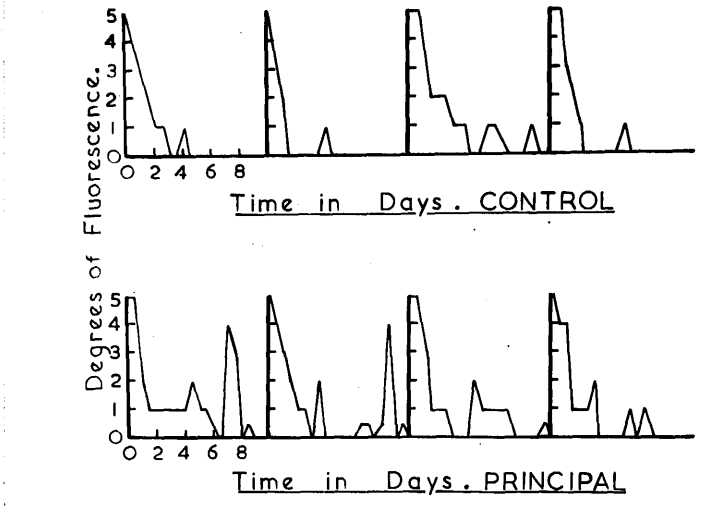


Figure 12.

The individual fluorescent gradients obtained from 4 eyes in the control group (above) and from 4 eyes from the sodium γ-resorcylate group (below).

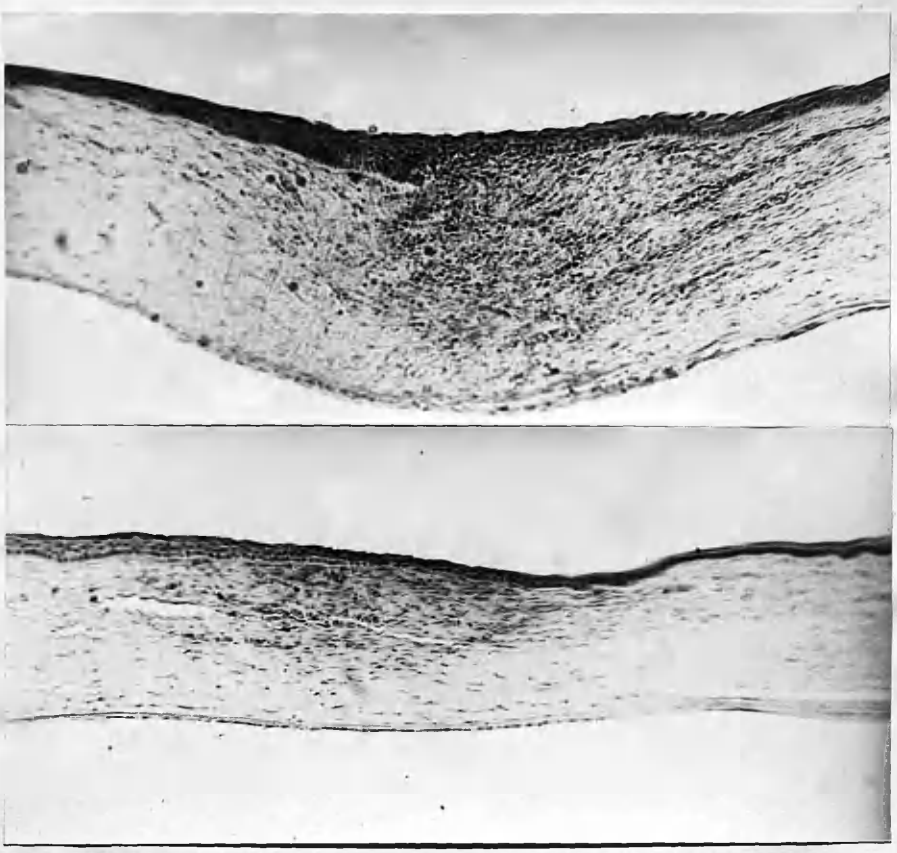


Figure 13.

The upper section is from a control guinea-pig after 11 days of healing. The lower section is from a sodium γ-resorcylate treated animal also after healing had progressed for 11 days. The epithelium is above and the endothelium below in both sections. Note the greater cellular reaction in the control cornea. (Magnification, 60).

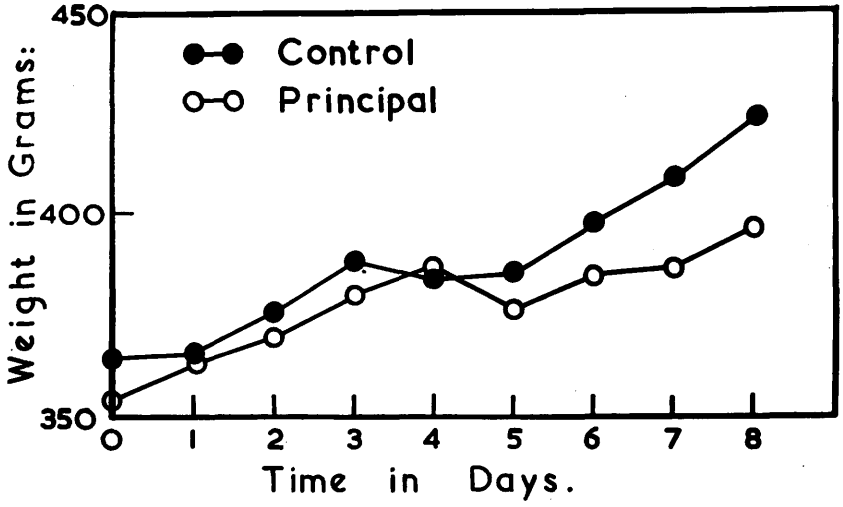


Figure 14.

The growth curves of the control and cortisone treated guinea-pigs.

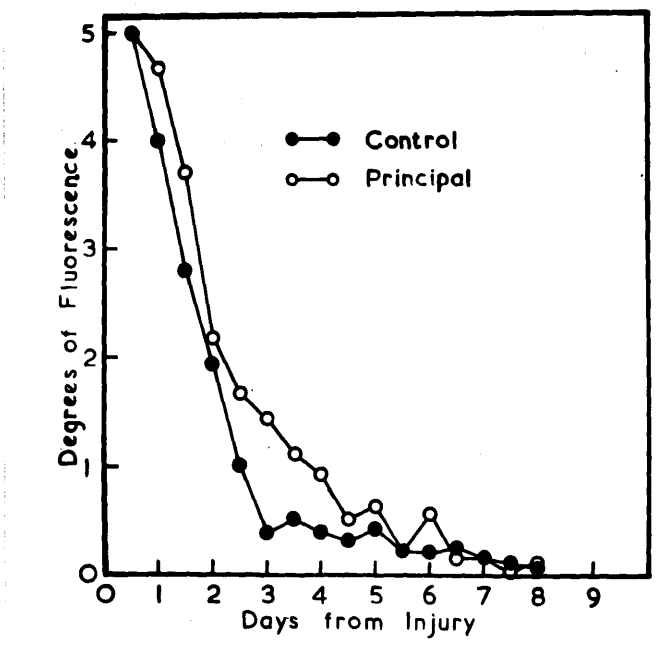


Figure 15.

The mean fluorescent gradients of the control and cortisone groups obtained from healing deep cofneal injuries in guinea-pigs.

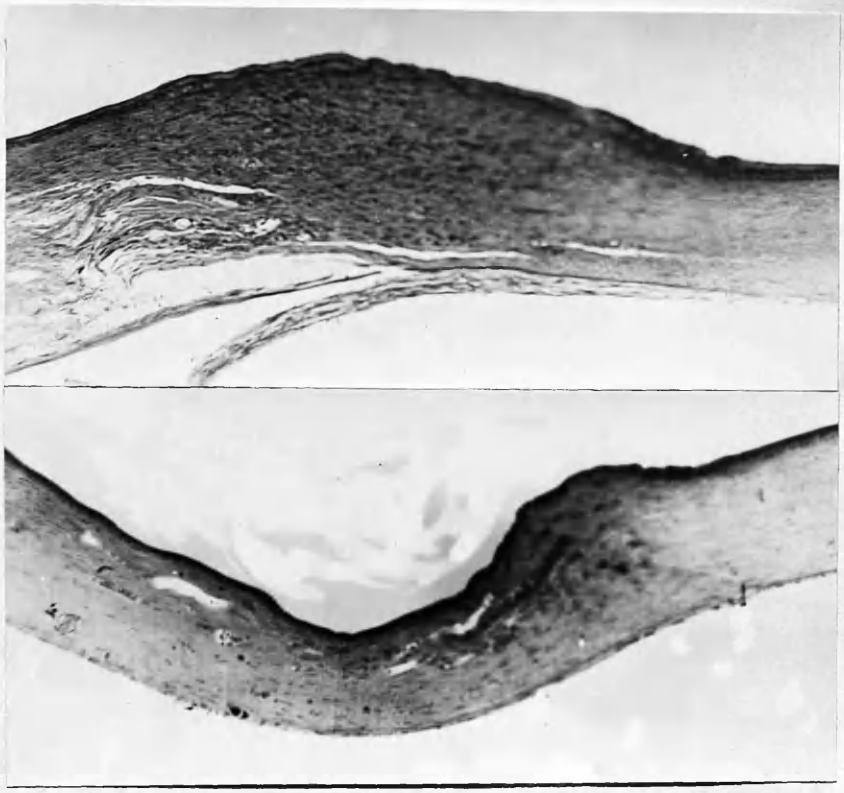


Figure 16.

The upper section is from a control guinea-pig and the lower section is from an animal treated with cortisone acetate. Both eyes were removed after healing had progressed for 9 days. (Magnification, 60).

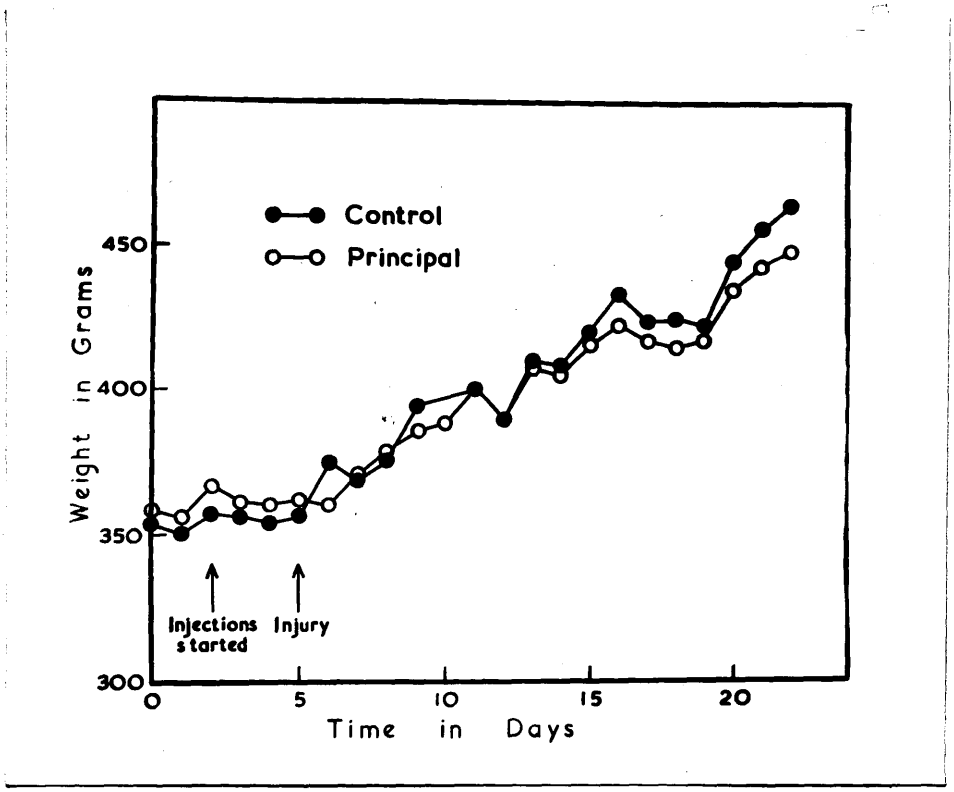


Figure 17.

The growth curves of the control and cortisone treated group of guinea-pigs (Superficial injuries).

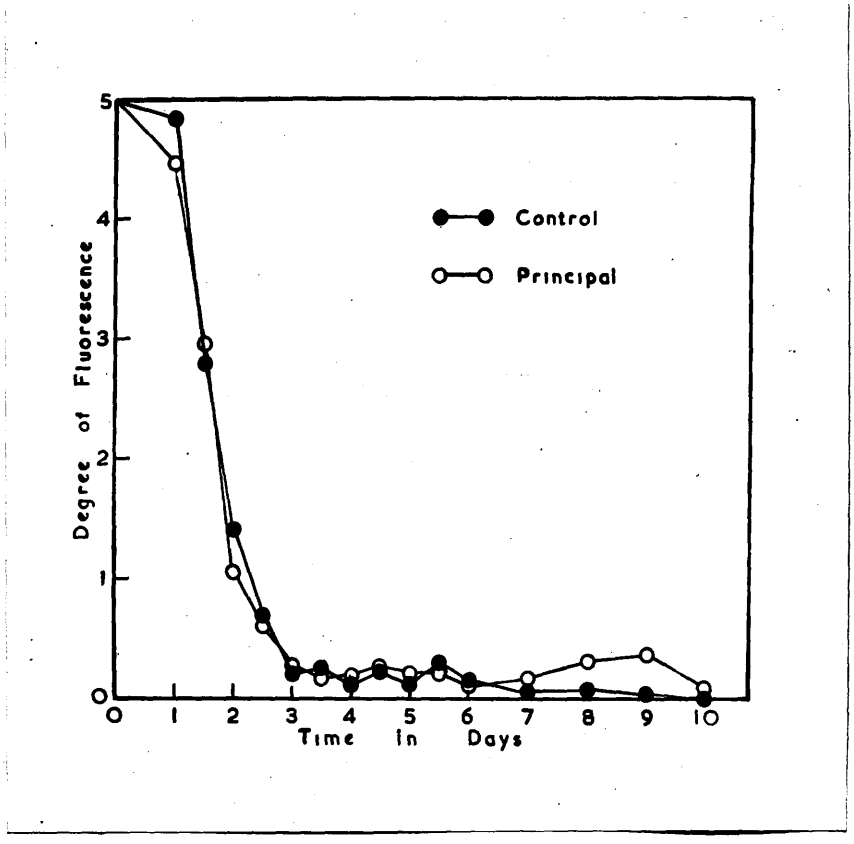


Figure 18.

The mean fluorescent gradients of the control and cortisone group obtained from healing superficial injuries in guinea-pigs.

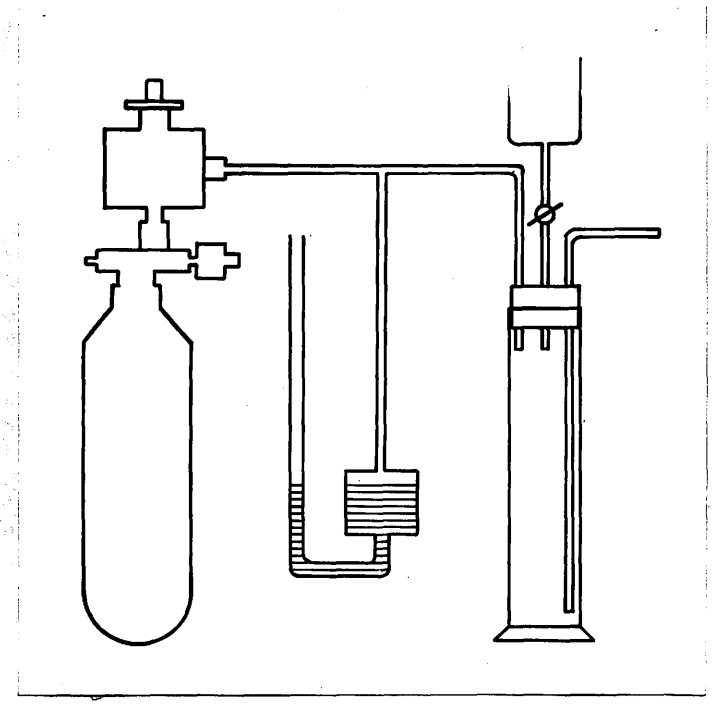


Figure 19

Apparatus for injecting Indian ink into the vascular system. See page 118 for description of apparatus.

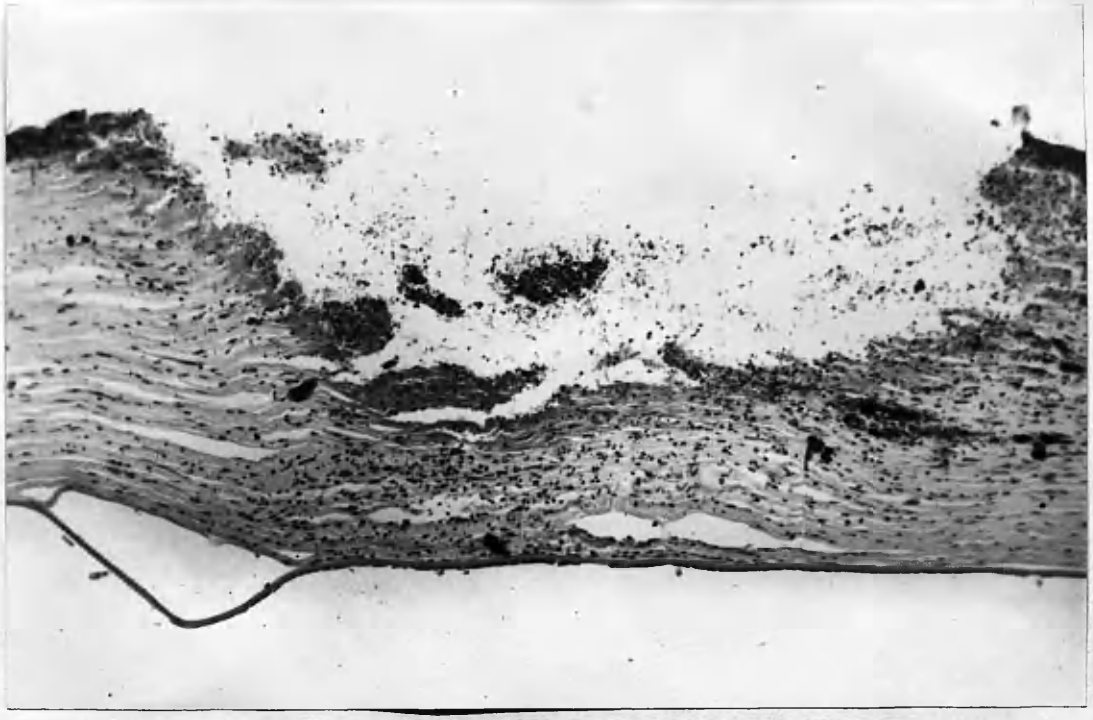


Figure 20.

Effect of repeated cauterisation of the cornea in a rabbit. The lesion involves the epithelium and the anterior two-thirds of the stroma. (Magnification, 50).

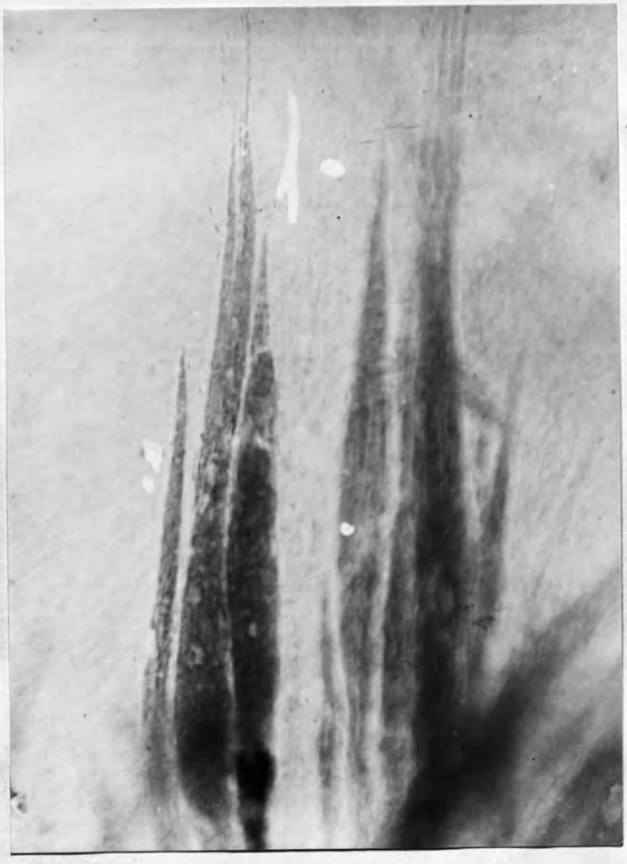


Figure 21.

Unstained flat preparation of rabbit's cornea showing the flame shaped haemorrhages into the corneal stroma formed during active vascularisation. Limbal vascular plexus below.

Figure 22.

Unstained flat preparation of rabbit's cornea showing the flame shaped haemorrhages into the cornea. Note that the Indian ink has not entered these haemorrhages and that they point towards the centre of the cornea and not towards the lesion (L).

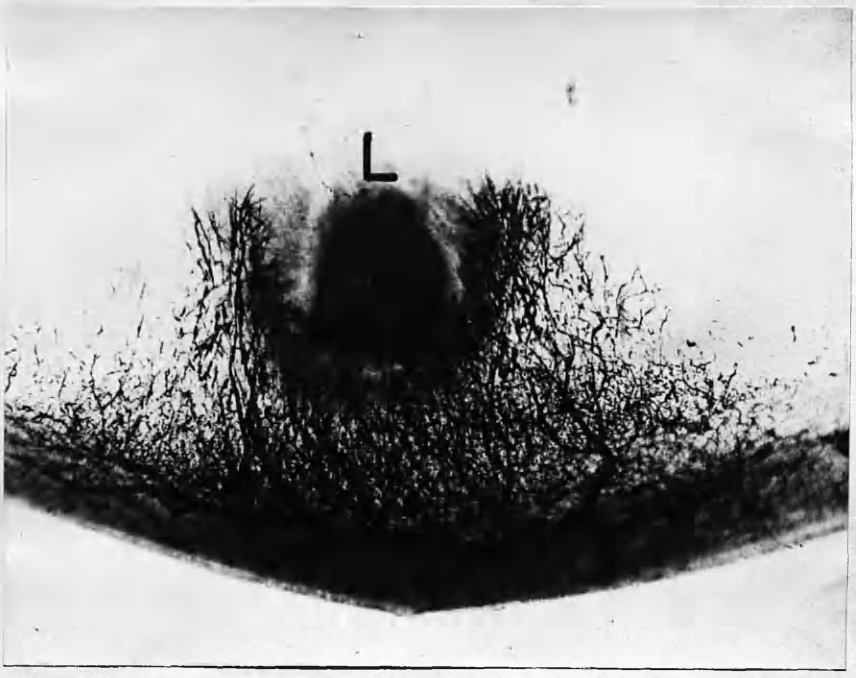


Figure 23.

New vessel formation in cornea of a rabbit as a result of repeated heat injury to the lesion (L). Vessels injected with a dilution of Indian ink after death. Note the isosceles triangular area of vascular infiltration from the limbal plexus above.

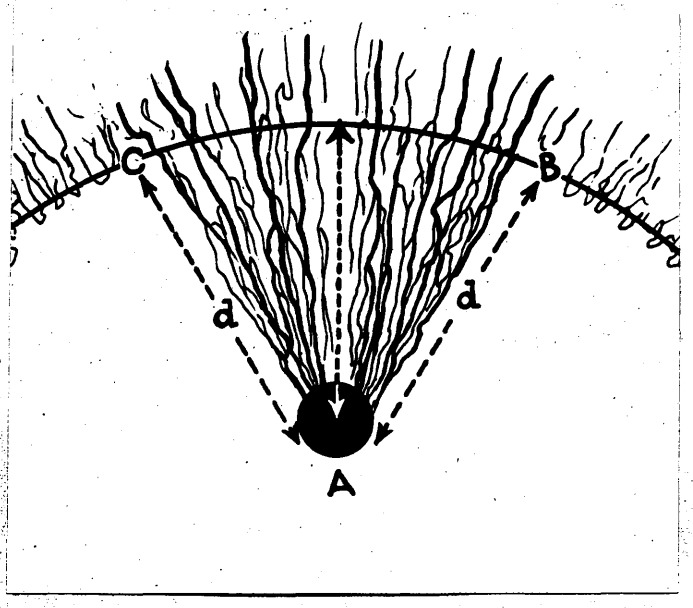


Figure 24.

A diagrammatic representation of the vascular triangle. The vessels do not all grow towards the lesion as shown, but tend to grow towards the centre of the cornea (Fig.23). The shortest distance from the lesion (A) to the limbus was noted in addition to the length of the side of the isosceles triangle (d).

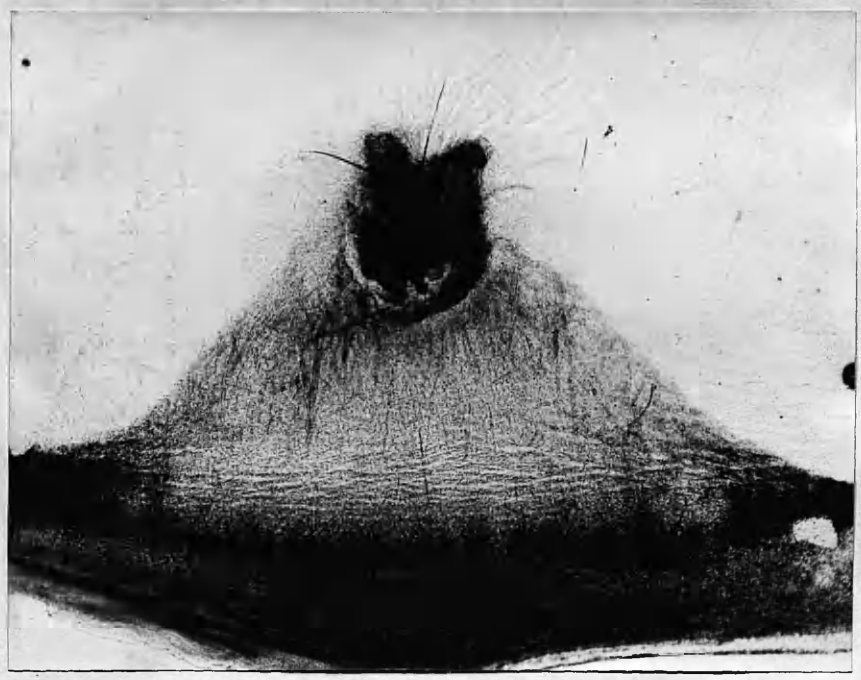


Figure 25

A flat preparation of rabbit's cornea mounted in 50% glycerol. The lesion (above) was produced by repeated heat injuries for 10 successive days. Note the migration of pigment from the limbal ring (below).

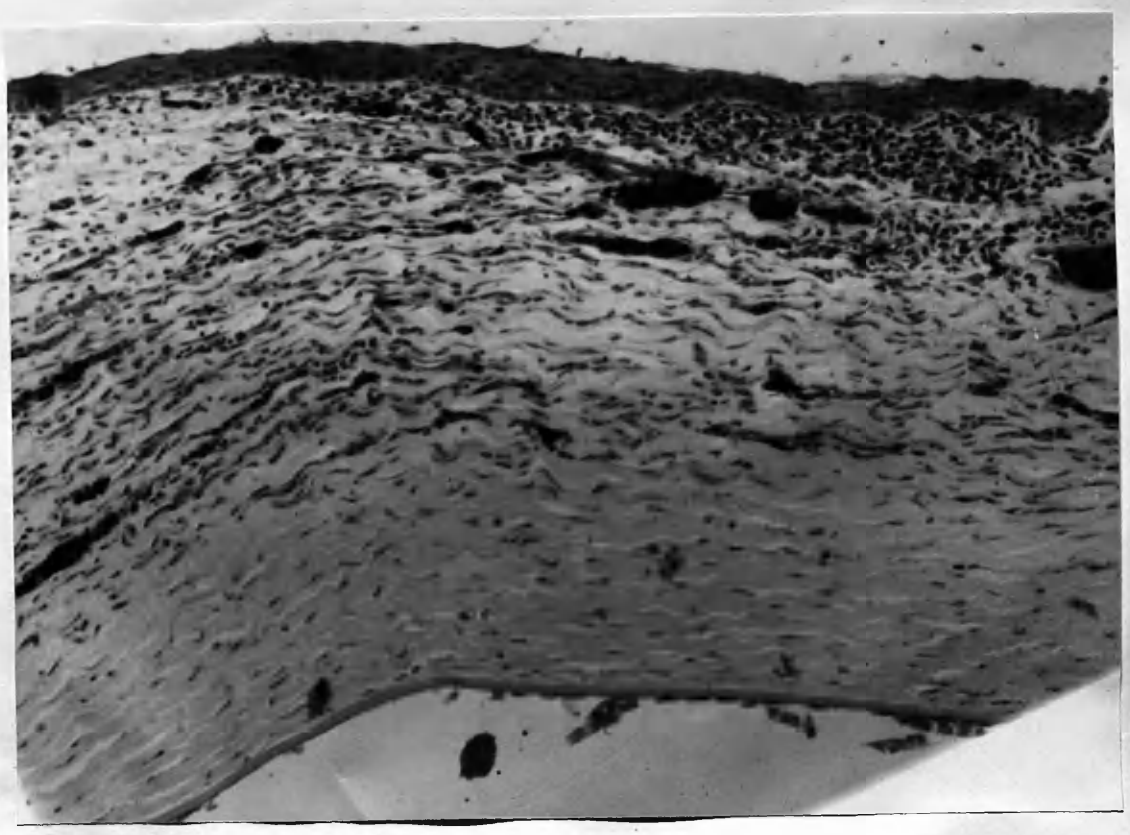


Figure 26

Section of rabbit's cornea after repeated cauterisation for ten successive days. The section was taken from an area between the limbus and the lesion. (Magnification, 60). The epithelium is above. The vessels occupy the anterior two-thirds of the cornea. There is also considerable cellular infiltration.

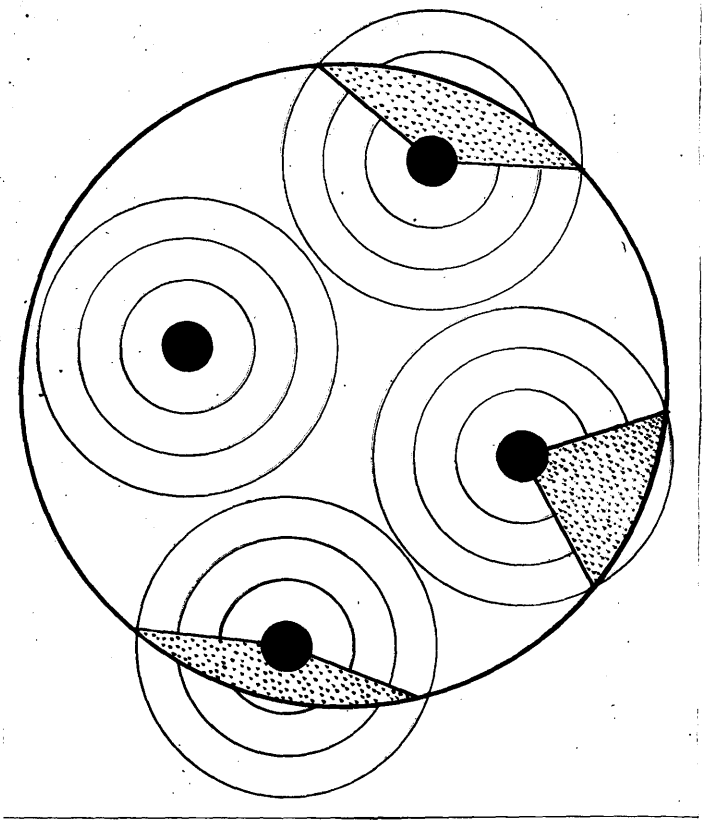


Figure 27.

Diagram to explain the effects of lesions of the cornea at different distances from the limbus. The black circle represents the standard lesion. The concentric lines are placed to scale at 1 mm. intervals. The shaded area indicates the theoritical shape of the area of infiltration.

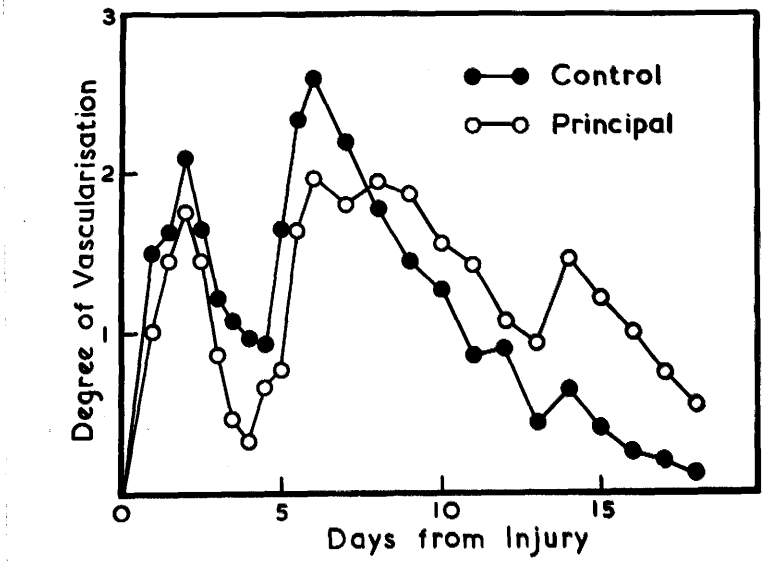


Figure 28.

Effect of cortisone on corneal vascularisation after injury. The mean degree of vascularisation in arbitrary units graphed against time in days in the control and cortisone treated group of guinea-pigs.

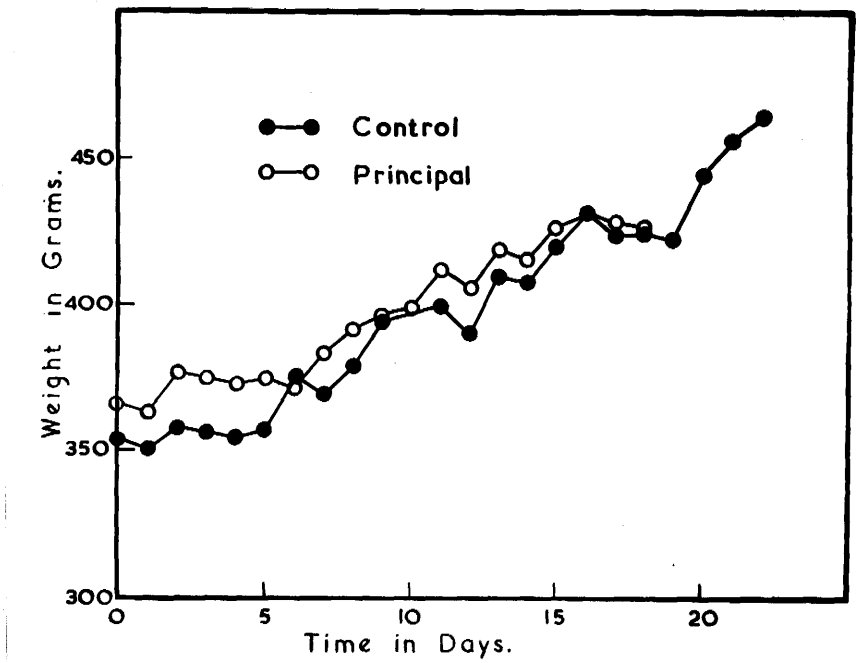


Figure 29.

The growth curves of the control group and deoxycorticosterone group of guinea-pigs (Chapter 10).

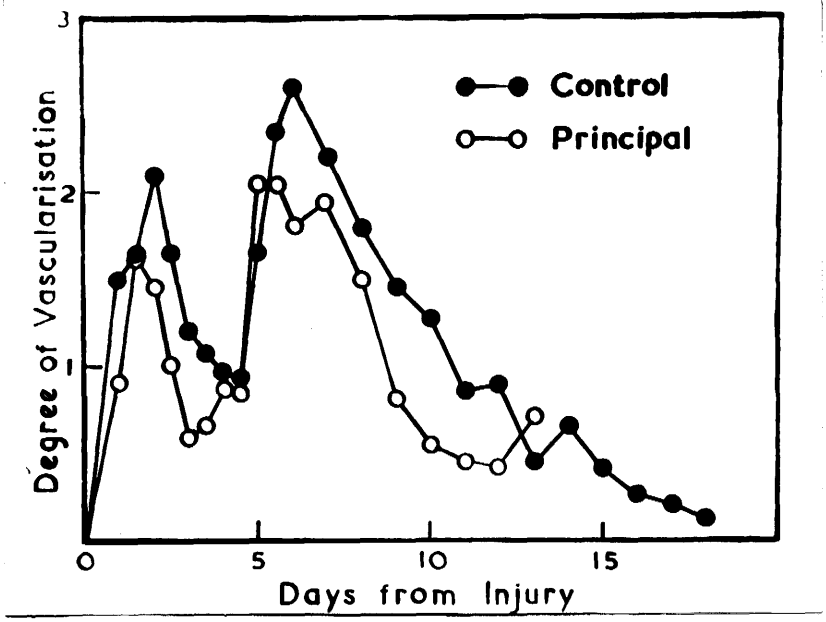


Figure 30.

The mean degree of vascularisation in arbitrary units graphed against time in days in the control and deoxycorticosterone treated group.

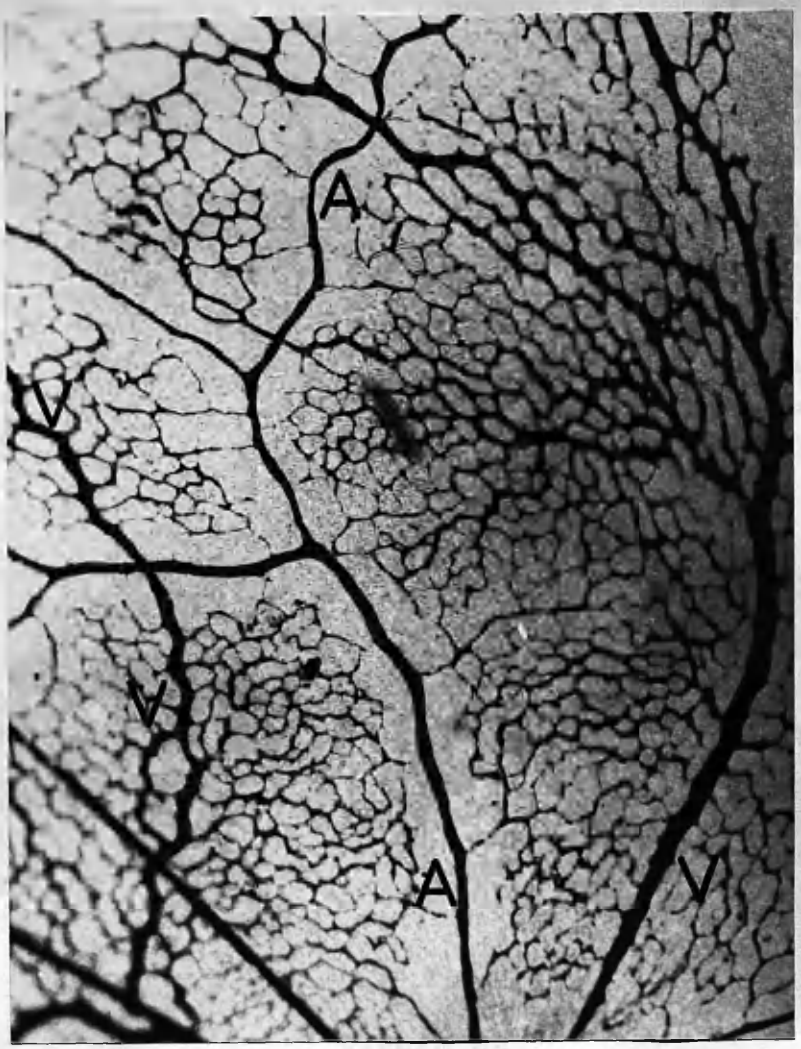


Figure 31

The retinal vascular system in a normal rat 150 hours old. A -- artery. V -- vein. The vessels are demarcated by injecting Indian ink into the left ventricle. (Magnification, 100).

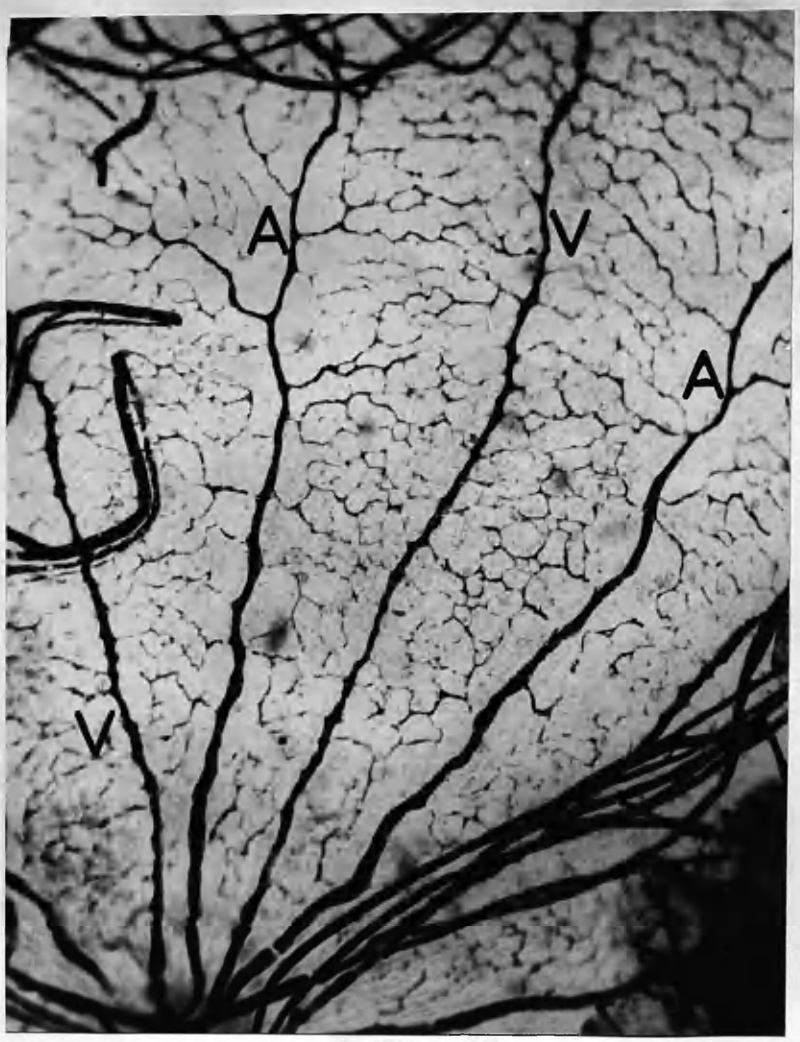


Figure 32

The retinal vascular system in a rat 220 hours old after 195 hours in the low pressure chamber. A -- artery. V -- vein. The vessels are demarcated by means of Indian ink perfusion. (Magnification, 100).

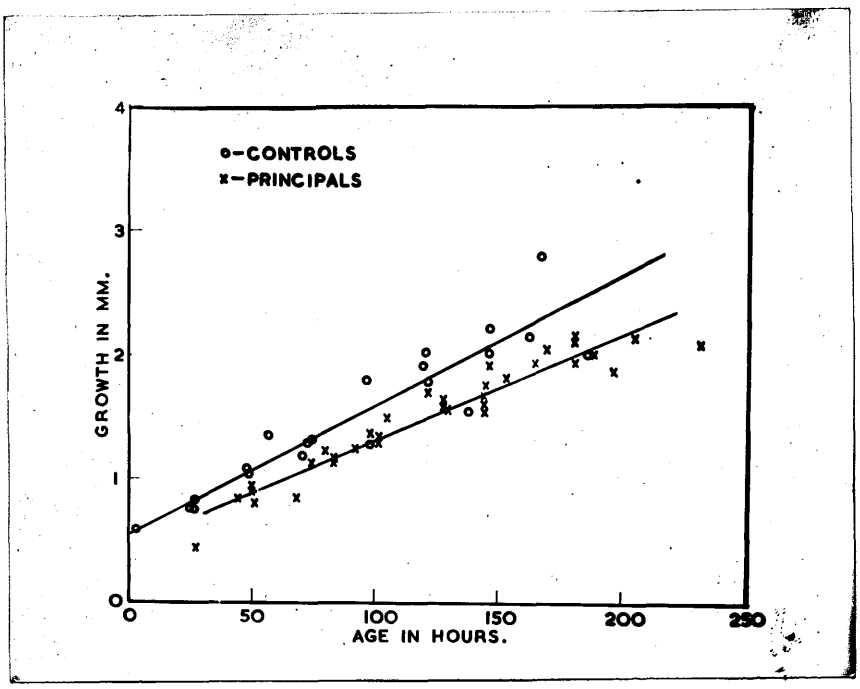


Figure 33.

The radial extent of retinal vascularisation in mm. measured from the optic disc to the periphery of the vascularised area plotted against the age of the rat in hours. Control group developed at normal atmospheric pressure. Principal group developed at half atmospheric pressure.

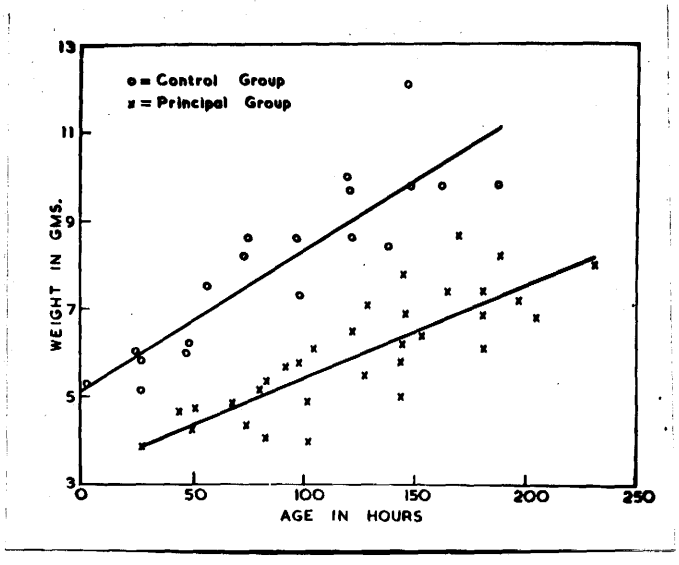


Figure 34.

The weight of the rat in grams plotted against the age in hours.

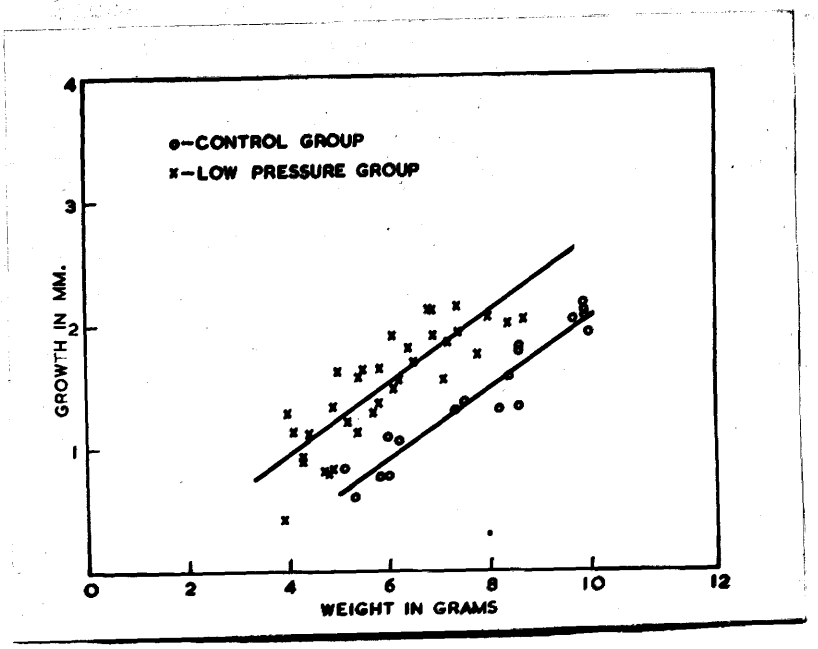


Figure 35.

The radial extent of retinal vascularisation in mm. plotted against the body weight in grams.

Table 1.

Relation of the Concentration of Sodium Fluorescein to the Intensity of Fluorescence from the Standard Strips of Filter Paper for Clinical Use.

Intensity of Fluorescence.	Concentration of Fluorescein in μg./cm.^{2} of Filter Paper.
0	No fluorescein
1	0.5
2	1
3	2
4	4
5	8

TABLE 2

Author	Method	Animal	Distribution of Ascorbic Acid					
			Epithelium	Bowmans M.	Superficial stroma	Deep stroma	Descemets M.	Endothelium
Henkes (1946)	Microtitration & Histochemical	Cow 24.1 mg%	+	0	+ + + Peak 70 mg%	+ +	+	+
		Rabbit 18.8 mg%	+	No membrane present	+ + + Peak 40 mg%	+	+ +	+
		Guinea-pig 18.9 mg%	+	-	+ + + Peak 30 mg%	+	+ +	+
Pirie (1945)	Microtitration	Cow 31 mg%	+ + + 72.5 mg%	-	+ 17 mg%		-	-
		Rabbit 55 mg%	+ + + 118 mg%	-	+ 25 mg%		-	-
Schmid and Bürke (1943)	Histochemical	Guinea-pig	+ + +	+ +	+		+ +	-

Table 3.

The effect of oral administration of ascorbic acid on the healing of corneal epithelium (Galloway, Garry and Hitchin, 1948).

		Mean healing time in hr.	Difference.
Control	(50)	45 (± 9.0)	
			1
Deficient	(50)	46 (±10.2)	

(P> 0.9)

Table 4

Time for Epithelial Healing of Superficial Corneal Heat Injuries in Guinea-pigs (180°C).

No. of wounds	Mean period for healing (hr.)	Difference between control and deficient animals
6 control	56.0 (±7.2) *	
		1.0
8 scorbutic	55.0 (±5.5)	

$P > 0.9$

* Standard Error of the Mean

Table 5

Time for Epithelial Healing of Superficial Corneal Heat Injuries in Guinea-pigs (120°C).

No. of wounds	Mean Period for healing (hr.)	Difference between control and deficient animals
10 control	26.4 (± 1.22)*	
		1.4
8 scorbutic	25.0 (± 1.81)	

$P > 0.5$

* Standard Error of the Mean

Table 6

Comparison of the Mean Times for Healing between the 120°C group and the 180°C group.

No. of Wounds	Mean period for healing (hr.)*
14 at 180°	55.43 ± 4.232
18 at 120°	25.78 ± 1.034

* Value with its standard error.

$P < 0.001$

Table 7.

Time for Epithelial Healing of Deep Corneal Heat Injuries in Guinea-pigs.

No. of Wounds	Mean Time of healing (hours)	Difference between controls and deficient animals (hours)
32 control	94.0 (±6.02)*	31.8
32 scorbutic	125.8 (±7.02)	(highly significant)

't' = 3.43 P < 0.01 * Standard Error of the Mean

Table 8.

Weights required to cause perforation of Cornea.

Interval after injury (hr.)	Perforation pressure. Controls (kg.)	Deficient animals (kg.)
218	0.8	---
218	0.7	---
242	0.8	0.5
242	0.9	0.7
264	1.1*	0.7
264	1.7*	0.7
288	1.4*	0.9
288	1.2*	0.3
288	0.3	0.8
288	0.7	0.6
314	1.4*	0.9
337	0.9	0.8
337	1.3*	0.9
528	1.2*	0.5
528	1.4*	0.9
552	1.2*	0.9
552	1.4*	0.9
631	1.5*	1.4*
631	1.4*	1.2
672	1.6*	1.4
672	1.6*	1.5*
672	1.7*	1.5
672	1.4*	1.6
696	1.7*	1.6*
770	1.3*	1.4
770	1.4*	1.5*
2004	1.4*	1.4
2004	1.6*	1.6*

* Indicates that the eye has slipped from the apparatus before perforation through the lesion had occurred.

Controls: 20 mg. ascorbic acid daily

Deficient animals: 0.5 mg. ascorbic acid on alternate days.

Table 9

Comparison of the Frequency of Perforation of the Eye in Control and Scorbutic Guinea-pigs with Deep Corneal Injuries.

No. of Eyes	Perforation	No Perforation
Control	7	21
Deficient	21	5

$Chi^2 = 14.64$

$P < 0.001$

Table 10.

Comparison of the Weight Required to Perforate the Eye on the Compression Balance in Control and Scorbutic Guinea-pigs.

No. of Wounds	Mean Weight for perforation (g.)*	Difference between control and deficient animals
17 control	1082 ± 85.9	349
15 scorbutic	733 ± 48.5	

* Value with its standard error.

$P < 0.001$

Table 11.

Comparison of the Weight Required to Rupture the Eye on the Compression Balance in Control and Scorbutic Guinea-pigs.

No. of eyes	Mean weight for rupture (g.)*	Difference between control and deficient animals
17 control	1700 ± 71.2	
		19
32 scorbutic	1681 ± 48.5	

* Value with its standard error

P > 0.8

Table 12.

Relationship of Mean Healing Time to Depth of Corneal Ulcers in Man.

Depth of Infiltration	Mean Healing Time in Days: Principals (Receiving 1.5g additional Ascorbic Acid Daily)	No. of Cases	Controls (No additional Ascorbic acid)	No. of cases
	A		B	
Superficial	3.63 (±0.54*)	11	3.80 (±0.50)	15
	C		D	
Deep	4.36 (±0.40)	11	6.15 (±0.50)	13

* Standard Error of Mean

Table 13.

Incidence of Pyorrhoea in Patients suffering from Corneal Ulcers compared with a Control Group with Non-Infective Eye Conditions

	No. of Patients with Pyorrhoea	No. of Patients without Pyorrhoea
Patients with ulcers	20 (69%)	9 (31%)
Patients without Ulcers (control group)	8 (28%)	21 (72%)

$x^2 = 8.4$ $P < 0.01$

Table 14.

Comparison of the Incidence of Recurrent Fluorescence in Control and Sodium γ-Resorcylate Treated Guinea-pigs.

	Simple Healing	Recurrent Fluorescence
Control	14	6
Principal	4	16

$x^2 = 8.18$

$P < 0.005$

Table 15

Effect of the Administration of Cortisone Acetate on Epithelial Healing Time.

	End Point of healing in hr.	Difference
Control (20)	101.4 (±6.8)	
		8.4
Principal (20)	109.8 (±7.2)	

$t = 0.6822$

$P > 0.5$

Table 16.

Dimensions of the Vascular Triangle resulting from Serial Cauterisation of the Rabbit Cornea.

Rabbit	Distance of lesion from limbus in mm.	Size of isosceles sides in mm.
A	1.7	3.3
B	1.7	3.3
C	2.1	3.2
D	2.1	3.2
E	4.2	No vascularisation

Table 17.

Dimensions of the Vascular Triangle resulting from Serial Cauterisation of the Rabbit Cornea.

Rabbit	Eye	Distance of lesion from limbus in mm.	Size of isosceles sides "d" in mm.
A	Rt.	1.4	4.0
E	Lt.	1.9	4.4
C	Lt.	2.1	4.3
D	Lt.	2.1	4.4
H	Lt.	2.1	3.9
B	Rt.	2.2	4.4
D	Rt.	2.2	4.4
E	Rt.	2.2	4.2
A	Lt.	2.3	4.3
H	Rt.	2.4	3.8
G	Rt.	2.5	3.9
C	Rt.	2.7	4.2
B	Lt.	3.3	4.3

Table 18

Incidence of Vascularisation in the Control and Scorbutic Groups of Guinea-pigs.

Eyes	No. of vascularised corneae	No. of non-vascularised corneae
Control	9	23
Scorbutic	19	13

$x^2 = 5.1$ $P < 0.05$ (Significant)

Table 19

Time for Epithelial Healing of Deep Corneal Heat Injuries in Guinea-pigs

No. of wounds	Mean time of healing (hours)	Difference between controls and deficient animals (hours)
32 control	94.0 ($\pm$6.02)*	31.8
32 scorbutic	125.8 ($\pm$7.02)	(Highly significant)

't' = 3.43 $P < 0.01$ * Standard Error of the Mean

Table 20.

Comparisom of the Mean Time of Wound Healing in the Vascular and Non-vascular Corneae in the Scorbutic Group

No. of Eyes	Mean time of healing (hours)	Difference in Means (hours)
19 vascular	127.6 (±9.3)*	4.5
13 nonvascular	123.1 (±11.5)	(Not significant)

't' = 0.79 P > 0.4 * Standard Error of the Mean

Table 21

Comparison of the Mean Time of Wound Healing in the Vascular and Non-vascular Corneae in the Control Group

No. of Eyes	Mean Time of healing (hours)	Difference in Mean (hours)
9 vascular	106.7 (±13.8)*	17.7
23 nonvascular	89.0 (± 6.3)	(Not significant)

't' = 1.33 P > 0.1 * Standard Error of the Mean

Table 22.

Comparison of the Progress of Corneal Vascularisation in Control and Scorbutic Groups.

Vascularisation	Mean Time (hours)		Difference in Mean (Hours)
	Control (9 eyes)	Scorbutic (19 eyes)	
Onset	33.7(±8.30)*	52.6 (±8.79)	18.9
Maximum	69.4(±6.99)	79.6 (±9.31)	14.7
Disappearance	134.2(±33.44)	146.5(±21.56)	12.3

* Standard Error of the Mean

Table 23.

Comparison of the Progress of Corneal Oedema in Control and Scorbutic Groups

Oedema	Mean Time (hours) Control (32 eyes)	Scorbutic (32 eyes)	Difference in Mean (hours)
Onset	17.3 (±2.53)*	16.5 (±1.83)	0.8
Maximum	25.8 (±2.78)	42.8 (±6.22)	17.0

* Standard Error of the Mean

$P < 0.05$

Table 24.

Incidence of Vascularisation in the later Stages of Healing of Deep Corneal Lesions in Control and Cortisone treated Guinea-pigs

	Vascularised.	Nonvascularised.
Control Group	6	14
Cortisone Group	12	8

$x^2 = 4.63$

$P < 0.05$

Table 25

Arbitrary Scale of extent of new vessels in the cornea

Limbal congestion	= ½
Vessels just within Cornea	= 1
Vessels half-way between limbus and lesion	= 2
Vessels touching lesion	= 3
Vessels encroaching on lesion	= 4

Table 26

The dimensions of the capillary-free zone in the control and low pressure group of rats.

	Mean breadth in μ	S.E. of mean.	Difference in mean.
Control (134)*	63.19	1.42	14.07 (Highly significant)
Low Pressure (116)*	49.12	1.17	

P 0.01 * Number of observations.

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Personal Publications

Many of the results presented in this thesis have been published, and a list of the papers in chronological order of appearance is appendaged. The conclusions expressed in this thesis are sometimes somewhat at variance with the views printed in the earlier papers. This is due simply to the inevitable advancement of knowledge and to a fuller understanding of the subject in the light of subsequent work.

1. CAMPBELL, F.W. & MICHAELSON, I.C. (1948). Heat Injury and New Vessel Formation in the Rabbit's Cornea. J. Physiol., 108, 19,P.

2. CAMPBELL, F.W. & MICHAELSON, I.C. (1949). Blood Vessel Formation in the Cornea. Brit. J. Ophth. 33, 248.

3. CAMPBELL, F.W. & FERGUSON, I.D. (1950). The Role of Ascorbic Acid in Corneal Vascularisation. Brit. J. Ophth., 34, 329.

4. CAMPBELL, F.W. & BOYD, T.A.S. (1950). The Use of Sodium Fluorescein in Assessing the Rate of Healing in Corneal Ulcers. Brit. J. Ophth., 34, 545.

5. CAMPBELL, F.W., FERGUSON, I.D. & GARRY, R.C. (1950). Ascorbic Acid and Healing of Heat Injuries in the Guinea-pig Cornea. Brit. J. Nutrit., 4, 32.

6. BOYD, T.A.S. & CAMPBELL, F.W. (1950). Influence of Ascorbic Acid on the Healing of Corneal Ulcers in Man. Brit. Med. J., (1950), 2, 1145.

7. CAMPBELL, F.W. & WYBAR, K.C. (1951). The Influence of Sodium γ-Resorcylate on Corneal Wound/

Wound Healing in the Guinea-pig. J. Physiol., 115, 11,P.

8. CAMPBELL, F.W. (1951). The Influence of a Low Atmospheric Pressure on the Development of the Retinal Vessels in the Rat. Trans. Ophth. Soc. U.K., 71, (in press).