COO-2401-67

COMBINED PROGRESS REPORT 2nd

PROPOSAL TO THE UNITED STATES

ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

CELLULAR PROLIFERATION AND REGENERATION FOLLOWING TISSUE DAMAGE

Contract E (11-1) 2401

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DISCLAIMER

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PROJECT ABSTRACT

This project involves a study of wound healing in tissues of the eye, particularly lens, cornea, and surrounding tissues. The reactions of these tissues to mechanical injuries, as well as injuries induced by chemotoxic agents are under study. It is felt that a better understanding of the basic reactions of the eye to injurious agents may be of importance in the evaluation of potential environmental hazards.

In order for a tissue to repair itself following injury, new cells must be produced, and considerable cellular migration and rearrangement occur. We need a better understanding of the mechanisms which control cellular proliferation in normal, injured, and repairing tissues. One important question, therefore, is what reactions trigger the cell cycle following injury, and turn off the cell cycle at a certain time during healing. Another is what factors control the migration and re-arrangement of cells during wound healing.

The reactions which trigger the cell cycle (promote the G-0 → G-1 transition) control the amount of cellular proliferation and tissue growth in normal and injured tissues. These reactions can be conveniently studied during the process of wound healing or exposure of the isolated whole rabbit lens to a serum mitogenic factor in organ culture. It has now been established in the lens, in tissue culture, that the triggering process can be effected by insulin in a completely defined medium. It is proposed to determine the components of the defined medium which are essential for the promotion of the G-0 → G-1 transition, and to determine if various phases and subphases of the cell cycle can be

characterized by the requirement for specific components of the defined medium. It is also planned to continue studies on the correlation of changes in fine structure (e.g., the burst of ribosome production which characteristically follows exposure of the lens to rabbit serum) with changes in macromolecular synthesis (RNA, protein); to investigate, through the use of specific anti-metabolites, the possible role of increased ribosomal and non-ribosomal RNA production in the stimulation of cellular proliferation during wound healing.

It is also planned to study the lens epithelial cell surface as well as the topography of the underlying lens fibers by means of the techniques of electron microscopy and scanning electron microscopy, before and after the addition of mitogenic agents (rabbit serum, insulin) to the culture medium, and at various times after mechanical and chemical injury in vivo. On the premise that cellular inter-relationships (e.g., cell communication, or coupling) play an important role in the control of tissue growth, particular attention will be paid to changes in the junctional complexes, as seen in replicas of freeze-etched preparations and in thin sections for T.E.M. The techniques developed during the past year here in the Kresge Eye Institute for studying the internal structure of the ocular lens with scanning electron microscopy (S.E.M.) will also help to serve as a basis for this portion of the program. Also, studies which have been initiated on the ultrastructure of the corneal surface utilizing the S.E.M. and T.E.M., will be expanded to include an analysis of the changes which

occur on the epithelial and endothelial cell surfaces after injury in the regenerating cornea. In addition it is proposed to study the stimulation of growth of new vessels (neovascularization) which can then invade the cornea following chemical injury, and reduce the possibilities of subsequent successful corneal transplantation. Within the last several months we have developed a method of isolating and culturing rabbit retinal vessels and have been able to demonstrate DNA synthesis in these cultures. It would seem then that this procedure could serve as a model for the study of what causes vessels to proliferate and therefore what factors might be involved in neovascularization, in much the same way that the cultured lens has been used to study those factors that regulate cellular proliferation in the lens epithelium. It is also planned to study the growth of new vessels into the cornea with the use of plastic corrosion casts, which can then be studied in detail with scanning electron microscopy.

It is expected that the proposed program will lead to a better understanding of some of the initial structural and biochemical changes which are essential to the mechanisms which control cellular proliferation, migration and re-arrangement following injury. The new knowledge gained from this study may be of use in helping to prevent or ameliorate the effects of various chemical and physical injurious agents on the eye.

INTRODUCTION

The present proposal is based on findings obtained during the past several years. The results of previous experiments are included in publications, the reprints of which are attached. The ultimate goal of this program is a further elucidation of the mechanisms that underlie the control of cellular proliferation and cellular migration in the normal and injured tissues of the eye. This should, in turn, lead to a better understanding of those phenomena which are basically related to cellular proliferation and migration: differentiation, regeneration, growth, wound healing, and the response to injurious agents. From a practical point of view, it is felt that a better understanding of the basic reactions of the eye to injurious agents may be of importance in the evaluation of potential environmental hazards.

Earlier work has shown that the central epithelial cells of the adult rabbit lens normally become "blocked" in the G-1 phase of the cell cycle (see attached reprint of review for International Review of Cytology). For convenience, this state of blockade has been referred to as G-0-1 (Srinivasan and Harding[1], 1965. See reprint, COO-2401-2). Following mechanical injury, many of these cells are triggered to enter the cell cycle and undergo DNA synthesis and mitosis. Some of these cells may go through a second, or possibly a third, cell cycle. At the end of the wound healing process, however, the cells presumably once again become blocked in the G-1 phase (enter G-0-1). The results emphasize that the key to understanding the mechanism(s) which controls cell division and the

1 Invest. Ophthal. 4:452-470, 1965

consequent tissue growth related to cell division, lies in an understanding of the reactions which trigger the cell cycle. These reactions can be conveniently studied during the process of wound healing or exposure of the isolated whole lens to a serum mitogenic factor in organ culture.

Recent work on the E.R.D.A. Contract indicates that such reactions can now be studied in the isolated lens maintained in organ culture in a completely defined medium.

The relationship of the G-0 → G-1 transition to the cell cycle is summarized in the accompanying diagram (Fig. 1). Data on the various topics listed in the diagram are described in detail in the publications (derived from the E.R.D.A. contract) listed in the bibliography on pages V-1 - V-5 . Photocopies of the majority of the publications (reprints or pre-prints) are also included. This also includes recent publications and manuscripts on the scanning electron microscopy of the ocular lens and the corneal surface, which we believe, will serve as a basis for studies of the effects of injurious agents on the lens, cornea, and surrounding tissues.

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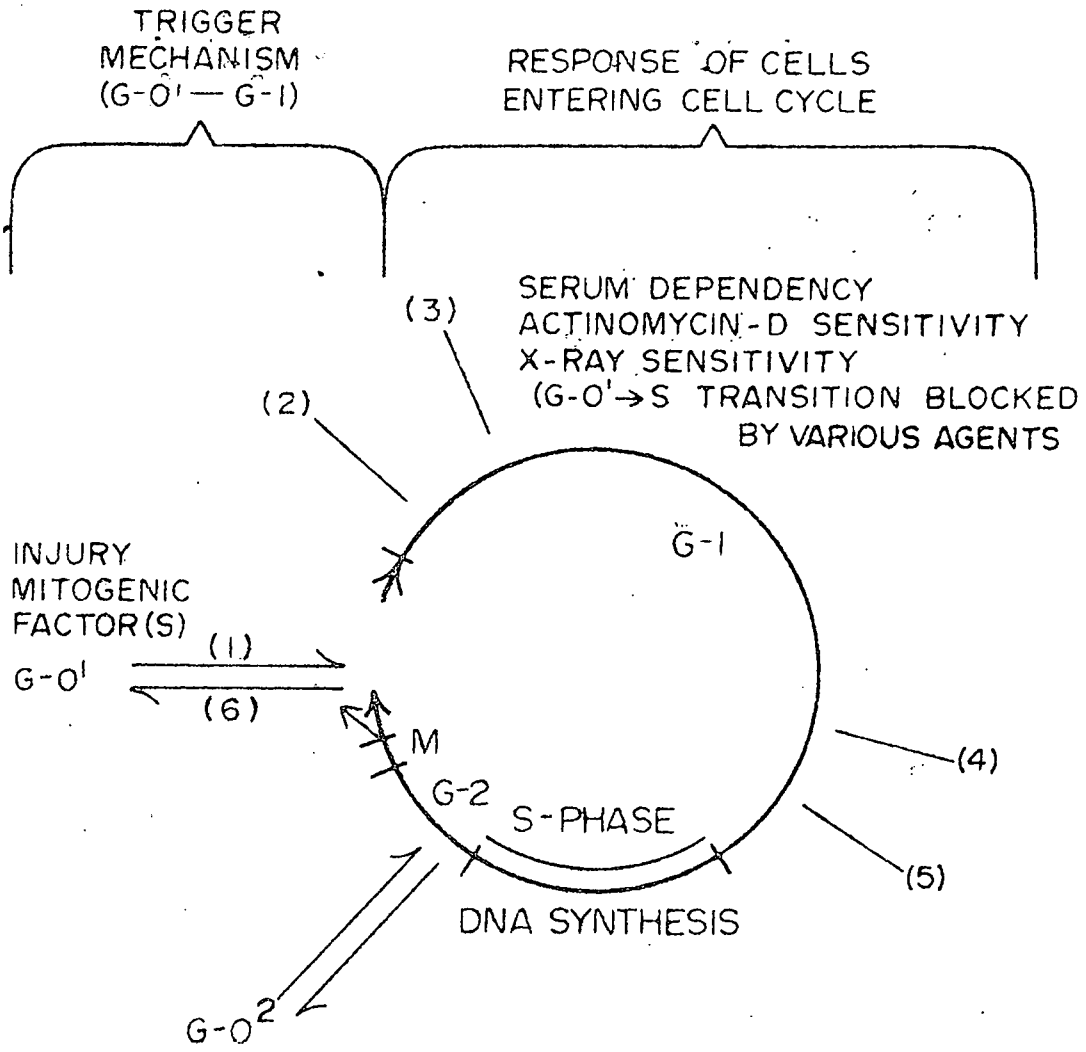


(1) INJURY
SERUM FACTOR(S),
INSULIN

(2) INITIAL CHANGE IN
TISSUE ORGANIZATION
(E.M. LEVEL)

(3) RNA AND PROTEIN
SYNTHESIS INCREASES
RIBOSOMES INCREASE

(4) CELLULAR REARRANGEMENT
EVIDENT AT HISTOLOGICAL LEVEL

(5) SERUM DEPENDENCY ENDS
ACTINOMYCIN-D SENSITIVITY ENDS

(6) POSSIBLE DIFFERENTIATION

FIG 1

Reaction to Injury

The ocular lens has proven uniquely suitable for studies on the initiation of cell division in an organized tissue (see attached reprints including review COO-2401-30. The epithelium which is situated as a monolayer on the anterior portion of the lens just subjacent to the lens capsule is very responsive to injury. A small mechanical wound to the anterior surface of the rabbit lens in vivo results in a wave of stimulation of DNA synthesis and mitosis that moves out from the wound site. X-irradiation of rabbit lenses two days after a mechanical injury can, within less than two hours, completely suppress the wave of mitosis, but allow DNA synthesis to continue. Additional studies on lenses x-irradiated at the time of mechanical injury suggest that radiation also tends to prevent the G-1 to S transition of the cell cycle. (For additional details, see Harding et al., International Review of Cytology, ~~Reprint COO-2401-30 attached~~).

Initiation of DNA Synthesis and Mitosis in the Cultured Lens

The lens maintained in organ culture has also proven useful in studies on the control of cellular proliferation. The lens is avascular, lacks a nerve supply, and is entirely enclosed within a membrane, the lens capsule. Because of these and other characteristics, it is relatively easy to isolate and study in organ culture with a minimal change in tissue organization. It has been found that the establishment of the whole lens in organ culture under a variety of

conditions can result in marked changes in the rate of DNA synthesis and mitosis. By suitable changes in the culture medium, it is possible to obtain either a marked stimulation of DNA synthesis and mitosis or a maintenance of the epithelium in a normal quiescent state. Rabbit lenses cultured in a medium consisting of medium 199 containing 23% serum (M-199-S) undergo a series of characteristic changes in epithelial mitotic activity. During the first 9 hours mitosis practically ceases. It subsequently resumes, reaches a peak at 20 hours, and then once again decreases to a low level. The above changes, all of which occur in the periphery of the epithelial layer, herald the initiation at approximately 22-24 hours of a pronounced stimulation of DNA synthesis (thymidine incorporation) in the central region. At 44 hours, the number of cells in mitosis in the central region increases enormously. At this and later times the epithelium appears as a multilayer of cells which have lost their original organization. (For details of these experiments see reprints COO-2401-30 and COO-2401-8).

The marked stimulation of DNA synthesis and mitosis which characterizes lenses cultured in M-199-S for 48 hours or more does not occur in the lenses cultured in medium 199 containing 23% serum dialysate (M-199-SD). In lenses cultured in this latter medium, the central epithelium is maintained in a non-proliferating state. This state of non-proliferation can be terminated by transfer of the lens to M-199-S. Thus lenses which have been cultured in M-199-SD for 48 hours and subsequently in M-199-S for an additional 48 hours, show a marked stimulation of cellular proliferation in the central epithelium.

It appears, therefore, that cellular proliferation in the cultured lens can be triggered at will by a change in composition of the culture medium. (For details of these experiments, see Harding et al., reprint attached COO-2401-21). Some characteristic, or combination of characteristics, of the serum-containing medium appears to be responsible for the stimulation.

The above experiments indicate that the central epithelium of the cultured rabbit lens can be kept in the G-0-1 state, characteristic of this tissue in the intact living adult organism. The subsequent addition of serum to the culture medium can trigger these cells to enter the cell cycle and divide. Cultured lenses, triggered by exposure to M-199-S during the first 24 hours of culture and then switched to M-199-SD, show extensive DNA synthesis at the end of 48 hours of total culture time, and very little DNA synthesis at 72 hours. These results also show that these cells have once again become "blocked" at some point in the cell cycle. The results also show that the addition of serum to the medium at 72 hours can cause a re-stimulation of these cells to divide. The cultured lens can, therefore, serve as a model system for studies on the control of cell division in an organized tissue in which the central epithelial cells can be maintained in the G-0 state (similar to the normal epithelium in the intact animal), or triggered at will to enter the cell cycle. It now appears that following a marked stimulation, the G-0 state can be re-established by removal of whole serum from the medium; and that once this "blocked" state has been achieved, the

cells can be re-triggered to enter the cell cycle upon the second addition of serum to the culture medium.

Serum is not required throughout the whole period of culture in order to elicit a stimulation of DNA synthesis, but only during an initial period which ends prior to the onset of both DNA synthesis and mitosis. Some of the characteristics of the serum factors which promote these reactions and the conditions of culture which affect their activity have been reported (see attached reprints). More recently, freshly isolated rabbit serum was fractionated by DEAE-cellulose column chromatography according to the methods of Sober and Peterson[1](1958). This fractionation procedure yields four major fractions; the first fraction, which according to its electrophoretic mobility and analytical ultracentrifugal analysis was tentatively identified as containing gamma globulin, showed mitogenic activity. Fraction one does not show cellular disorganization and multilayering comparable to whole serum, although it does show some cellular disorganization. Mitogenic activity of the first fraction is significantly higher than that of whole serum, when activity is expressed on the basis of milligrams of protein per milliliter in the culture medium.

Recent experiments have shown that in a chemically-defined medium, KEI-4 as developed in Kinsey's laboratory (Compare with COO-2401-60), insulin can replace the serum mitogenic factor. This seems to depend on the integrity of the insulin molecule, since neither the α nor β chains evoked the mitotic response. Therefore, we have now reached the point where the cell cycle can be chemically triggered in an organized tissue maintained in a completely defined medium in organ culture.

1 Fed. Proc. 17(4), 1116-1126, 1958.

Period of Serum Dependency and Actinomycin D Sensitivity

Previous experiments have shown that in order to obtain a pronounced serum-induced stimulation of mitosis at a total of 48 hours of lens culture, it is not necessary to have the serum in the culture medium beyond the first 20 hours of culture. It is possible that this "period of serum-dependency" is a reflection of special requirements of the cells for a portion or all of the G-1 phase, since serum dependency ends just prior to the initiation of DNA synthesis. The need for specific serum components may change at this time (and, indeed, each phase of the cell cycle may eventually be characterized by its own set of specific requirements).

It appears, therefore, that there is a discrete and definable period of serum dependency, which may reflect specific requirements for the G-0 to G-1 transition, and perhaps for the G-1 phase itself. Serum may not be required during all portions of this 20 hour period. Lenses which have been cultured 24 hours in M-199-SD and which do not, therefore, show a stimulation, can be subsequently stimulated by the addition of whole serum to the medium (transfer to M-199-S). Under these conditions, however, the time between addition of serum and the initiation of DNA synthesis is significantly reduced. One possible explanation for this effect is that the triggering of the cell cycle (G-0 → G-1 transition) and perhaps some of the initial reactions of the G-1 phase can occur in M-199-SD, (i.e., whole serum is not required during the beginning of the first 20 hours of culture).

This assumes that the pre-synthetic phase (the period between explanation and the initiation of DNA synthesis) can be divided into sub-phases, characterized by their dependence on the presence of specific components in the culture medium (see COO-2401-34 for a further discussion of these experiments). Another possible explanation is that the preincubated lenses have had time to "recuperate" at least partially from the trauma of isolation and establishment in culture. If this trauma delays the mitogenic action of serum, the shorter period of time required for the serum factor(s) to stimulate DNA synthesis in the preincubated lenses, which have had time to "recuperate", can be explained. Recent evidence, however, indicates that there is indeed an initial sub-phase which is serum-independent (COO-2401-49). The results of this investigation, indicate that approximately the first 4 hours of the 20 hour period are also serum-independent. It is of interest that the end of the period of serum dependency corresponds to the end of the time during which the mitotic-stimulatory mechanism is actinomycin D sensitive. Exposure of the freshly-isolated lens to actinomycin D as early as the first 4 hours of culture in M-199-S is sufficient to completely inhibit the DNA synthesis and mitosis which normally follows. However at intervals beyond 18 hours, actinomycin D (0.03 mg/ml) has little or no effect on the entrance of cells into DNA synthesis. It is apparent, therefore, that the initiation of the DNA synthetic phase is preceded by a phase characterized by the production of RNA which is necessary for the epithelial cells to subsequently undergo DNA synthesis and mitosis. (For additional details, see reprints COO-2401-27 and COO-2401-30).

Necessity of RNA Synthesis for Subsequent DNA Synthesis and Mitosis

There is a significant increase in RNA synthesis (as evidenced by the DNA-ase resistant incorporation of tritium-labeled uridine) which commences at 7 hours and remains at elevated levels from 7-25 hours. Moreover, the inhibition of DNA synthesis and mitosis which is brought about by exposure of the lenses to actinomycin D during the early hours of incubation (see COO-2401-27) results in a 20-50% reduction in the incorporation of tritium-labeled uridine. It is during this period of increased RNA production that the system is dependent upon the presence of the serum. As mentioned above, pre-incubation for 24 hours in the non-stimulatory medium M-199-SD, decreases the time required between the addition of serum and the initiation of DNA synthesis. Preincubation also shortens the time required for the serum-induced increase in RNA production and ribosome formation, (see COO-2401-34 for details of this experiment). Serum-induced ribosome formation in preincubated lenses is relatively intense (as compared with the freshly-isolated lens). The preincubated lens, therefore, appears to be an excellent system in which to study induced ribosome formation, which has been suggested by Yamada to characterize the G-0 $\rightarrow$ G-1 transition.

Changes in Ultrastructure

Other studies on the ultrastructure of the cultured and wounded lens showed a distinct pattern of changes, a detailed description of which appears in (COO-2401-28). Scanning electron microscope studies have been initiated to follow changes in the cell surface at various times after injury. Preliminary observations on the dogfish lens epithelium show a striking orientation of the cells toward the site

of injury within 24 hours after injury. Among the earliest changes which have been detected following injury in vivo or (exposure) of the lens in culture to mitogenic agents, are changes at the ultrastructural level and, in particular, changes in cell-to-cell relationship (COO-2401-30).

This is of interest since there is increasing evidence that alterations in cell-to-cell relationships may play an important role in the control of cellular activities, including cellular proliferation and tissue growth. In the lens correlations can be made between the distribution patterns of the stimulated cells and the observed changes in structural organization of the epithelium; that is, both tend to assume concentric patterns relative to the center of the epithelial layer. This correlation between proliferation and tissue structural organization is of particular interest in relation to the concepts of contact inhibition (Abercrombie[1], 1970; Abercrombie and Ambrose[2], 1962; Eagle[3], 1967) and cell coupling (Loewenstein[4], 1968a,b,1975[5]) Observations on the growth of cultures of dissociated cells have shown that when the cultured cells come in contact there are distinct changes in cellular properties and activities. When a sufficient degree of contact is made among the originally dissociated cells, cellular motility is markedly reduced and mitosis decreased.

The observations of Loewenstein on cell coupling may be importantly related to contact inhibition. When dissociated cells in culture come in contact, the junctional membranes show a decrease in electrical

1 In Vitro 6:128-142, 1970.
2 Cancer Res. 22:525-548, 1962.
3 Growth Regulating Substances for Animal Cells in Culture, Philadelphia, Wistar Inst. Press, 1967.
4 Dev. Biol. 19, Suppl. 2:151-183 and Perspect. Biol. Med. 11:260-272, 1968.
5 Cell Membranes, New York, H.P. Publishing Co., Inc., 1975.

resistance and an increase in permeability to the extent that ions and relatively large molecules can diffuse freely from cell to cell. This relatively high permeability of junctional membranes has also been described for epithelial cells in their normal tissue organizations (Loewenstein[1], 1968a,b, 1975[2]).

It has been suggested that this "communication" among the cells of a tissue affords a mechanism for the coordination of cellular activities, and it presumably may be through this mechanism that a tissue can control proliferative activity (Loewenstein[1], 1968a,b, 1975[2]). The concepts of contact inhibition and cell coupling offer a highly attractive hypothesis to explain the relation between changes in structural organization in the lens epithelium and the triggering of the cell cycle.

At the ultrastructural level, the gap junctions, which make up part of the junctional complexes between cells, have been implicated as the structures responsible for cell coupling. Considerable experimental evidence indicates that at the gap junctions, there is relatively free diffusion of ions and molecules up to about 200 M.W., from cell to cell. If the gap junctions are the structures responsible for cell communication, and cell communication is involved as a primary mechanism in the control of cellular proliferation, there should be changes in the ultrastructure of the gap junctions following injury, that might be observed in replicas of freeze-fractured preparations.

1 Dev. Biol. 19, Suppl. 2:151-183 and Perspect. Biol. Med. 11:260-272, 1968.
2 Cell Membranes, New York, H.P. Publishing Co., Inc., 1975.

The Kresge Eye Institute will soon have a new Balzers 301 freeze-etch unit with all accessories (a gift of the Kresge Foundation). This will be installed in our cell biology research laboratory, close to the E.M. facilities. Also, newer techniques of examining the ultrastructure of the lens using a combination of scanning and transmission electron microscopy have been established in our laboratory during the past year. A preliminary report of this study is presented in reports COO-2401-68 and COO-2401-66. It is felt that the results presented in COO-2401-68 and COO-2401-66 can serve as a basis for further experimental studies at the ultrastructural level on wound healing, cataract formation, development and aging.

Studies on Other Tissues. Injury-induced Growth of Vascular Endothelium; Neovascularization.

While we plan to continue our studies on the control of cellular proliferation in the ocular lens, we would like to expand the program to studies on other ocular tissues. Neovascularization following injury or certain diseases occurs in the cornea and the retina and can lead to blindness.

In many cases, the loss of sight, due to a chemically-induced opaque cornea, can be corrected by means of corneal transplantation. However, as Duke-Elder has pointed out "..a deep injury involving the entire thickness of the cornea, particularly when the tissue is replaced by fibrous tissue and is heavily vascularized, provides a more difficult problem. Unfortunately, many chemical injuries belong to this category, and most authorities have found that such corneae provide unfavorable material for full-thickness grafting owing to the tendency for the transplant in its turn to become vascularized and opaque." (Duke-Elder, 1972)[1].

Therefore, a blind eye due to a heavily vascularized opaque cornea resulting from chemical injury has a much reduced chance of successful corneal transplantation due to the increased tendency of the transplant to become vascularized and opaque. Very little is known about why blood vessels do not grow into a normal cornea, but do grow into corneae following certain kinds of chemical injury. The growth of new vessels (neovascularization) is dependent in part upon the production of new cells that make up the vascular system.

1 System of Ophthalmology, Vol. XIV Injuries, Part II. Non-Mechanical Injuries, St. Louis, The C.V. Mosby Co., 1972, p. 1035.

We know very little about the factors which control the production of the vascular endothelium. A better knowledge of the factors which control neovascularization in the chemically-injured cornea should serve as a basis for the development of new procedures to control vascularization, and increase the probability of restoration of sight through successful corneal transplantation. In spite of the important and extensive investigations on neovascularization little is known about the initial events that trigger the growth of new vessels. It is generally assumed that a proliferation of vascular endothelial cells in one of the earlier events in neovascularization (Futterman,[1] 1975). For example, it has been observed that "In human retrolental fibroplasia, where its early stages have been studied, multiplication of endothelial cells in patent superficial retinal capillaries is the earliest visible evidence of new vessel formation." (Wise, Dollery, and Henkind,[2] 1971a,b) Very little, or virtually no cellular proliferation occurs in the retinal vessels of the normal adult. In certain disease states, however, vascular endothelial proliferation and neovascularization can be a complication leading to blindness. These include diabetes mellitus, sickle cell disease, and other disorders.

Compared with other tissues, we know relatively little about the factors which initiate the cell cycle in vascular endothelium, and the ultrastructural and molecular changes which accompany it. For example, does blood serum contain a mitogenic agent for vascular

1 Invest. Ophthal. 14:4-6, 1975.
2 The Retinal Circulation, New York, Harper & Row, 1971.

their passage through leaky vessels. Enzymes such as peroxidase and myoglobin have been used to establish the ultrastructural basis of capillary permeability in various tissues and to detect alterations in the permeability of the capillary endothelium under abnormal conditions. The "leakiness" of neovascular formations is also of interest from the point of view of contact inhibition (Abercrombie,[1] 1970; Abercrombie and Ambrose[2], 1962 and Eagle[3], 1967) and intercellular communication (Loewenstein[4], 1968).

Tissue Culture of Retinal Vessels: The techniques do exist for a study of the effects of serum and other possible mitogens on the isolated retinal vessels in culture. The entire "retinal" vasculature of the young rabbit can be isolated and established in tissue culture (Ashton,[5] 1966; Tripathi, Knight, and Ashton,[6] 1974). During approximately the first three weeks after birth the "retinal vasculature" develops along the surface of the retina. Since during this period of time it does not become firmly attached to the retina, it can be dissected out essentially in its entirety and established in tissue culture. Ashton and his co-workers have utilized this in vitro system for studies on the direct effects of oxygen on the retinal vascular endothelium. Tripathi et al, 1974[6] were able to maintain these cultures for over 90 days. Growth in these cultures is very complex, involving several different kinds of cells, macrophages, mesenchyme-like cells derived from endothelium and pericytes but "mature endothelial cells could also be seen proliferating...without passing through a mesenchymal phase" (Ashton,[5] 1966; Tripathi, Knight, and Ashton,[6] 1974).

1 In Vitro 6:128-142, 1970
2 Cancer Res. 22:525-548, 1962
3 Growth Regulating Substances for Animal Cells in Culture, Philadelphia, Wistar Inst. Press, 1967.
4. Dev. Biol. 19, Suppl. 2:151-183 and Perspect. Biol. Med. 11:260-272, 1968
5. Am. J. Ophthal. 62:412-435, 1966.
6. Exptl. Eye Res. 19:449-475, 1974.

Within the last several months we have developed a method of isolating and culturing rabbit retinal vessels and have been able to demonstrate DNA synthesis in these cultures. It would seem then that this procedure could serve as a model for the study of what causes vessels to proliferate and therefore what factors might be involved in neovascularization. The studies on the cultured vessels which have been initiated could be also carried out on excised "buttons" of cornea which have undergone some neovascularization. Using these two systems much might be learned about this process.

In addition, preliminary experiments have shown that plastic corrosion casts can duplicate the vasculature of experimental animal eyes (e.g. rat, rabbit, dog). The procedures required for this study have been used extensively here at Wayne for studies on splenic vasculature. (Barnhart and Baechler,[1] 1974). We feel that these corrosion casts, as observed with scanning electron microscopy, can be used for studies of neovascularization of the cornea following injury. Preliminary experiments, carried out in collaboration with Dr. Charles Baechler and Dr. Marion Barnhart of the Department of Physiology at Wayne State University, School of Medicine, have yielded replicas of the ocular vasculature.

1 Scanning Electron Microscopy, Part III, IITRI, 705-712, 1974.

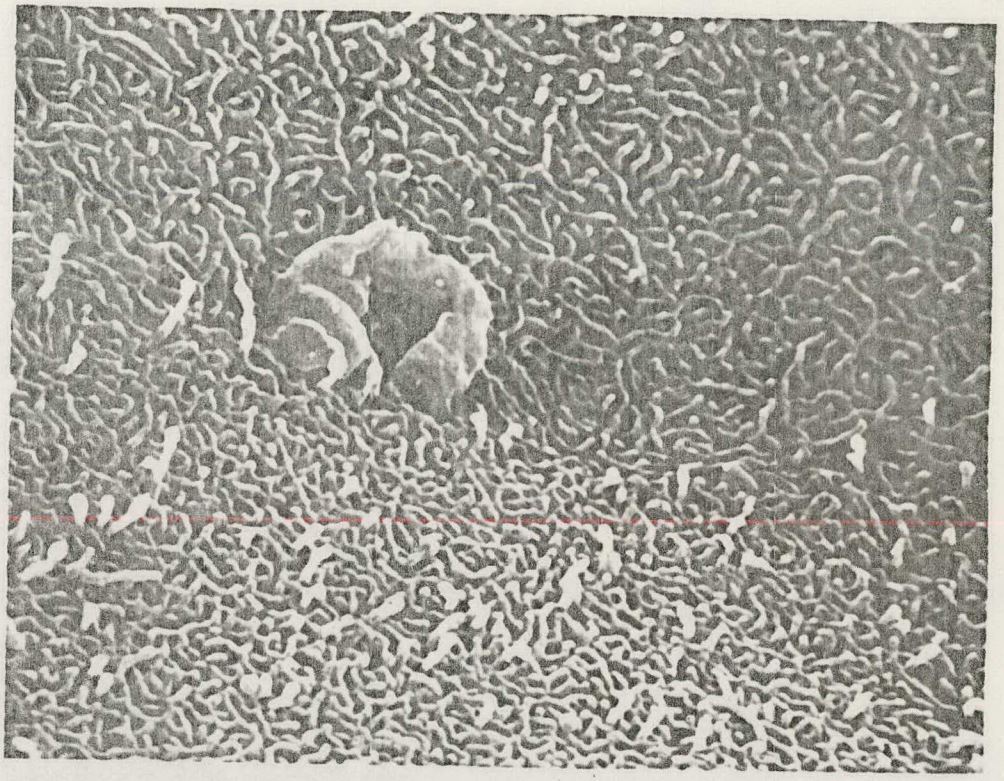
Other Studies on the Cornea

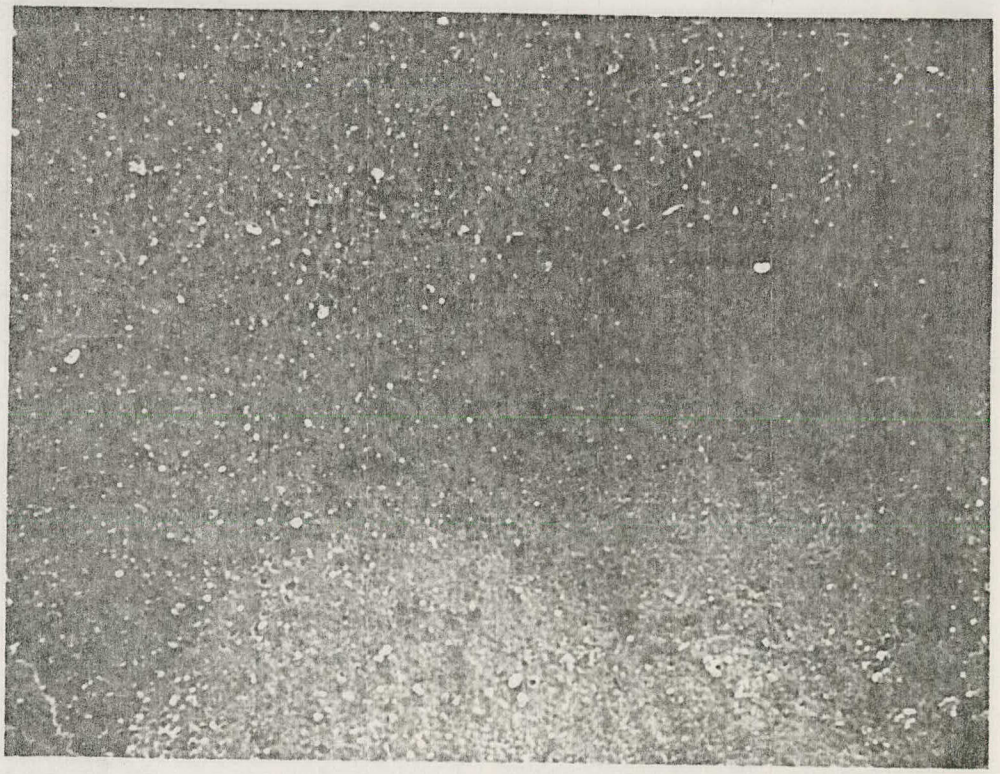
Since the outer surface of the cornea is the most exposed part of the eye, it is most susceptible to physical and chemical injury. S.E.M. experiments in our laboratory have shown a variety of micro-structures on the corneal surface. Comparative studies of these structures have been fruitful in furthering our understanding of the relationships of their role in the human. What the meaning of these microprojections is and what effect toxic agents and physical injuries might be on them is as yet unknown. It is known, however, that in the rabbit the application of a local anesthetic, proparacaine hydrochloride, causes these microprojections to be greatly altered (see next page). Also, there is a strong indication that the pattern of the epithelial microprojections may react sensitively to environmental factors.

Legend for photographs on following page:

The scanning electron micrograph at the top is of a normal rabbit cornea (epithelial surface). Portions of the two cells with their numerous microprojections can be seen. 10,000 X.

The second micrograph is of a rabbit cornea 10 mins. after dropping a solution of proparacaine hydrochloride on the corneal surface. The microprojections are greatly altered. 5,000 X.





Summary of Results Obtained During the Last Nine Year Period

The following is a summary of some of the results obtained during the past nine year period. The details of these results plus additional experimental data are given in the reprints which are included in this report. The number in parentheses refer to publications from this laboratory which have resulted from this project (see Ref. pgs V-1 - V-5).

1. Mechanical and chemical injury to the lens, in vivo initiates the cell cycle and is accompanied by propagated waves of DNA synthesis and mitosis that move out slowly from the site of injury. The injury response, and the effect of x-irradiation on the propagated injury response and wound healing has been determined (1,2,25,41,43,47).

2. A determination of the in vitro culture conditions which bring about a reproducible stimulation of DNA synthesis and mitosis in the cultured mammalian lens has been accomplished. It has been found that the lens in culture provides an excellent model system for studies on the response of tissue to injury under carefully controlled and defined conditions (3,6,9,10,12,26,32,34,36).

3. A system has been established for the procurement and processing of fresh rabbit serum in this laboratory (13,25,26).

4. A chronological pattern of RNA, DNA, protein synthesis and mitosis and the effect of selected antimetabolites on these processes has been characterized in lenses cultured in serum-containing media (4,6,7,8,17, 23,31).

5. The mitogenic serum factor(s) has been partially characterized and the effect of various treatments on the mitotic-stimulating activity of serum has been determined (11,12,13,14,26,27).

6. Rabbit serum was fractionated on DEAE-cellulose. Preliminary experiments indicate that fraction one, which is primarily γ - globulin, triggers a central mitotic stimulation. Moreover, fraction one brings about mitosis without the pronounced cellular migration that typifies the response realized in the presence of whole rabbit serum. Fraction one is more mitogenic on a mg protein/ml of culture medium basis than whole rabbit serum. The second fraction, i.e., beta globulin, gives a limited mitotic response. Cohn fraction IV, a mixture of alpha and beta globulin, also evoked limited mitotic response.

7. Modification of the time required for the serum-induced metabolic and ultrastructural changes in lenses which are preincubated in a non-stimulating medium has been determined (23). The results of these experiments have provided evidence that the pre-synthetic period can be divided into three sub-phases (22,23,25,40).

8. Cell division can be initiated in the ocular lens in a completely defined medium. Insulin added to a specific formulation of medium 199 or to medium KEI-4 brings about a central mitotic stimulation. Recent findings suggest that KEI-4 alone may stimulate cell division at a low pH (15,16,19,20,33,37,44,49).

9. Chemical injury induced by the injection of methylene blue into the anterior chamber of the rabbit eye causes extensive stimulation of DNA synthesis and mitosis in the lens epithelium. This and other effects of chemotoxic agents have been reported in a recent publication (47).

10. Wound healing in a teleost ocular lens has been described. Seasonal variations in mitotic activity have also been described in this species.

11. Changes in tissue organization and cell structure which precede and accompany DNA synthesis and mitosis in the cultured rabbit lens have been partially characterized at the ultrastructural level (18,21).

12. A number of fine structural changes which precede and accompany tissue repair in the rabbit lens <u>in vivo</u> have been characterized (27,28,29,30,38,48).

13. Techniques and procedures have been worked out to visualize the lens epithelium, capsule, and fibers using the scanning electron microscope (30,35).

14. Scanning electron microscope observations of the corneal surface, have revealed intricate patterns of microprojections on the epithelial cell surface. (39,42,45).

15. A technique has been worked out in which lenses which have been fixed, dehydrated, and dried by a critical-point drying method, can be fractured through the cytoplasm of the differentiating lens fibers,

exposing the cell nuclei. The fracture, under these conditions, causes a complete separation of the two membranes of the nuclear envelope from one another, thus exposing entire membrane surfaces (those which line the perinuclear space). These surfaces are not seen in their entirety in typical freeze-fracture or freeze-etch preparations, and consequently have not been described previously. The exposed membrane surfaces which line the perinuclear space have numerous convex structures of approximately 1,000 Å, and some larger more irregularly shaped structures. These appear to be fragments of the nuclear pore complexes. Differences in these structures between young fibers and those nearing completion of differentiation is suggested. The application of this technique to studies on changes in the nuclear membrane during wound healing, involving lens fiber cell regeneration is suggested. (52).

16. The scanning electron microscope has been applied to a study of a human disorder, which affects the lens capsule and other tissues as well (The Capsular [Iridocapsular] Exfoliation Syndrome: A Review) (51).

17. Spherical structures in the inner part of the lens have been discovered with scanning electron microscopy and described. This region is among the oldest parts of the lens, derived from early stages of development. Some of these structures, embedded and thin sectioned for transmission electron microscopy showed that they were

made up of varying numbers of multiple membranes. One hypothesis is that they are fiber cell fragments, derived from an injury sustained during early development, and, because of the nature of development in the lens, were preserved.

18. Some basic tissue preparative techniques for the ocular lens have been developed, which allow a study of the lens fiber cells at various locations in the lens, with a combination of scanning and transmission electron microscopy. The structure of the rabbit lens cells at different stages of maturation is presented as a basis for experimental studies on wound healing, cataract formation, development and aging. (53).

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WHICH ARE ASSOCIATED WITH THE CURRENT CONTRACT

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PROPOSED EXPERIMENTS

As indicated in the previous section, because of a combination of properties, the cultured ocular lens can be conveniently used as a model system for studies on certain aspects of wound healing: e.g., those factors responsible for initiation of the cell cycle and cell migration, and the optimal conditions for mitogenesis. It has been possible to characterize a sequence of events between explantation to a suitable medium and the initiation of DNA synthesis. Ultrastructural changes (changes in cell-to-cell relationship) occur within the first hour and increased ribosome production occur by 7 hrs. Macromolecular changes such as increased RNA and protein production (as determined by autoradiography and liquid scintillation counting) also occur by 7 hrs., and the activation of DNA synthesis follows at 25 hrs. and mitosis at 40 hrs. This period between explantation and DNA synthesis can be broken down into subphases, a, b, & c as defined by the requirement for serum or other mitogenic agents, and the effectiveness of certain metabolic inhibitors. The mitogenic agent of serum has been partially characterized, and it has also been found that insulin can replace this serum mitogenic agent. Thus, the cell cycle in the cultured whole lens can be initiated in a completely defined medium.

It is planned to continue the study of the $G_0 - G_1$ transition, attempting to more closely define the essential composition of the culture medium required for this transition, with a further characterization of the subphases.

As indicated above, the results thus far show that alterations in intercellular relationship are among the earliest observable changes. Since cell communication (cell coupling) may play a role in the regulation of tissue growth (Loewenstein[1], 1975), and since the gap junctions are presumably the means by which cell coupling (communication) takes place it might be projected that injurious agents (physical injury, or chemotoxic agents) which stimulate cell proliferation may have a primary action on the junctional complexes. Thus, alterations in the junctional complexes (notably, gap junctions) might well be observed after injury, as part of a primary mechanism in the stimulation of cellular proliferation.

The junctional complexes (particularly gap junctions and tight junctions) can be visualized very well in replicas of freeze-etched preparations. It is proposed, therefore, to analyze the junctional complexes of the lens epithelial cells at different distances from a small mechanical wound in the rabbit lens at different times after injury. This would be done, utilizing replicas of freeze-etched preparations obtained in the Balzers-301 unit. It is also proposed to make a similar analysis of the junctional complexes in the epithelium of rabbit lenses which had been isolated and exposed in culture to mitogenic agents, such as serum, insulin, and proteolytic enzymes. The analyses could be made on the cultured lens before the addition of a mitogenic agent, at different times after the addition of a mitogenic agent, as well as at different times after the removal of the mitogenic agent. Any correlations between changes

1 Cell Membranes, New York, H.P. Publishing Co., Inc., 1975.

in the junctional complexes and the effectiveness of the mitogenic agent will be sought. A freeze-etch unit has been donated to our laboratory by the Kresge Foundation and will be delivered shortly to aid in this phase of our experiments.

Wound Healing of Lens Fiber Cells

It is proposed to study the response of the lens fiber cells to physical and chemical injury, as seen with S.E.M. and T.E.M. The structural 'map' of the normal rabbit lens, as seen in S.E.M. has now been established (see plates and legends, starting on next page), and it is proposed that this serve as a basis for studies on wound healing of the lens. It would be of particular interest to note any differences in response to injury between the nucleated and non-nucleated lens fiber cells. The ability to visualize such differences might also be of importance in the study of cataracts and their development, which appears to involve a swelling and rupture of some of the fiber cells

Studies on the Cornea

We feel that information gained from the lens model system can be applied to other tissues. There has been much study over a period of many years on the growth of new vessels into the normally avascular cornea following various kinds of injury, particularly chemical injuries. Still, we are far from an understanding of what triggers this renewed growth (neo-vascularization). From a practical point of view, a better understanding of the mechanism(s) of new vessel growth and invasion into the cornea, and the means for pre-

Legends:

Fig. 1

a. Diagrammatic representations of the arrangement of the mature fibers relative to the sutures in the adult rabbit lens (the differentiating fibers are not shown). The endings of the mature fibers throughout the entire sutural arrangement define a roughly triangular single plane in the anterior part of the lens, and another such plane in the posterior part. These two suture planes are at right angles to one another.

b. Low magnification stereo S.E.M. of an adult rabbit lens, fractured through the anterior suture (toward left). The roughly triangular suture area consists of mature fiber endings (seen head on). The suture in the posterior part of the lens is seen in cross-section toward the right of the preparation. This appears at this magnification, as a horizontal split. A roughly spherical structure at the center of the lens may represent the embryonic nucleus. 16 X.

c. Diagram, summarizing appearance of rabbit lens, fractured through the suture in such a way that opposing fiber endings are separated, exposing an open 'face' of the anterior suture (left portion of diagram). The roughly triangular area consists of mature fiber endings. A 'cross-section' of the posterior suture is seen toward the right, as a line where mature fibers meet. Nuclei of the bow region are seen at the equator.

Fig. 2

a. 'Cross-section' of suture, showing fibers, from left and right, ending at the suture in the middle. 10 μm marker, 725 X.

b. Portion of an 'open-face' of a suture plane. Fibers can be seen coming in from the left to end at the suture plane, in the right half of the photograph. The rough appearance of the suture plane is due to the numerous, relatively large interdigitations which characterize the mature fiber endings. 50 μm marker, 360 X.

c. Fiber endings, showing large terminal interdigitations. Fibers at the right have been pulled away from the suture, exposing the numerous interdigitations. 10 μm marker, 1,450 X.

Fig. 3

The numbers in the diagram at lower left indicate the relative locations at which a series of S.E.M. photographs were taken. These numbers correspond to the numbers shown on the eight photographs in Figs. 3 and 4. In each case, the fibers are oriented so that the narrow sides of the hexagon are exposed. Cases in which the fibers fractured, revealing their cross-sectional dimensions, are summarized in drawings A-G.

1 μm marker. All micrographs are 7,500 X.

Fig. 4

S.E.M. photographs of lens fibers at different regions within the lens. See Fig. 3 for explanation. 1 μm marker, 7,500 X.

Fig. 5

a. Generalized diagram of hexagonal lens fibers. The width and thickness of the fibers, as seen in cross-section are defined. (Measurements of width and thickness of fibers at different locations within the lens are plotted in Fig. 6).

b. Superficial fibers. The narrow sides of the hexagons are exposed. There are no prominent interdigitations at points a and b of the hexagon (see a). 10 μm marker, 1,600 X.

c. Superficial fibers. The wide sides of the hexagon are exposed. Ball and socket interdigitations are seen along wide sides. No prominent interdigitations are seen at other points. 10 μm marker, 1,600 X.

d and e. Cortical fibers (deeper than the superficial fibers seen in b and c do not have interdigitations along wide sides, but do have prominent interdigitations at points a and b of the hexagon (see a). These interdigitations create a 'zipper-like' appearance between fibers. 5 μm marker, 3,000 X.

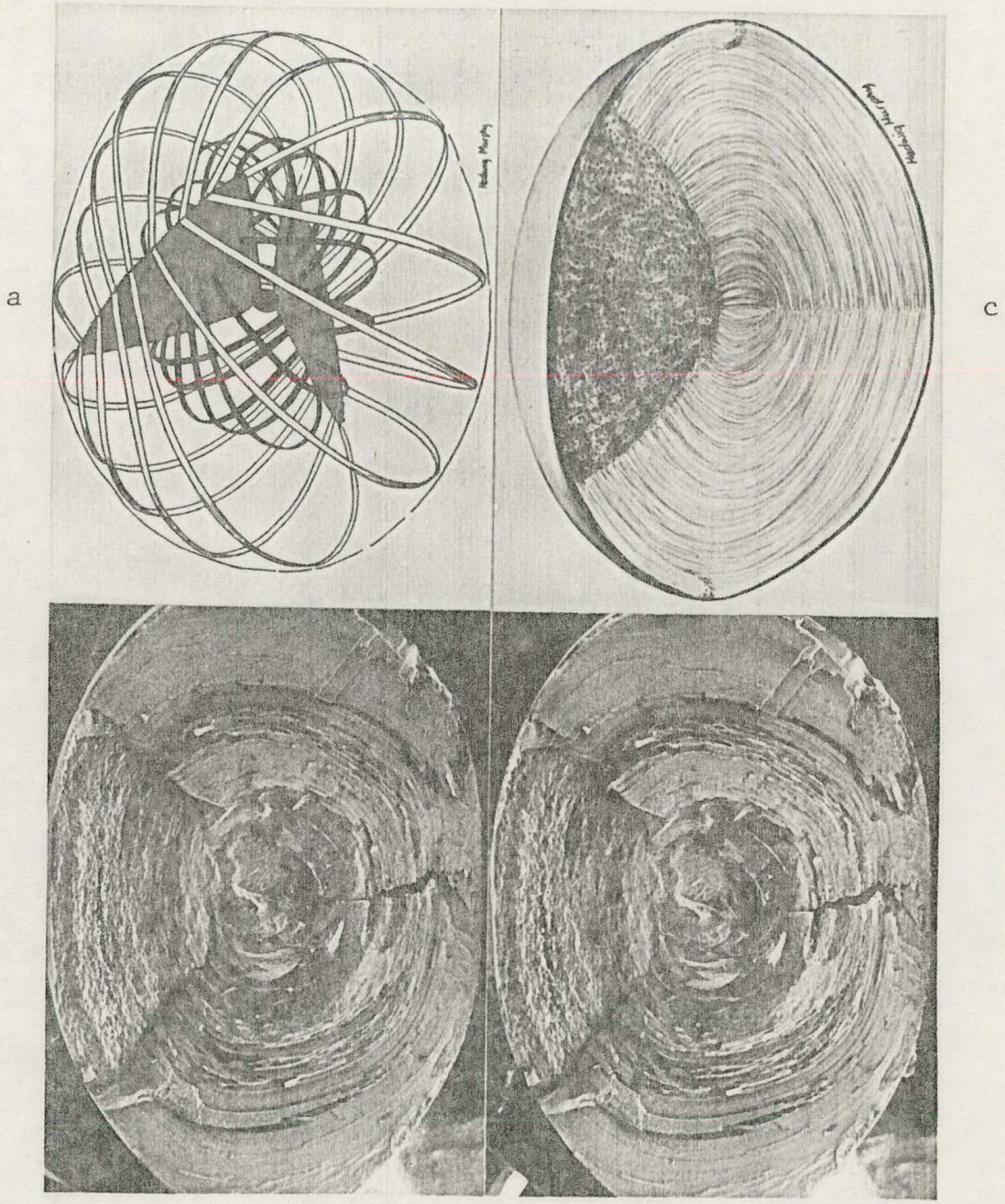
Fig. 6

Thickness and width of lens fibers as a function of distance from center to equator. See Fig. 5 for a definition of thickness and width of the lens fibers.

Fig. 7

a. Bow region under low magnification. 100 μm marker, 180 X.

b. Higher magnification of lower left portion of 7a showing exposed nuclei. 10 μm marker, 1,400 X.

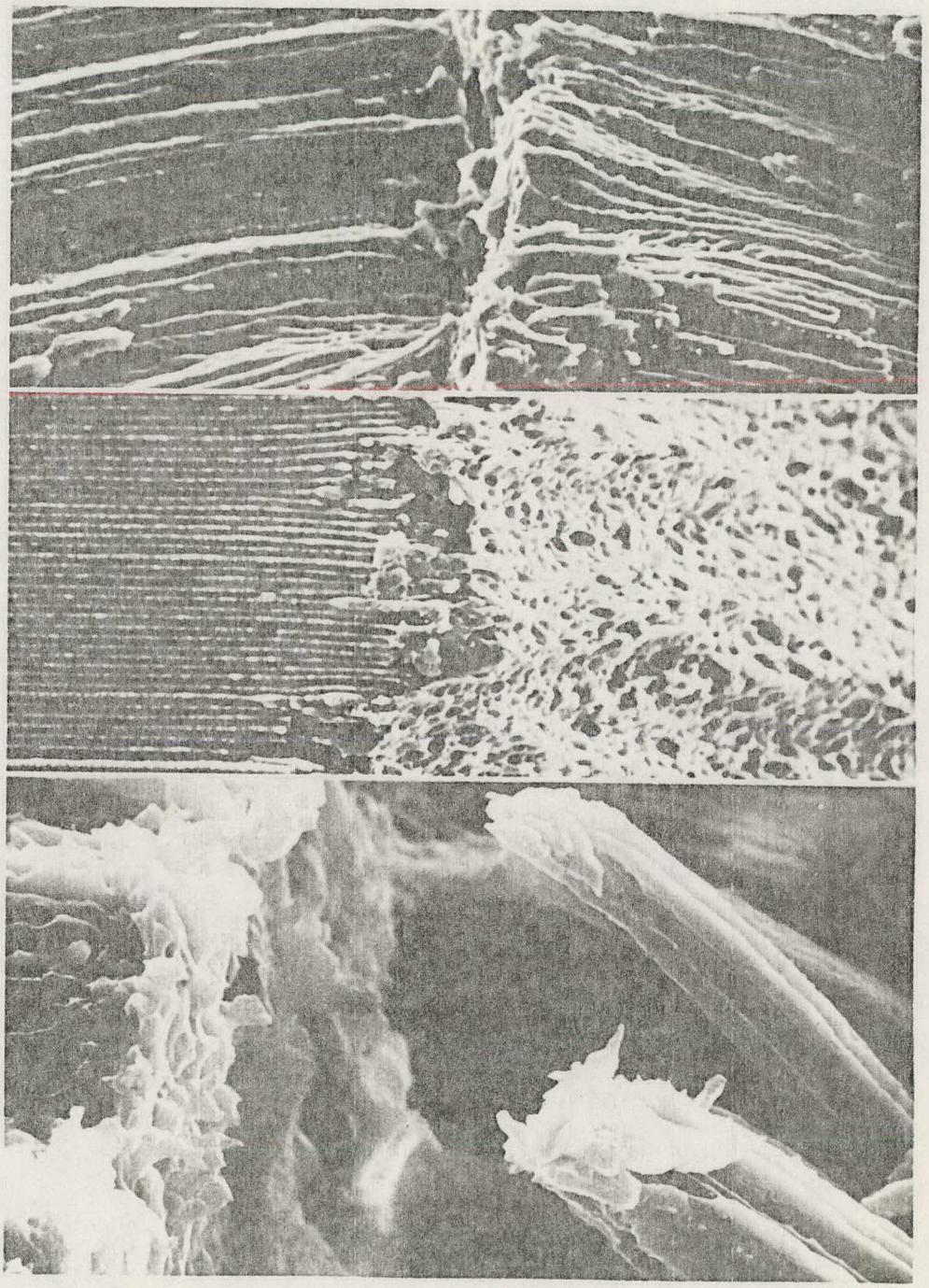


a

c

b

Fig. 1



a

b

c

Fig. 2

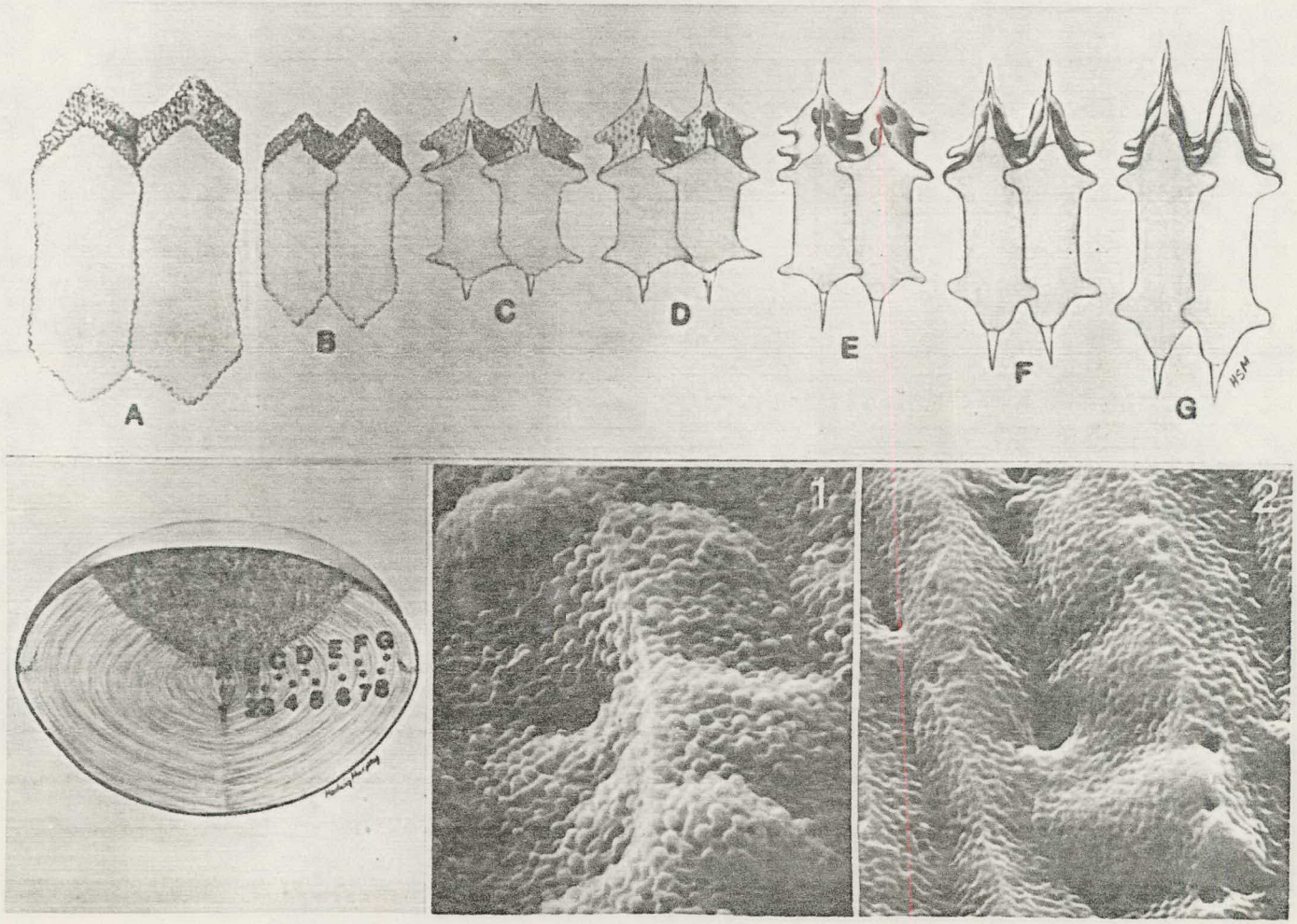


Fig.3

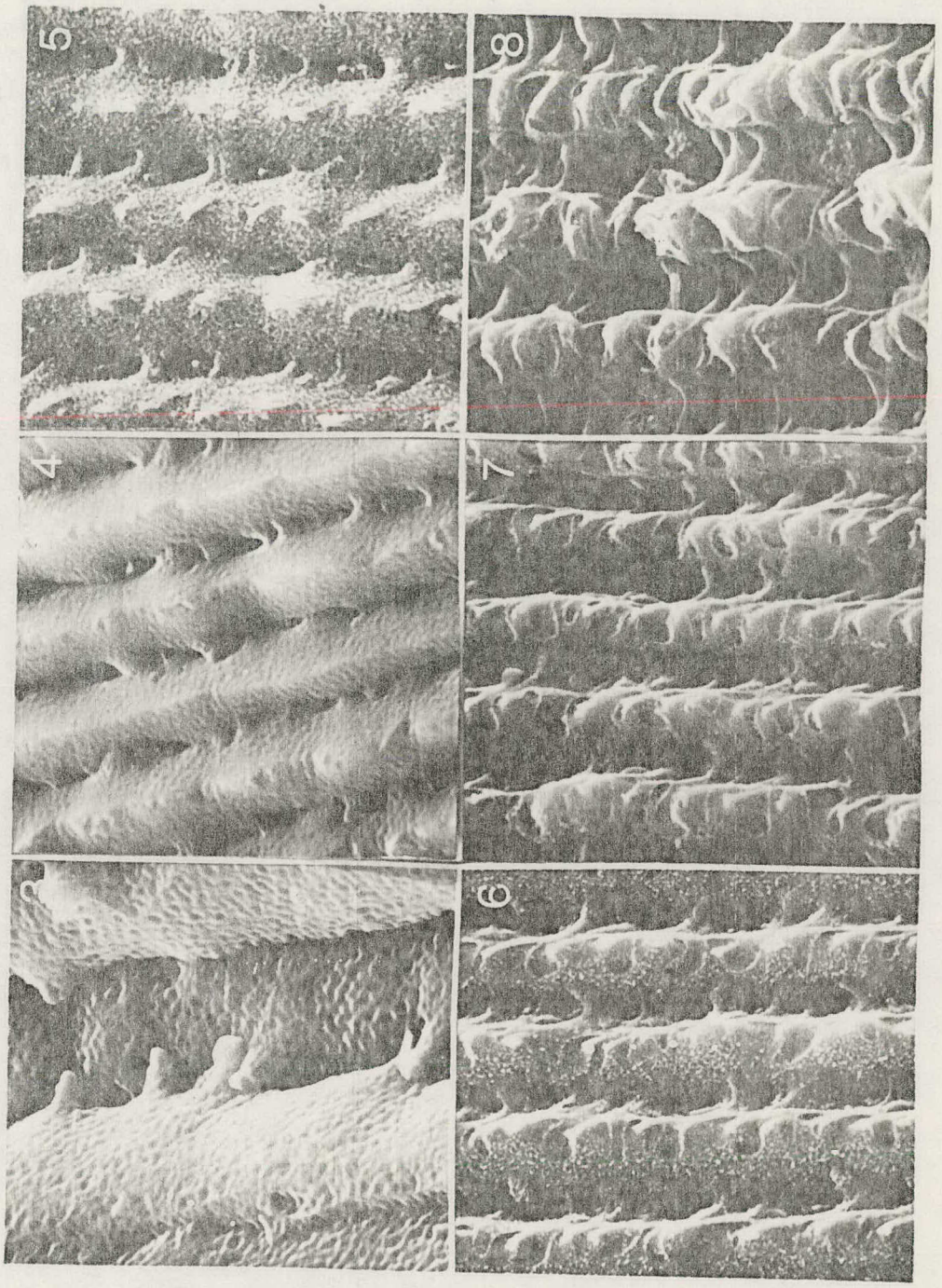
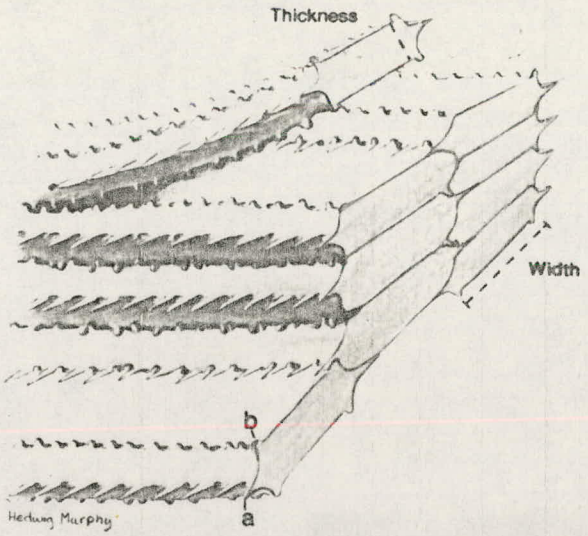
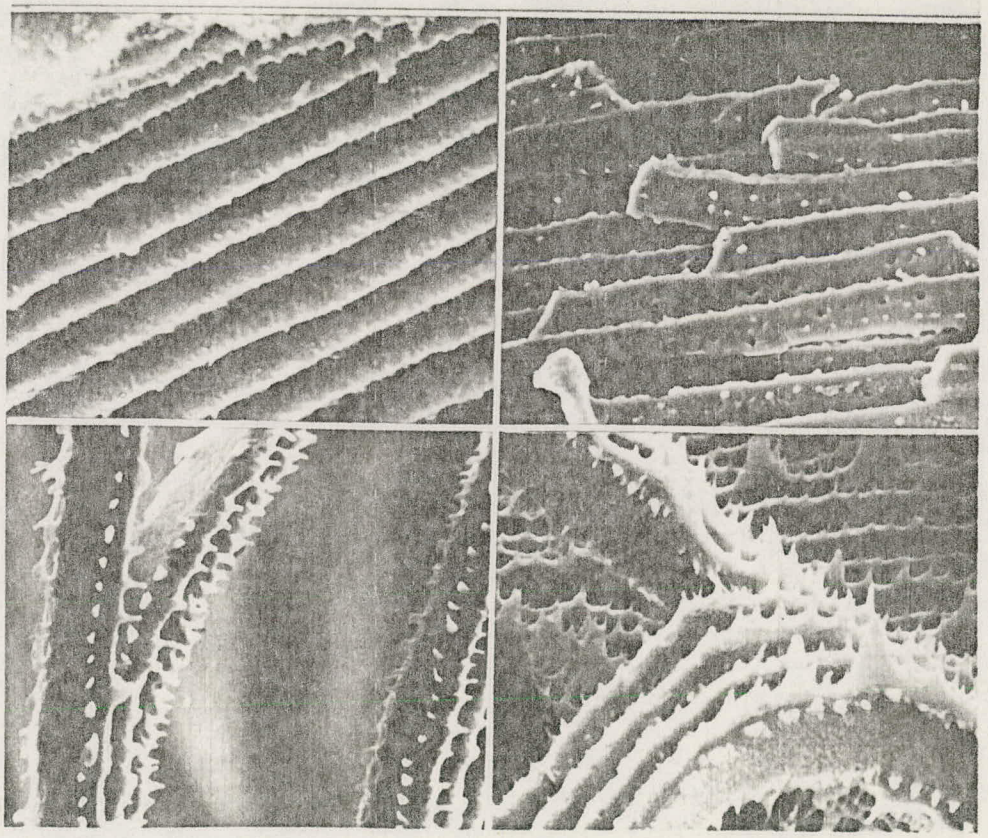


Fig.4



a



b

c

d

e

Fig.5

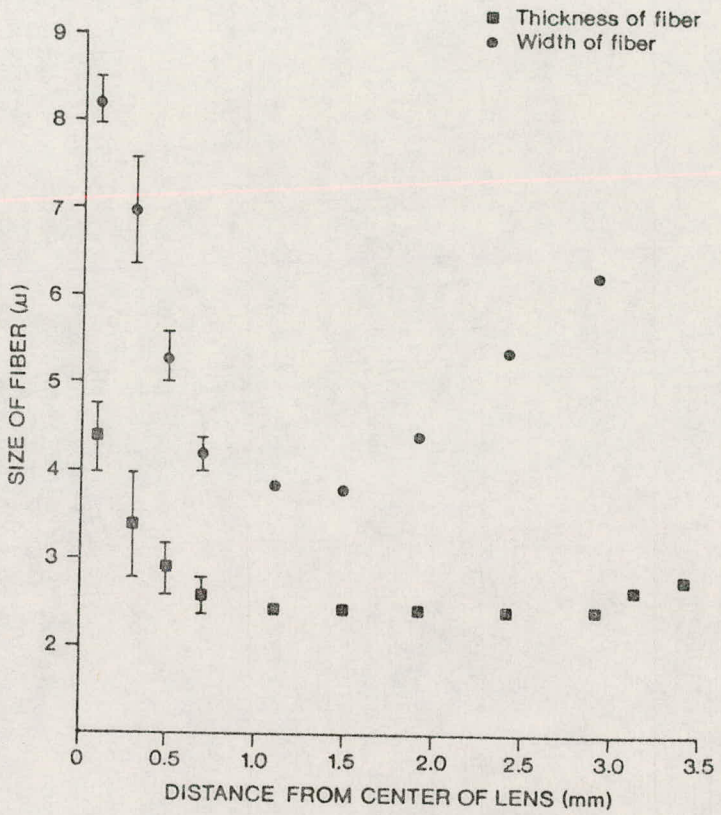
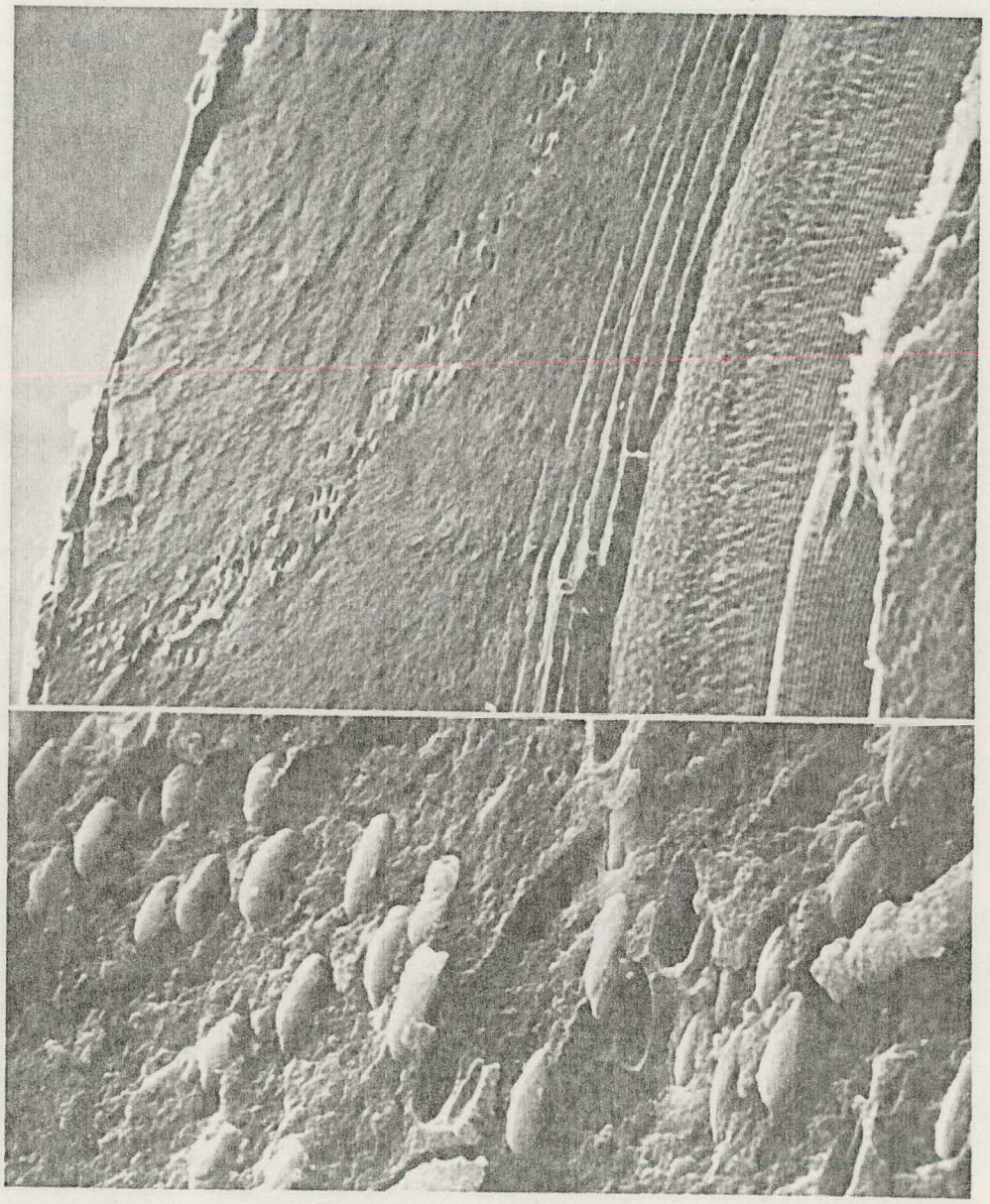

Thickness of fiber
Width of fiber
SIZE OF FIBER (μ)
9
8
7
6
5
4
3
2
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
DISTANCE FROM CENTER OF LENS (mm)

Fig.6



a

b

Fig. 7

venting it, are important, since the eye with a neovascularized cornea, has a greatly reduced chance of successful corneal transplantation. More knowledge of the basic mechanism of corneal neovascularization would appear to be an important base from which to better understand the way in which injurious chemicals cause neovascularization, and how to prevent it. Langham[1] (1953). has devised an experimental technique for the induction of corneal neovascularization in the rabbit cornea. This involves the injection of a solution containing alloxan into the anterior chamber. A corneal edema is induced, and this is followed by the growth of new vessels into the cornea. We plan to use this technique to stimulate the growth of new vessels. The stimulation of vascular endothelial proliferation is a prerequisite for neovascularization.

On the assumption that changes in junctional complexes may be part of a primary control mechanism for cellular proliferation, we propose to study the junctional complexes of the vascular endothelium at different times after the injection of alloxan. Replicas of freeze-etched preparations of vascular endothelium, as seen in T.E.M., and thin-sections for T.E.M. will be used. Any changes in cell membranes or cell-to-cell relationships (for example, intercellular spaces or junctional complexes) will be recorded. Changes in permeability of the vessels to protein, as indicated by the movement of enzyme markers such as horseradish peroxidase and myoglobin through the endothelial lining, will be studied. The rationale for using these enzymes as tracers was discussed above.

1 Brit. J. Ophthal. 37:210-222, 1953.

An activation of ribosome production (which characterizes the G-0 → G-1 transition in other cells) and the timing of such an activation relative to the initiation of DNA synthesis in the outgrowing vessels will be sought.

Related changes in macromolecular synthesis, as indicated by altered rates of incorporation of labeled precursors (e.g., uridine and amino acids) in RNA and protein will be determined by means of autoradiography.

In addition, preliminary experiments have shown that plastic corrosion casts can duplicate the vasculature of experimental animal eyes (e.g. rat, rabbit, dog). The procedures required for this study have been used extensively here at Wayne for studies on splenic vasculature. (Barnhart and Baechler,[1] 1974). We feel that these corrosion casts, as observed with scanning electron microscopy, can be used for studies of neovascularization. We therefore plan to study replicas of the vasculature at various times after the induction of neovascularization. These will then be examined with the scanning electron microscope, in order to study changes in the three-dimensional pattern of the growing, invading vessels.

It is also planned to expand other aspects of the work on the cornea. Scanning electron microscopy of the corneal surface has revealed the presence of numerous microprojections. (See figure on p. III-18 and also figures in appended publication COO-2401-61). The patterns formed by these microprojections varies from cell to cell in the same cornea, and it has been suggested that the various

1 Scanning Electron Microscopy, Part III, IITRI, 705-712, 1974.

patterns reflect stages in the maturation and senescence of the surface epithelial cells. Also, these microprojections appear to be sensitively reactive to chemical agents (e.g. proparacaine hydrochloride) (See figure on page III-18) and it is planned to investigate the effects of potentially toxic chemical agents on the surface fine structure of the cornea. This would involve the use of procedures already established in our laboratory (Investigative Ophthalmology, Vol. 13, 1974, COO-2401-61).

Studies on human corneal endothelium in rejected corneal transplants have already been initiated. The corneal endothelium plays an important role in maintaining the normal state of hydration of the cornea, and hence its transparency. In two preliminary studies, human corneas which had been transplanted and subsequently rejected by the human recipients were examined under the scanning electron microscope. The inner (endothelial)surface of the cornea was found to have uniformly distributed, but completely separated cells (page VI-18, top micrograph). Each cell appeared to be active, with many processes (page VI-18, bottom micrograph). The number of endothelial cells in the human cornea appears to play a crucial role in the maintenance of a normal transparent human cornea and some problems with the use of intraocular lens prostheses following cataract surgery may be due to their injurious effects on the corneal endothelium (Kaufman, personal communication).

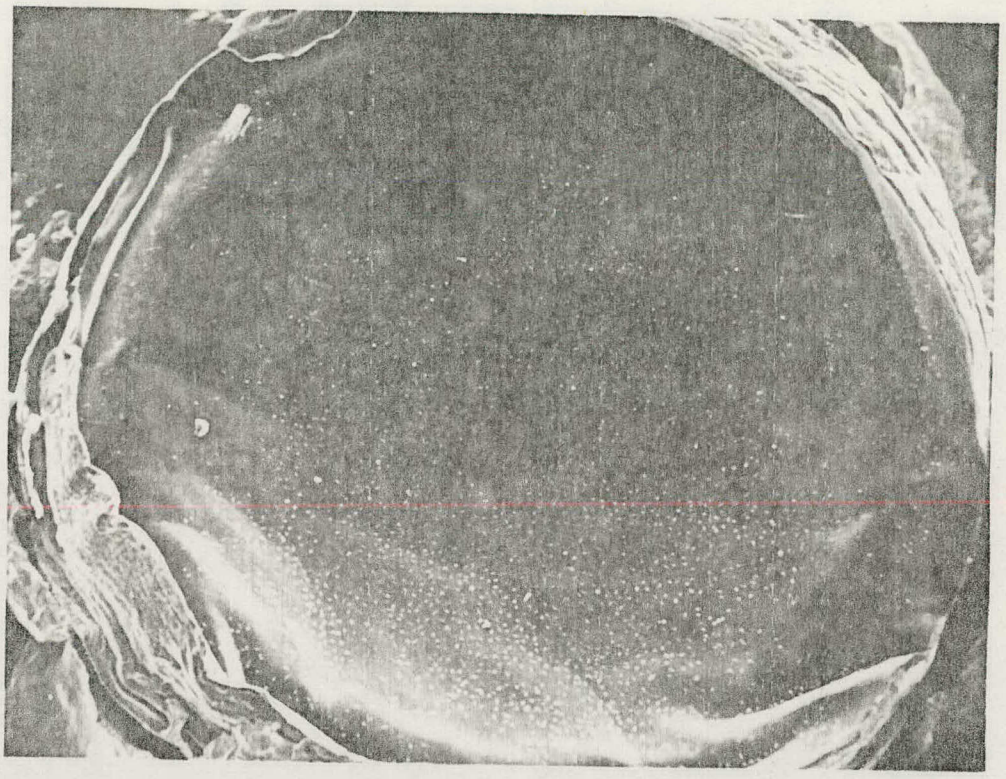
The Vascular Endothelium <u>In Vitro</u>: Much could be learned about the factors which control the initiation of the cell cycle in vascular endothelium if the process could be studied in cultured vessels. The living "retinal" vasculature of the young rabbit (up to about 3 weeks

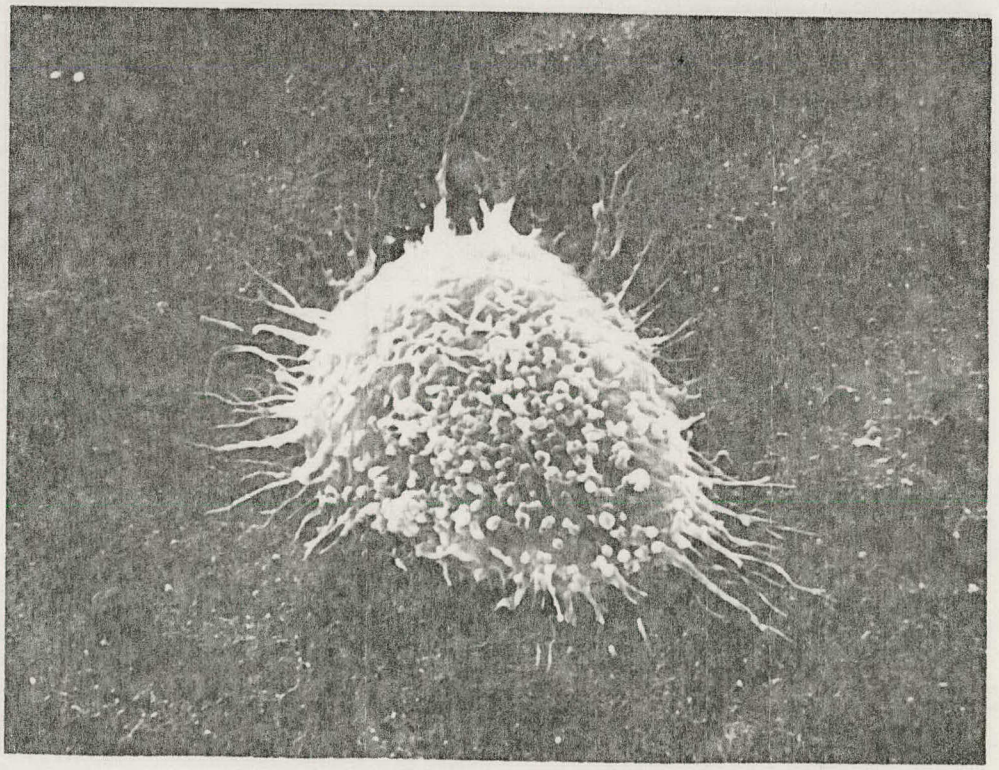
Legends (for figures on page VI-18)

Human cornea (endothelial surface) which had been transplanted and subsequently rejected.

The scanning electron micrograph at top shows the cornea under very low magnification. The white dots are cells, which are relatively uniformly distributed. 25 X.

The micrograph at bottom shows an individual cell at higher magnification. All of the cells appear to be very active, with many processes, but do not appear to come in contact. 3,000 X.





of age) can be isolated and established in culture. Ashton[1], 1966; Tripathi, Knight, and Ashton[2], 1974 have used this preparation to study the effects of oxygen on the vascular endothelium. We plan to use the young rabbit retinal vasculature in culture for short term studies on the cell cycle and the factors which initiate it.

Within the last several months we have developed a method of isolating and culturing rabbit retinal vessels and have been able to monitor DNA synthesis in these cultures. It would seem then that this procedure could serve as a model of the study of what causes vessels to proliferate and therefore what factors might be involved in neovascularization, in much the same way that the cultured lens has been used to study those factors that regulate cellular proliferation in the lens epithelium.

Since little is known about the characteristics of the cell cycle in the endothelium of cultured retinal vessels, it is proposed to study the distribution of H^3- thymidine-incorporating cells in young rabbit retinal vasculature which have been freshly explanted, or maintained in culture from 1 to several days. In the initial experiments, three culture media will be compared: Ashton's medium, unsupplemented M-199, and M-199 supplemented with fresh whole rabbit serum (approximately 23% by volume). The results will be compared with what we already know about the effects of two of these media on the cultured rabbit lens.

1 Am. J. Ophthal. 62:412-435, 1966.
2 Exptl. Eye Res. 19:449-475, 1974.

A serum factor(s) can turn on the cell cycle in the central lens epithelium. If serum does not have a similar effect on the cultured vascular endothelium, it may indicate that some extra-vascular factor is required to initiate the cell cycle in these cells, which would be of considerable interest (these possibilities have been considered under background). The results of Buzney, Frank and Robison[1](1975) showed in cultured retinal microvessels that the pericytes grew profusely, for long periods of time. The lack of growth of the endothelium here might indicate the absence of endothelial cell mitogenic activity in serum. It is possible however that the endothelial cells require high concentrations of serum, which could account for the lack of proliferation of these cells.

Studies on newly-stimulated vessel growth in tissue culture will also be carried out on excised "buttons" of cornea which have been induced to initiate neovascularization.

1 Science 190:985-986, 1975.

Additional experiments: It is also proposed to follow up on some of the observations made in the course of this study. For example, it is proposed to study further the 'spherical' structures observed in the nuclear (central, oldest) region of some of the rabbit lenses studied. Our observations suggest that these may be fragments of fiber cells that may have originated as an early developmental abnormality or injury. Thin sections of these 'spheres' have shown that they consist of multiple unit membranes which could have arisen by the folding of fiber cell membranes following injury, and which were then 'preserved' within the developing lens. If this is true, the multiple membranes would have to form a spiral (jelly-roll) structure. In our thin sections, we find it difficult to prove the spiral structure without the use of a goniometer stage in the T.E.M. A recent gift from the Kresge Foundation which provides funds for the purchase of a goniometer stage for the Philips - 301 - T.E.M. should enable us to answer this.

Procedures: Most of the procedures referred to in this proposal are already in routine use. The details are included in the appended manuscripts. Because of the new direction of the program several new procedures are being added.

Tissue that is to be freeze-etched will include both fixed and unfixed material. 2.5% glutaraldehyde buffered with phosphate or cacodylate buffer (pH 7.4) will be used. The preserved or the fresh specimen will be permeated with a cryoprotective agent, [glycerol (20-30%) or DMSO (30-40%)] in a buffer or culture medium at lowered temperatures. When the cryoprotectant has completely infiltrated the tissue it will be frozen rapidly by plunging it into liquid propane or isopentane, cooled by liquid nitrogen. At this point the specimen will be transferred to the cooled stage of the vacuum evaporator, the chamber evacuated and the replica made. The replica will then be viewed in the transmission electron microscope after the tissue has been dissolved.

Corrosion casts of eyes are prepared by injecting a modified formulation of Batson's #17 plastic into the carotid arteries of the anesthetized experimental animal. Both jugular veins and carotid arteries are ligated after the plastic injection and subsequently the eyes are excised after the polymerization process is complete. The monomer cross-linker, and catalyst are formulated in a manner which not only allows a very low viscosity of the injection material but also provides for a relatively short period of polymerixation, i.e. 1-2 hours. The tissue is removed from the cast by alkaline digestion in 10% KOH (potassium hydroxide) at 60° C. for 12 hours.

"Leakiness" to fluorescein is characteristic of neovascular vessels. Changes in permeability of the vessel walls to protein will be studied during the induction of neovascularization of the cornea. Enzyme markers, such as horseradish peroxidase (Sigma, Type II)(Anderson,[1] 1972) mol. wt. of 40,000 and myoglobin (Miles Lab., Kankakee, Ill.)(Graham,[2] 1966) mol. wt. of 17,500 etc. will be injected intravenously at different times after the injection of alloxan into the anterior chamber (Langham,[3] 1953). Thirty seconds to 45 minutes later the tissues will be fixed in 3% glutaraldehyde. For light microscopic cytochemistry, 10 μ frozen sections, prepared on a Leitz freezing microtome, will be incubated in diaminobenzidine containing-media (Anderson,[1] 1972, Graham,[2] 1966) for the demonstration of peroxidase activity, then rinsed and mounted in glycerin jelly on glass slides. For electron microscope cytochemistry, 20 - 40 μ, non-frozen sections, prepared on a Smith-Farquhar tissue sectioner, will be incubated in the same media, rinsed, fixed in osmium tetroxide, and embedded for electron microscopy.

1 J. Histochem. Cytochem. 20:672-684, 1972.
2 J. Histochem. Cytochem. 14:291-302, 1966.
3 Brit. J. Ophthal. 37:210-222, 1953.

Premises, Facilities, Equipment and Materials to be Furnished by the Contractor: The new facilities of the Kresge Eye Institute were opened in the spring of 1974. The Institute contains modern research laboratories. Appropriate facilities are available for the proposed project. The electron microscope facilities are now completed, with a new Philips 301 transmission electron microscope, and a new Philips 500 scanning electron microscope.

In addition to the T.E.M. and S.E.M. facilities mentioned above, the Kresge Eye Institute will soon receive several pieces of equipment as a result of a grant from the Kresge Foundation.

These include a Balzers freeze-etch installation with ancillary equipment, a goniometer stage for the existing Philips 301 transmission electron microscope, and equipment for time-lapse photomicrography of tissue cultures.

The Relationship of the Proposed Research to Plans for the Development of the Institution: This is a very important time in the development of the Kresge Eye Institute, with its new facilities and expanding faculty and staff. This contract is playing a crucial role in the development of new research programs at the Kresge Eye Institute.

Also, it is expected that there will be a continuing collaboration with the people at Oakland University, where a very strong program in ocular research has developed. The principal investigator (C.H.) retains an appointment of Adjunct Professor at Oakland.

Also, the present program is playing an important role in the training of graduate students and post-doctoral fellows. With the

fine cooperation of the Departments of Basic Medical Sciences the faculty of the Kresge Eye Institute can have graduate students, doing thesis research in the Kresge Eye Institute. It is planned to increase the emphasis on graduate training, as well as post-doctoral training in the Kresge Eye Institute. We believe that the central location of the Kresge Eye Institute within the new Detroit Medical Center should facilitate the interchange of ideas among students and faculty of the basic and clinical departments. The new Kresge Eye Institute is adjacent to the newly-constructed section of Harper Hospital; Grace Hospital (under new construction); Detroit General Hospital (under new construction); Children's Hospital (recently re-constructed), and Hutzel Hospital. At the current time, one student, Mrs. Hedwig Murphy, working for her Ph.D., is doing research related to the E.R.D.A. contract. During the tenure of the current contract and the two which preceded it, a number of pre- and post-doctoral trainees have worked in our laboratory on research related to the E.R.D.A. contract. The following is a list of some of these people and their current positions:

FORMER TRAINEES	CURRENT POSITIONS
Dr. Carl Feldherr	Prof. of Anat. and Path., Univ. of Florida, Gainesville
Dr. Howard Rothstein	Prof. of Zoology, Univ. of Vern
Dr. Dobli Srinivasan	Faculty Member, Ophthalmology Columbia University

Dr. Laslo Bito	Research Prof., Ophthalmology Columbia University
Dr. Maynard Wheeler	Faculty Member, Ophthalmology Univ. of Connecticut
Dr. Jane Kaltenback Townsend (when on sabattical)	Prof. of Biological Sciences Mount Holyoke College
Dr. John Reddan	Prof. of Biological Sciences Oakland University
Dr. Allan Weinsieder	Assist. Prof. Anatomy Wayne State Univ.
Dr. Mihir Bagchi	Assist. Prof. Anatomy Wayne State Univ.

CURRICULUM VITAE

NAME: Clifford V. Harding [redacted]

EDUCATION:

A.B.	Brown University, 1946
M.S.	Yale University, 1948
Ph.D.	University of Pennsylvania, 1950

EXPERIENCE:

1950-52 Research fellow, Wennergrens Institute (University of Stockholm) and Zoological Station, Naples, Italy.

1952-54 Assistant Professor of Zoology, University of Southern California.

1954-56 Assistant Professor of General Physiology, University of Pennsylvania.

1956-57 Physiologist, Division of Biology and Medicine, U.S. Atomic Energy Commission, Washington.

1958-61 Assistant Professor of Physiology, Columbia University, College of Physicians and Surgeons.

1961-64 Associate Professor of Physiology, Columbia University, College of Physicians and Surgeons.

1964-73 Professor of Biology and Chairman of Biological Sciences, Oakland University.

1973- Professor and Director of Research, Kresge Eye Institute, Wayne State University, School of Medicine.

PUBLICATIONS:

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Effect of ultraviolet light (2357 A) on cleavage time in centrifuged Arbacia eggs. (with L. Thomas) Biol. Bull., 97:241, 1949.

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The nuclear envelope in the crystalline lens fiber cell. (with S. Susan). Invest. Ophthal. 15, no. 5:433-437, 1976.

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Scanning electron microscopy of the adult rabbit lens. (with S. Susan and H. Murphy), Submitted for publication, 1976.

CURRICULUM VITAE

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Education:

B.A. Asbury College, Wilmore, Kentucky, 1966.
M.S. Wayne State University, Detroit, Michigan, 1972.

Experience:

1969-1974: Research Technician.
Department of Anatomy, Wayne State University, Detroit, Mich.

1974: Research Technician.
Kresge Eye Institute, Wayne State University, Detroit, Mich.

Publication:

(with D. B. Meyer and Linda Hazlett). The fine structure of the retina in the Japanese quail (coturnix coturnix Japonica). I. Pigment epithelium and its vascular barrier. Tissue and Cell, 5:489-500, 1973.

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