THE ORIGIN OF NEW CONNECTIVE TISSUE CELLS IN THE CORNEAL STROMA DURING WOUND REPAIR

by

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PMN	Polymorphonuclear
Tdr	Thymidine
^{14}C-Tdr	Carbon-14 Thymidine
^{3}H-Tdr	Tritiated Thymidine
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
E_{mean}	Mean Energy
E_{max}	Maximum Energy
VSF	Vessel Stimulating Factor

INTRODUCTION

Wound repair is the process whereby dead or damaged cells are replaced by healthy cells. This ability to renew injured tissue is essential for the preservation and evolvement of multicellular organisms and represents a biological adaption of paramount value (1). Wound repair is not a process purely local in character, but involves the entire physiological armamentarium of the organism (2).

Although the general features of the wound healing process have been known and recognized for centuries, it was not until the observations of Hunter, published in 1794, that the cellular participation in the repair process was established (3, 4). During the middle of the past century the noted pathologist Virchow formulated the concept that all cells arise from previously existing cells (5). This important doctrine introduced new possibilities in the study of the inflammatory and wound healing processes (6). In 1867, Cohnheim, a former pupil of Virchow, demonstrated the migration of blood leucocytes by diapedesis from blood vessels into inflammatory exudates (7). He suggested that these leucocytes might become transformed into proliferating connective tissue cells and aid in tissue repair (8). Since the original work of Cohnheim, thousands of observations that relate to the specific aspects of wound healing have been published (9). Despite this vast accumulation of data many basic problems in wound

healing remain to be solved (10). Dunphy (11) stated as recently as 1960:

> Even though there has been remarkable progress in surgical techniques we remain painfully ignorant of the nature of the healing process itself.

Russell and Billingham (12) commented in a review of the repair process in mammals,

> ...probably no aspect of medicine has a longer history or more copius literature than wound healing, but despite all the work carried out on wound healing to date, it is probably fair to say that the total progress made amounts to little more than the provisions of a rational basis for current surgical practices which have evolved in a more or less empirical manner.

Schilling (9) noted that one of the goals of past research on wound repair has been to

> ... discover information that when applied will accelerate wound healing, but today this may be an unrealistic and unattainable objective.

However, the author stated that investigation of the basic mechanisms of wound healing may eventually provide the necessary information to allow the clinician to control the variables that retard or complicate wound healing:

> Any new knowledge about any of the several closely integrated processes of repair is adequate justification for continued research

according to Schilling (9).

Cutaneous Wound Healing

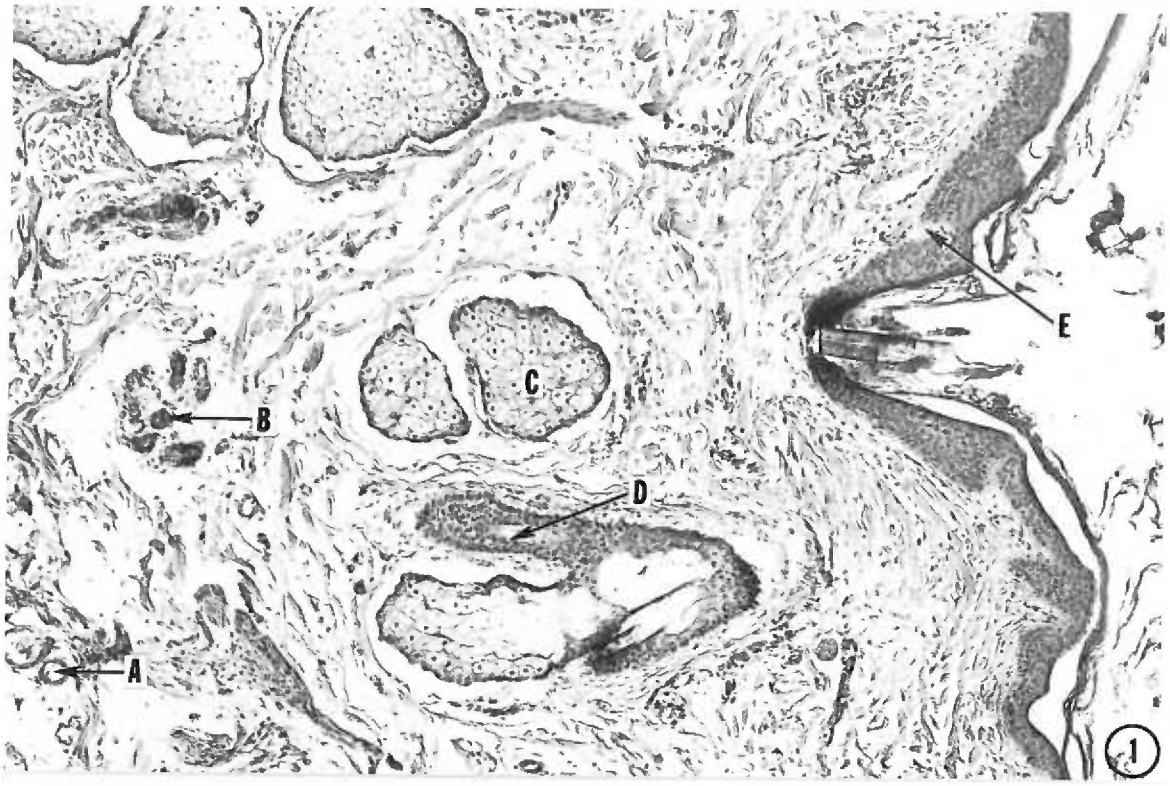
To most people wound healing is synomymous with the repair of cutaneous lesions, because they have personally experienced cuts and deep abrasions and subsequently have observed their repair. The skin has been used in the majority of studies in wound healing owing to its convenience for production and observations of experimental wounds. It is a complex organ comprised of multiple specialized cellular layers, and within these layers are a variety of skin appendages including: capillary beds, nerves, hair follicles, muscle fibers, adipose cells, lymphatic vessels, sebaceous, and sweat glands (figure 1).

The biological processes set in motion in response to a cutaneous wound, which will ultimately result in restoration of the tissue, are with certain qualifications common to injuries involving other tissues and organs in the body. Therefore, the following review of the general features of inflammation and wound repair in the cutaneous lesion is presented as a basic orientation to the healing process. This information will allow for a later discussion of the similarities and differences in the healing processes of the skin and cornea.

Hemostatic response: Following an accidental cut which transects the full thickness of the skin, an inflamatory response is produced which is accompanied by bacterial contamination and

Figure 1. A section of human skin (scalp) is shown. This section illustrates the complexity of this organ which is comprised of multiple specialized cellular layers and a variety of skin appendages including: A. arteriole, B. secretory section of a sweat gland, C. sebaceous glands, D. hair follicle, E. epidermis.

Figure 2. Meridional paraffin section through the rat cornea. This tissue is avascular and relatively acellular and lacks the histological complexity of the skin. In the transparent rat cornea five structual zones can be distinguished in cross section when viewed with the light microscope: A. epithelium, B. area of basement membrane, C. stroma including the fibrocytes, D. Descemet's membrane, E. endothelium.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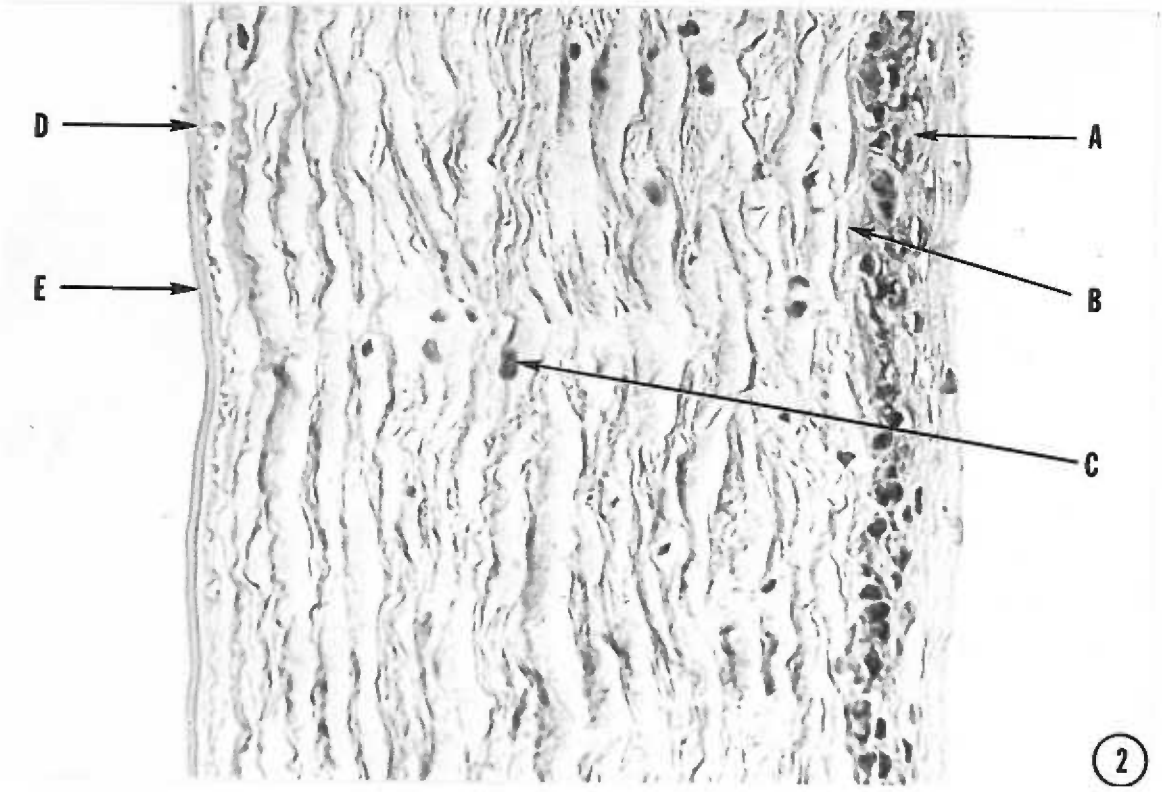
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devitalized or necrotic tissue. Inflammation is a tissue response to injury in which cellular and humoral elements act to destroy, neutralize or otherwise restrict the action of injurious agents, subsequent to wound repair (13). Inflammation and repair are concurrent processes, but repair is not prominent until most of the inflammatory reaction has been completed (13). In the immediate reaction to injury there is a sequence of vascular changes at the wound site. The first phase is vasoconstriction, beginning within a few seconds and lasting a few minutes. The second phase is vasodilation accompanied by increased permeability to plasma proteins and attains a maximum response in about 10 minutes. Following this stage of acute hyperemia, a third change appears after approximately 30 minutes. The rate of blood flow begins to decrease and resulting several hours later in vascular stasis, infiltration of the tissue with leucocytes and local hemorrhage. This influx of blood leucocytes and vascular fluid is accompanied by edema and the formation of an inflammatory exudate.

In the cutaneous lesion, the early inflammatory response to injury is dominated by the presence of polymorphonuclear (PMN) leucocytes which appear to migrate more quickly and in greater numbers than mononuclear leucocytes (13). The PMN leucocytes comprise a family of three cell types. The most numerous is the neutrophilic granulocyte. These cells are highly susceptible to chemotactic influence and are produced in large numbers by the bone

marrow during the inflammatory response. The neutrophils have a half-life of only a few days and are incapable of division. During these few days they actively digest or ingest foreign material, phagocytize bacteria, and degranulate releasing proteolytic and other enzymes. The eosinophilic granulocyte resembles the neutrophil except that its cytoplasmic granules are considerably larger and stain brilliant red with Wright's stain. These granules are lysosomes which contain a variety of enzymes. The eosinophils comprise 2-4 percent of the circulating leucocytes, but are increased with hypersensitivity and parasitic infections. The basophilic granulocyte or mast cell accounts for only 0.3 - 1 percent of the circulating leucocytes, and does not appear to be an important participant in most inflammatory reactions. The cytoplasmic granules have been shown to contain heparin and histamine, but the precise function of these cells remains obscure.

The lymphocyte seldom accumulates in large numbers in the inflammatory zone, although in chronic inflammation these cells become more numerous. There is general agreement that the lymphocyte is mildly phagocytic and that its most important function is the release of antibodies in the wound. There is evidence that the lymphocyte does lyse and release DNA and RNA as intact fragments which may be incorporated into the nucleic acids of proliferating cells at the wound site (14).

The mononuclear phagocytes consist of two cell types. The monocyte which is produced in the bone marrow and comprises

4 - 6 percent of the circulating white blood cells is infrequently present in normal tissue. The macrophage, on the other hand, is normally found only in tissue. This cell has a much longer life span than the PMN leucocyte and may survive for a period of months. The morphology of the macrophage and monocyte are similar. As the pH decreases below 6.8 in later stages of acute inflammation, the mononuclear phagocytes remain active and persist after the PMN leucocytes have been destroyed by the progressively increasing acidosis. One of the principle functions of the monocyte and macrophage is to phagocytize bacteria, necrotic debris, and dying cells after the PMN leucocytes are lysed. An additional function attributed to the monocyte by many investigators is its ability to undergo transformation and act as a precursor source for newly forming fibroblasts (15, 2). This concept, however, is controversial. The extensive literature on the biochemical aspects of the inflammatory reaction has not been reviewed since it is not directly relevant to this thesis problem.

Cellular response: After the inflammatory response to the cutaneous lesion, there is a proliferation of cells that are responsible for repair. Most tissues, including the skin, replace dead or damaged cells following injury by proliferation of fibroblasts which ultimately lead to scarring. These newly formed fibroblasts are usually structurally and functionally quite different from the original tissue cells, resulting in a permanent loss of specialized function in

the involved area. Scarring is irreversible and remains as a residual of previous damage. If the skin incision resulted only in minimal loss of tissue without significant bacterial contamination the wound will heal by the process referred to by the surgeons as, "primary healing" or "union by first intention". In this type of wound the different layers of the skin are able to coapt neatly one by one, leaving only a narrow space between the opposing tissues. By contrast, had the injury left a gaping wound, the healing would occur by "secondary union" or "union by secondary intention". Such a wound fills in by granulation, which produces greater amounts of scar and results in a slower replacement of the destroyed tissue elements.

Initially in the incised skin wound there is a flow of blood from the severed capillaries and after a few moments thrombosis occurs. The extravasated blood fills the tissue defect, clotting to form a matrix of fibrin strands which will serve later as a framework to orient migrating fibroblasts and developing capillaries. There is a slight decrease in the size of the wound as the fibrin clot contracts. By the end of the first hour inflammatory reaction is evident and there are leucocyte aggregates within the blood vessels at the margin of the incision. Within 24 hours, moderate numbers of leucocytes are present interstitially, and there is moderate edema in addition to the hyperemia. Several hours after the wound is made, the epithelium at the margins of the wound begins to migrate out over the surface of

the wound beneath the fibrin clot. Between the second and fifth days, granulocytes become less numerous, and lymphocytes and macrophages become prominent. By the fourth or fifth day, fibroblast activity is much in evidence and appears to be migrating into the clot. The erythrocytes within the clot lose much of their pigment and are no longer distinct. The clot has served several important functions up to this time: 1. Holding the margins of the wound together; 2. Protecting the underlying defect from bacterial invasion; 3. Contributing nutritive substances for the proliferating cells. An additional service the clot provides is a fibrin scaffolding for fibroblast proliferation and production of a firm network of collagen fibers. Newly formed blood and lymphatic vessels may be seen by the fifth day. During the seventh to eighth day the wound space will have been filled in with newly formed, highly vascular granulation tissue which is ordinarily completely covered with new epithelium. In succeeding weeks the deposition of collagen by the fibroblasts exerts mechanical pressure upon the newly formed walls of the capillaries and vascularity diminishes. By the fourteenth day this continued deposition of collagen has materially strengthened the wound (16).

Regeneration

An alternative process of wound healing is observed in select tissues of the mammalian system. This process is

regeneration and results in replacement of destroyed cells by cells of similar structure and function. The power of regeneration is basic and universal amongst living organisms. The capacity for cellular growth is essential for life. Maintenance growth is a normal mechanism seen in tissues where there is a continuous replacement of lost cellular elements, such as blood cells, epithelial cells of the skin, and of the cornea. In contrast regenerative growth is a response to massive loss of cells with replacement of whole tissues. The capacity for regenerative growth in general decreases as the complexity of the organism increases.

Regeneration of tissue is not only the replacement of cells which are destroyed, but these regenerated cells must be organized to replicate the architectural pattern of the original tissue in order for function to be restored. Based on their capacity to regenerate, the cells of the body can be divided into three groups: labile, stable, and permanent cells. Labile and stable cells retain the ability to regenerate throughout life, in contrast to permanent cells which cannot. Complete restoration of function following injury can take place only in tissue containing labile or stable parenchymal elements. Nerve and muscle cells are examples of permanent cells, and repair of these tissues must occur by proliferation of the simpler, less differentiated cells from connective tissue. Mild thermal burns and superficial abrasions of the skin are healed by regeneration of labile epithelial

cells. Bone marrow and lymphoid tissue also have great regenerative capacities. The bone marrow remains in a state of active proliferation throughout life in response to peripheral blood needs. When large amounts of bone marrow are destroyed, new hematopietic cells can be formed from persistent, primitive reticuloendothelial cells found in the marrow sinuses. Splenic and lymphoid tissues are similar to the bone marrow in that they can regenerate lost elements from the persistent, primitave reticuloendothelial cells. Perhaps hepatocytes are the best representatives of the category of stable cells. Hepatocytes do not ordinarily multiply in significant numbers; however, they retain a latent capacity for mitotic division and following injury can regain complete function by proliferative regeneration. As long as the supporting liver stroma is left intact following injury, regeneration will take place with the parenchymal cells utilizing the stroma as a scaffolding.

Wound Healing In The Cornea

Weimar in 1957 (17) selected the corneal stroma to study wound healing. The author noted that this tissue is avascular and relatively acellular and that these properties enhance the value of the cornea for studying the role of connective tissue cells in wound healing. The cornea lacks the histological complexity of the skin. In order for the reader to appreciate healing problems in the cornea, the anatomy of

this tissue must be well understood. In the transparent cornea five structural zones can be distinguished in cross section when viewed with the light microscope (figure 2). These zones from the anterior to the posterior surfaces are: 1. the corneal epithelium, which consists of five or six layers of nucleated epithelial cells constituting about 10 percent of the total corneal thickness in many mammals. The epithelial cells at the base are columnar, but as they are pushed forward by new cells they become flattened and are labeled basal, wing-shaped, and squamous cells. The fine structure of the epithelial cell reveals a cytoplasm poor in organelles, but with a well developed Golgi apparatus (18). The basal cells of the epithelium rest, as do those of other stratified squamous epithelia, on a basement membrane. 2. Immediately beneath the epithelial basement membrane is the anterior limiting or Bowman's membrane. Under the light microscope utilizing ordinary staining techniques this membrane appears structureless. However, in the electron microscope it is seen to consist of closely but randomly packed collagen fibrils and is not sharply delineated from the underlying stroma (19). In the rat this membrane is absent, and only the basement membrane separates the epithelium from the stroma proper (20). 3. The stroma accounts for approximately 90 percent of the corneal thickness in most mammals (21). It is divided into sheets of lamellae, which run parallel with the surface. The lamellae are made up of microscopic

collagenous fibers (21). Apparently there is no interweaving between fibers, so that in any given lamella the fibers are arranged parallel with themselves (20). Between the lamellae lie the corneal corpuscles which are equivalent to fibrocytes of other connective tissue. The lamellae are cemented into a matrix which contains the flattened fibrocytes. 4. The posterior limiting or Descemet's membrane is an elastic sheet bounding the inner surface of the stroma and is seemingly secreted by the endothelium (20). The electron microscope reveals an organized meshwork of collagen fibers arranged in a geometrical pattern of equilateral triangles (22). In the rat this mesh arrangement is sparse and scattered (21). 5. The endothelium of the anterior chamber is comprised of simple low cuboidal cells. Endothelial cells are normally a non-renewable population, but following injury there is considerably mitotic activity and complete restoration of this layer (20).

It is apparent from the foregoing histological description that the cornea does have definite advantages over skin as a model system to study the cellular aspects of wound healing. The investigator need deal with only three distinct native cell populations: thus, any influx of foreign cells during wound repair is more readily perceived. In addition, the low cellular density of the stroma allows for improved visual observations of individual fibrocytes because of the relatively wide intercellular spacing. Definitive interpretation of

autoradiographic and histological procedures are more easily made with this cellular separation. The transparency of the cornea has provided an easy site in which to observe pathological changes and for this reason was often chosen by early pathologists as the tissue in which to study the fundamental processes which accompany inflammation and repair (23). Duke-Elder and Leigh have stated that the tissues of the cornea have formed the battleground for some of the historial controversies in medicine (23).

The avascular and relatively acellular nature of the stromal tissue of the cornea does not seem to interfere with the healing of corneal epithelium or the stroma itself (24, 25). In fact the cornea may be better prepared to cope with tissue injury than the skin. If the corneal injury is not extensive, the wound will close by primary healing, which generally results in the formation of a transparent and avascular cornea. Very small lesions of corneal epithelium are rapidly repaired. The repair process begins immediately following injury by neighboring cells extending cytoplasmic pseudopodia into the area and covering the defect (26). Larger corneal defects are covered by migration and flattening of surrounding epithelium. After corneal wounding, Arey and Covode (27) observed that initially there was a marked decrease in the number of mitotic cells in the basal epithelial cell layer over the entire cornea, when compared to the control cornea. The authors noted that in these wounds the

epithelial cells covered the denuded area in 12 hours while the epithelial cell mitotic rate did not go above the prewound value until the fourth day.

When there is an extreme loss of corneal epithelium the cornea can be assisted in covering the denuded area by the migration of cells from the nearby conjunctiva. These cells maintain their original characteristics for a considerable time following corneal restitution (25). An interesting phenomenon takes place during the re-epithelialization process; the cells surrounding the denuded area migrate to cover the defect by "epithelial slide". Apparently epithelial sheets move as a cellular unit by ameboid motion into the denuded area (26). Accompanying the destruction of the epithelium there are slight, but significant, changes in the underlying stroma (23). These changes, in the absence of infection, lead to the accumulation of considerable numbers of leucocytes in the interlamellar spaces after the first 24 hours. The inflammatory reaction then progressively decreases and the stroma returns to its normal appearance in about four days.

Taylor, Goldsmith and Bevelander (28) suggested that the infiltration of the phosphatase rich leucocytes from the bloodstream into the avascular corneal stroma may be concerned in the process of phosphorylation, which is apparently necessary for the repair of tissues. Weimar (29) has documented that injury to the epithelial cells activates proteolytic enzymes liberating chemotactic

substances which induce PMN leucocyte invasion. The chemotactic substances are presumably polypeptides produced by the hydrolysis of proteins from the injured tissue by the activated proteolytic enzymes. Complete inhibition of the invasion of PMN leucocytes was achieved by the topical application of sodium salicylate and/or soybean trypsin inhibitor to the injured cornea (29). In these studies the inhibitors were administered during the second hour following injury and the inflammatory response was abolished for at least six hours postoperatively. Weimar's studies (29) indicated how essential the epithelium is in the early reactions to injury: 1. the growth activities of the epithelium seem to be the cause of transformation of corneal stromal cells into fibroblasts, 2. the epithelium is apparently the source of the proteolytic enzymes involved in the early response to injury, and 3. the presence of epithelium is necessary for the development of fibroblasts at the wound edge.

Within a few minutes after making an incised wound to the depth of the corneal stroma, there is set in motion a complex chain of events that will, in the uncomplicated wound, ultimately lead to complete repair. The repair processes are relatively more involved when the stroma is injured as opposed to those processes initiated by a simple corneal epithelial abrasion. An injury to the stroma not only damages or destroys the cellular elements, but also disrupts the architecture of the lamellae and ground substance. Depending on

the type of injurious agent and the amount of tissue destruction, the stroma will heal by one of two processes. In an uncomplicated wound avascular healing takes place by regeneration of corneal fibers or cells; but, in the presence of infection or in destructive lesions, vascularized healing occurs. Avascular healing is unique to the cornea, while the presence of blood vessels in the cornea corresponds in general to healing found elsewhere and results in the formation of granulation and fibrous tissue (23).

A superficial defect in the stroma is rapidly covered by a combination of epithelial slide and thinning of the entire epithelium (30). In a short while the cavity of the defect is filled with proliferating epithelial cells so that the surface again becomes smooth and regular. The inflammatory response in this type of wound is minimal and the wound will heal without scarring. Dunnington (31) noted that in no case is Bowman's membrane ever regenerated and therefore it plays a relatively unimportant role in the reparative process.

In the case of a large defect involving a considerable amount of stromal tissue destruction, the edges of the wound increase in thickness (29). The next event in reparation is an invasion of the cornea by PMN leucocytes. This phase of repair constitutes the phagocytic exudative stage with a duration of about three days before the formation of new connective tissue. The leucocyte arrives rapidly and may reach a central lesion in the rabbit cornea in about one hour (32).

Weimar (33), working with centrally placed knife incisions in the rat cornea described how the PMN leucocytes entered the damaged tissue during the fifth postoperative hour. They steadily increased in number until they reached a maximum between 24 and 36 hours and returned to normal by the sixth day.

How do these inflammatory cells reach the stromal wound? It is apparent that since these cells are of hemopoietic origin they must be transported to the eye via the vascular system. The limbus is the term that denotes the transitional zone of the corneoscleral junction. This region has a rich vascular plexus which surrounds the cornea with a system of marginal capillary loops entering a short distance into the stroma in the superficial layers (21). Soon after a destructive lesion blood leucocytes are seen to swarm in the tissue around the vascular plexus nearest the effected area. From these capillary loops the leucocytes have two and possibly three ways to reach the site of injury. They can escape from the conjunctival vessels near the limbus, traverse the conjunctiva and pass superficially to the corneal wound by way of the tear film (34). This concept is not new. Robb and Kuwabara (34) have discussed the important findings of Julius Cohnheim in 1867 (7), who observed that inflammatory cells not only migrate through corneal stromal tissue from perilimbal vessels, but also reach the edges of corneal wounds by passage from "conjunctival secretions" directly into the wound defect. If

following injury the precorneal film is continously washed away with a saline drip no PMN leucocytes are seen at the wound margins through the first 16 hours following injury.

The second and perhaps the most important route that the blood leucocytes take from the perilimbal vessels to the wound is through the stromal tissue (15, 24, 34, 35). Leucocytes have been observed as nearly continuous stream of cells migrating from perilimbal vessels to the wounded area in an eight hour peripheral corneal incision (34).

A third means whereby inflammatory cells can reach the injured cornea is via the anterior chamber. Recent evidence (36, 37) indicates that subsequent to a central linear incision in the corneal endothelium, inflammatory cells reach the wound without first passing through the stroma. Evidence of a marked increase in the number of inflammatory cells within the anterior chamber of the wounded versus control eyes was derived from aqueous humour cell counts. The cell counts were made by passing samples of aqueous fluid through membrane filters which were later stained and examined under the optical microscope. The invading leucocytes become deformed into long spindle shapes as a result of movement through the compact lamellae of the corneal tissue. This alteration in cell shape must be considered when morphological studies are performed utilizing the cornea.

Almost concurrently with the invasion of PMN leucocytes and

the re-epithelialization of the corneal surface, considerable changes in the stromal cells are seen. In the rat cornea the stromal cells undergo marked morphological changes and become fibroblastic in appearance during the first 24 postoperative hours following a standard penetrating incision (38, 39). Microscopic examination of the wound edge from a period of 4 through 24 hours after wounding shows a gradual transition of the corneal stromal cells into fibroblasts (40). This transition is morphologically marked by an increase in the number of nucleoli per cell nucleus and an increase in the size of nucleoli. This modification of cells was present only in a narrow border, approximately 200μ from the wound edge (40).

Accompanying the changes seen in morphology following injury, are remarkable alterations in the physiology and enzymology of the stromal cells. It is now generally agreed, based on many different lines of evidence, that the major role of this metabolic activation of the stromal fibrocytes is for the purpose of fibrogenesis (12).

The site and sequence of collagen fibril formation has been debated for many years and has recently been reviewed by Ross (8). Most of the available evidence seems to favor the cell surface of the fibroblast as the primary site of collagen formation (41). New ground substance appears to be synthesized together with the formation of collagen fibrils. Employing radioautographic techniques, Dunnington and Smelser (42) have shown that the sulphated mucopolysaccharides

appear very early in a healing corneal wound and that their rate of synthesis is, and remains, high for a long time in the area of regenerating connective tissue. The fresh collagen fibers layed down by the fibroblasts are coarser and are disorganized in reticular fashion (43, 44). With time the fibers gradually conform to the configuration of the normal corneal lamellae (23). This conformity may be due to the mechanical stress and tension placed on the cornea by the intraocular pressure.

Shortly following the invasion of the injured rat corneal stroma by PMN leucocytes, the monocyte makes its appearance (15,. 24). This phenomenon is similar to the cellular events of acute inflammation in the skin (45). The extent of the monocyte invasion is dependent on the degree of injury. In small aseptic wounds only a few monocytes are seen, but in destructive lesions there is a marked increase in their numbers. As is the case with healing skin, the initial function of the monocytes apparently is to act as scavengers removing connective tissue debris following injury. At a later stage the monocytes have been observed by Weimar (15) and others, to undergo transformation to stromal fibroblasts. This important process for renewal of damaged or destroyed stromal cells will be discussed in detail later. Mast cells are found in large numbers in the limbal tissues but are not seen in the corneas of normal or wounded eyes (15). Pullinger and Mann (24) reported that only PMN leucocytes

and "wandering cells" entered the cornea from the limbal tissue following injury to the stroma. The authors noted that fibroblasts and lymphocytes are never seen entering the injured cornea, unless blood vessels also invade the wound. In the uncomplicated wound the regenerated tissue may be distinguishable only with difficulty from the normal, but as is true with all wound repair the replacement tissue never reaches the functional standards of the original tissue.

More severe corneal damage may result in vascularized healing. When the cornea has sustained a large destructive injury or the corneal wound becomes infected, leading to keratitis or corneal abscess, the ensuing inflammation is characterized by a massive influx of PMN leucocytes as well as macrophages and lymphocytes (23). During the period of inflammation an ingrowth from the limbus of new blood vessels takes place mainly in the superficial layers of the stroma. Cogan (46) described the development of stromal vascularization in a classic study in 1949. After experimental lesions were made on rabbit corneas, the limbal vessels engorged and formed saccular aneurysms. These aneurysms eventually ruptured and caused localized hemorrhage into the stroma. The hemorrhagic areas in turn became sites for new capillaries. Most of these capillaries receded leaving a few loops which repeated the invasion process and with each cycle the newly formed loops moved closer to the lesion.

Generally two theories have been proposed to explain the

etiology of corneal vascularization (47): 1. A chemical substance or group of substances termed vessel stimulating factor (VSF) is formed in the damaged tissue, and upon contact with the capillaries provokes a budding or growth phase. An alternate postulate may be that VSF neutralizes a normally present growth-inhibiting substance. 2. Normally vessel ingrowth is prevented by the compactness of the stroma, but the corneal edema which accompanies inflammation following injury is a necessary but not sufficient condition to open pathways to vessel growth. The exact mechanism of neovascularization is at the present poorly understood (48).

The end result of vascularized healing in the cornea is similar to a repaired skin lesion. Granulation tissue is laid down which consolidates into scar tissue. There is wound contracture and most of the blood vessels disappear, although with the aid of the microscope endothelial tubes or columns may be visible for a time. A degree of permanent opacity will always persist; however, the connective tissue fibers of the repaired stroma gradually may assume the appearance of normal corneal fibers (49).

Small defects in the corneal endothelium are covered by a migration of neighboring cells (50). Mitosis, which is normally absent in the endothelium, takes place in large numbers in the region of the lesion after 24 hours, but disappears after five days (51). As a result of cellular migration to fill the defect, the diameter of

the endothelial cells can approach twice their normal value (52). Descemet's membrane is repaired by a slow secretion of material from regenerated endothelium (53). In the event the endothelial defect is too great, and cannot be covered by migration or replication of neighboring cells, the cornea becomes edematous and permanent loss of transparency results (48).

Wound Fibroblasts

What is a fibroblast? The term fibroblast is used to denote a cell which is actively engaged in the formation of collagen and glycoproteins (8). Viewed at the level of the light microscope the fibroblast appears as a long spindle or star-shaped cell with an oval nucleus which contains one or more large nucleoli (54). The nuclei are oval and larger than any other found in connective tissue cells (8). In thin sections the cytoplasm is often obscure, so that only the nuclei are seen. Ross (8) gives in detail the fine structural characteristics that are used to recognize and classify the fibroblast when the electron microscope is utilized. In mature or repaired connective tissue the fibroblast is relatively quiescent metabolically and almost immobile. In this state the fibroblast is commonly referred to as a fibrocyte.

Interest in determining the exact source of fibroblasts in wound repair has existed for many years. The derivation of these cells has remained controversial since Cohnheim (8) and later Maximow

(55, 56, 57) challenged the classical concept that they were derived solely from local connective tissues of the wound. The controversy has continued for over 100 years without final resolution. Generally two theories have been advanced to account for the appearance of wound fibroblasts: 1. Mononuclear blood cells enter the wound site from capillary loops and transform into proliferating fibroblasts and 2. The fibroblasts are derived from the connective tissues of the wound including the adventitia of small blood vessels and capillaries. Allgöwer (2) in his monograph reviews much of the earlier controversy regarding the origin of the fibroblast. Historically two main experimental approaches have been employed by both proponents to support their conflicting theories of the origin of the fibroblast. The first group has utilized classical histological techniques, while the second group has made use of tissue culture methods. The two camps of investigators have often had similar experimental protocols; however, their results and their interpretation of the data have been in complete opposition. Recently there has been renewed interest in this problem because of the availability of new methodology (58).

Proponents of the Hematogenous Origin of the Fibroblast

There has never been any strong evidence to include the PMN leucocytes and mast cells among those cells capable of undergoing metaplasia into fibroblasts (59). Likewise, intensive work carried

out in recent years has all but eliminated the lymphocyte as a contender for being a fibroblastic precursor cell. The lymphocyte has been eliminated on the basis that it does not appear to transform directly into a fibroblast. What may take place is a lymphocyte-to-macrophage transformation. Most of the available data are consistent with the concept that some of the lymphocytes can transform into macrophages under the homeostatic control of PMN leucocytes or their degradation products (60). The newly formed macrophages then, under the proper stimulation following tissue injury, may differentiate into fibroblasts (61). Based on evidence from tissue culture studies, Yoffey (62) has suggested that there may be transformation of lymphocytes into monocytes and monocytes into macrophages and "... possibly a fibroblast." Maximow (57) and later Allgöwer (2) regarded the blood monocyte as one of the principle contenders for the role of the fibroblastic precursor. Gillman and Penn (63) have stated that, following injury to the dermis in human skin, there is an invasion of monocytes from the blood-stream to the area immediately underlying the regenerated epithelium. In the authors opinion these monocytes do undergo metaplasia and become fibroblasts.

Allgöwer (64) some 25 years ago forgot to remove from the incubator a buffy-coat culture used to study leucocyte migration. Unaware of the work of Bloom (65, 66) and Maximow (55, 56) Allgöwer

was surprised to find a dense fibrocytic network in these cultures after an incubation period of about ten days. The evaluation of the importance of the circulating blood cell in connective tissue repair has been pursued intensely by Allgöwer since his initial observations with buffy-coat cultures. A considerable amount of indirect evidence in favor of the hematogenous origin of the fibroblast was presented by Allgöwer from experiments conducted upon healing wounds in rabbit ears (2). The author induced leucopenia by total body irradiation and found that granulation tissue subsequently formed in the shielded ear was reduced by fifty to seventy percent. The repair process is unaffected if the irradiation is carried out four days after wounding. Evidence was presented that suggested this reduction in wound healing following irradiation was not secondary to formation of circulating toxins. In this same series of experiments, Allgöwer remarked that both human and rabbit leucocytes are capable of producing connective tissue networks when cultured in vitro for several weeks. The author further stated

> These networks imitate in vitro certain phenomena of wound repair and particularly that of 'organizing' the plasma medium, in a better fashion than similar networks formed from loose and dense connective tissue and small vessel sections, all being obtained from adult animals.

A review of the literature pertaining to the cellular aspects of wound repair through 1958, showed that the majority of the data was based on morphological identification. The identification of cells is

somewhat subjective when only morphological criteria are used because the cells may assume atypical forms in culture as well as in traumatized tissue.

To overcome this discrepancy, Allgöwer and Hulliger (67) designed an experiment to determine whether blood cells that had transformed into fibroblasts in culture were capable of synthesizing extracellular connective tissue elements. Biochemical identification of fibroblasts was made possible by the analysis of material containing hydroxyproline, the amino acid characteristic of collagen, which is a specific product of functioning differentiated fibroblasts. Studies were conducted utilizing cultures from blood buffy-coat of the rabbit. Blood was taken by several different procedures to test the possibility that tissue fragments were not inadvertently introduced into the cultures. Samples were obtained by cardiac puncture, repeated cardiac punctures in exsanguinated animals, and by prior cannulation of the carotid artery. Hydroxyproline was estimated utilizing a colorimetric method on samples of supernatant culture medium and cultured fibrocytic colonies. Cultures from blood buffy coat formed considerable amounts of hydroxyproline over a period of 28 days and the authors concluded from these findings that the monocyte can produce a collagen constituent in culture. They further stated that the monocyte probably performs the same function in wounds and thus is a "functional" fibroblast. Strangely enough, blood samples taken via

cannulation of the carotid artery produced approximately twice the amount of connective tissue in culture as those cultures arising from cardiac aspirated blood. However, contamination by cells picked up as the needle passed through the chest wall or myocardium during cardiac puncture apparently did occur, but the amount of contamination was such that it accounted for only a small percentage of the proliferating connective tissue cells.

In later work Hulliger and Allgöwer (68) studied quantitative aspects of the leucocyte contribution to fibroblastic repair. Leucocytes were combined with fibrocytes to determine the possible reciprocal influences between the different cell types. To differentiate both cell types quantitatively, sex chromatin was chosen as a suitable marker. Fibrocytes were obtained from subcutaneous connective tissue that was excised from the abdomen of rabbits, while the source of leucocytes was from blood drawn by carotid artery cannulation from a rabbit of the opposite sex. Pieces of connective tissue and buffy coat were explanted either singly, or in combination, in one diffusion chamber. The chambers were then implanted into the peritoneal cavity of a rabbit of the same sex as the donor of the connective tissue. Quantitative cell analysis of the explant material prior to placement in the diffusion chambers was performed. Connective tissue pieces were stained, their size determined, and total number of cell nuclei were then calculated using a microscope. The total leucocyte number

in buffy coat pieces was determined by incubating them in four percent acetic acid for 24 hours. The acetic acid dissolves the fibrin clot and releases the cells enabeling them to be counted using a hemocytometer. The ratio of fibrocytes to large mononuclear cells (monocytes and large lymphocytes) in the mixed chambers was between 1:5 and 1:2. Chambers were removed from the rabbits 14-21 days following implantation. Cell population counts in 10 out of 22 vascularized chambers revealed that 17.7 percent of cells present were fibrocytes. In chambers containing connective tissue only one out of 22 vascularized chambers grew abundant fibrocytes. However, a break in this chamber was found and invading macrophages were seen passing through this defect. In chambers containing connective tissue and buffy coat in combination, there was abundant growth of fibrocytes mixed with macrophages. Cell counts made on 33 out of the 41 vascularized chambers indicated 50 percent of the fibrocytes as determined by sex chromatin were of hematogenous origin. The authors imply that combined cultures produce more fibrocytes, but they failed to state the amount of the increase. They did speculate on why fibrocytes did not grow if explanted alone in diffusion chambers. It appears that the addition of leucocytes brings to the culture growth promoting substances which stimulate fibrocytic reproduction (69). On the basis of their investigations they concluded

> ...the contribution of hematogenous monocytes to fibrocytic

> repair is considerable and appears to be in the order of magnitude of 50 percent or more of the final fibrocytic population.

Petrakis, Davis and Lucia (70) used the diffusion chamber method to explore the potentialities of the nongranulated leucocytes to differentiate into fibroblasts in healing wounds. In these studies modified Algire diffusion chambers containing autologous and/or homologous human leucocytes obtained by venapuncture were implanted subcutaneously into volunteer subjects. The chambers were removed at weekly intervals up to six weeks and histologically analyzed. A gradual disintegration of neutrophilic granulocytes and eosinophils was seen occuring over a two to three week period, and the authors noted no granulocytes were found beyond three weeks. During the first two weeks, the mononuclear leucocytes appeared to hypertrophy into cells resembling macrophages and histiocytes. There was such a variety of morphological forms of these cells that they were designated as polyblast macrophages (after Maximow). These cells, at three weeks, assumed the stellate or spindle-shaped appearance of fibroblasts and within another week dense sheets of fibroblasts were seen in the chambers. To confirm the transformational sequence from polyblast macrophage to fibroblast, small amounts of India ink were added to the cell suspensions at the time when the chambers were sealed. At two weeks the ink particles were found to be present in the macrophages and by four weeks, the

ink particles were seen in the cytoplasm of the fibroblasts. By staining sections of the plasma clots within the chambers with Mallory's or Van Giesen stains, the presence of a considerable amount of collagen in some of the chambers was observed. The authors concluded that they had demonstrated experimentally the mesenchymal potentialities of the circulating nongranular leucocytes to eventually differentiate into fibroblasts capable of collagen and reticulin production.

Moen in 1934 (71) observed that individual guinea pig mononuclear exudative cells in tissue culture go through sequential developmental steps to form pure colonies of fibroblasts. The mononuclear exudate consisting almost entirely of monocytes and macrophages was produced by the injection of fluid parowax into the pleural cavity. After three to 14 days the mononuclear exudate was removed and the suspension was found to contain almost entirely mononuclear cells chiefly of the monocytic type with a small percentage of lymphocytes and a few macrophages. Cell types were determined by the uptake of the vital stains neutral red and Janus green. Some one hundred to several thousand exudate cells were placed in each of a series of Carrel's micro flasks and incubated. The ratio of the surface area of the culture flask to the initial number of cells was such as to allow isolated adherent cells to be identified under the microscope. These isolated cells were designated by marking their location on the outer surface of the flask with black enamel paint. A number of the

isolated cells were observed undergoing the transition from the typical macrophage to the stellate and spindle-shaped fibroblast. Many of these fibroblasts went on to form proliferating colonies. Moen concluded,

> ...when environmental conditions are favorable, and when manipulative trauma is reduced to a minimum, certain isolated cells will proliferate to form pure colonies of fibroblasts.

Recently Stirling and Kakkar (72) have essentially repeated the earlier work of Allgöwer and Hulliger (67), and Petrakis et al (70). This study was undertaken to help eliminate the usual criticism made of work in which leucocytes are claimed to have transformed into fibroblasts. Opponents to the hematogenous origin of the fibroblast have stated: 1. The fibroblasts are contaminants derived from venapuncture or cardiac puncture. 2. Fibroblasts are present in the tissue extract when this is used to stimulate growth. 3. In those studies employing diffusion chambers, the host cells may penetrate into the interior of the chambers. 4. The identification of fibroblasts is based on their morphology only. To meet these criticisms the authors cannulated the jugular vein of the dog and utilized specially constructed diffusion chambers to rule out cellular penetration. Morphological identification of cells was eliminated

> ...by regarding as positive only those chambers containing compact material with regular orientation of the cells and fibers, readily recognizable as tissue with the light microscope and containing fibrils staining as for reticulin and

collagen.

Autologous buffy coat was placed in the chambers which were then implanted in the peritoneal space of the experimental animals. A second group of identical chambers was implanted with blood buffy coat obtained by venapuncture instead of cannulated blood buffy coat. Three weeks later the chambers were removed and their contents histologically examined. Alternate adjacent serial sections were stained with hematoxylin-eosin or stained for reticulin fibers and collagen. Combined data from all chambers implanted revealed 98 percent had isolated cells resembling fibrocytes or fibroblasts. Twenty percent of those chambers originally containing venapuncture-derived buffy coat showed evidence of connective tissue formation, while 16 percent of those chambers originally containing cannulated-derived buffy coat showed the presence of connective tissue. These results were interpreted by the authors as evidence that certain cells in the circulating blood are capable of forming connective tissue. They concede that the leucocyte may not be the only source of fibroblasts. in the blood and that connective tissue cells derived from blood vessel walls or other sources including the bone marrow could in part supply the pre-fibroblastic cells.

A recent significant contribution to the understanding of the role of the mononuclear blood cell in wound healing and the fibrogenic processes has been made by Gillman and Wright (61). These authors

have employed in vivo techniques as part of a series of investigations into the timing of the cellular changes preceding the onset of fibrogenesis in wounds and around implanted foreign bodies. They noted that several recently published reports (73, 74) have indicated that when cells labeled with tritiated thymidine (^{3}H-Tdr) were injected immediately before killing a previously wounded animal, the isotope was first observed in perivascular cells close to the wound site. Hadfield (75) and others have also noted that mitotic figures first appear near vessels that are adjacent to the site of injury. These cells undergoing mitosis have been labeled perithelial cells or local fibrocytes and apparently the possibility that they are derived from mononuclear blood cells has not been considered (73, 74).

Two sets of experiments were conducted by Gillman and Wright (61) to determine if

> ...pre-operatively labeled mononuclears (that is, lymphocytes and monocytes) mitose and undergo transformation into macrophages and other cell forms.

In addition it was anticipated that these experiments would test the generally accepted view that perithelial cells play a significant part in fibrosis. In both experiments ^{3}H-Tdr was used as the tracer material. Full thickness wounds, one centimeter in diameter, were excised from the dorsal skin of rats and small polyvinyl sponges and 'millipore' filters were implanted in a distant site, either subcutaneously and/or intraperitoneally. The experiments differed in that one

series of animals received ^{3}H-Tdr pre-operatively and the other post-operatively. Imprint or touch preparations were made of portions of the granulation tissue which formed in the excised wounds, and of the sponges, filters, spleen, lymph nodes, and bone marrows of all experimental animals. Paraffin sections also were made of all the aforementioned tissues. All preparations were then processed for autoradiographic analysis. In the group of animals that were post-operatively injected with isotope two hours before killing, labeling was first seen between 24 and 48 hours after wounding. Lymphocytes and monocytes located around small venules in the sites of injury were obviously labeled by the third day. Also at this time occasional labeled mononuclears were seen within vessel lumina in the injured areas. These findings strongly suggested to the authors that the labeled perivascular cells might well be a mixed population, composed of not only local cells entering mitosis, but already labeled emigrating blood-borne mononuclear cells. By the end of one week large numbers of cells were labeled, which included not only inflammatory cells, but also spindle-shaped fibroblastic and fibrocytic cells. However, no more than 15 to 20 percent of the round cells in these injuries were labeled. In contrast, based on the experimental group of animals in which isotope was injected postoperatively, shortly before the rats were killed, 40 to 80 percent of the perivascular round cells were labeled. By comparing imprints, blood smears,

and hematopoietic organs from the same animals, the investigators found that by using hematological criteria they could easily classify lymphocytes and monocytes in the healing wounds. These cells were then observed two to three days post-operatively to be in mitosis and to acquire morphological features indistinguishable from those described as typical of small lymphocytes activated in vitro (61). At this time, or even up to fourteen days after the last dose of isotope, fibroblasts and fibrocytes identified on morphological grounds with labeled nuclei were seen. Samples were taken from other connective tissue sites at the time of injury and the appearance of label was extremely rare. Thus, the increased radioactivity found in those pre-operatively labeled rats could be accounted for in only one way according to the authors, and that was by emigration of labeled blood mononuclears. Once the pre-operatively labeled round cells arrived at the wound site they assumed a number of different roles: some became phagocytic, others remained in the wound for weeks and transformed into fibroblastic and mitotic cells, and some moved away from the site of inflammation. A major portion of the up-take of ^{3}H-Tdr in the wound was by the blood-borne mononuclears preparing to undergo mitosis. Gillman and Wright concluded that their evidence

> ... seems to provide objective support for Maximow's contention that at least one pluripotential cell does indeed exist in circulating blood.

They state that fibroblasts are not solely derived from circulating

mononuclears; however, these cells do seem to give rise to a proportion of fibrocytes in sites of repair.

A fascinating study was reported by Barnes and Khrushchov (76) using syngeneic mouse radiation chimaeras prepared with the cytological marker T_6 chromosome to distinguish cells of host and donor origin. The purpose of the investigation was to provide additional evidence that the fibroblast can be derived from hematopoietic tissue. Male CBA or CBA-T_6T_6 mice were used as recipients and received either singularly, or in combination, fetal liver, lymph node cells and bone marrow of CBA-T_6T_6 or CBA mice, respectively. Mice were X-irradiated to suppress their hematopoietic activity and then received intravenously the appropriate donor hematopoietic cell suspension. Some weeks after irradiation and seeding, four pieces of a sterile glass cover slip were implanted under the dorsal skin of each animal to collect fibroblasts. The animals were then intraperitioneally injected with "Colcemid" (desacetyl-n-methyl colchicine) five hours before they were killed. The glass pieces had been in place for a week at this time. The author had already shown that with normal animals five to seven days after surgery was the optimal time for obtaining sufficient mitosis among fibroblasts attached to the glass. After removal the glass pieces were incubated for 1 hour in a 1 percent "Colcemid" medium. The glass pieces were fixed and stained. Some glass pieces were removed from the mice and the

adhering cells served as a source of cells for tissue culture. By this method the authors were able to recognize the karyotype of cells in metaphase from the sterile inflammatory area. Mitotic figures containing the T_6 chromosomal marker were seen in those CBA mice that received bone marrow and/or fetal liver, but not from lymph node cells. The investigators felt that most of the cells in these preparations were fibroblasts. They admittedly had difficulty distinguishing between fibroblasts and macrophages in division. However, previous experiments revealed intense proliferation of fibroblasts occurred at this time period after onset of inflammation,and mitoses were not found before fibroblasts had appeared on the coverslip. The cells attached to the pieces of cover slips when placed in tissue culture, yielded T_6 marked fibroblasts. The authors concluded that their

> ...results provide evidence that the fibroblast can be added to the list of cells of blood, lymphoid and macrophage systems which are derived from hematopoietic tissue.

A less convincing study was reported by Stromberg, Woodward, Mahin and Donati (77) in support of the bone marrow as a source of new fibroblasts. The purpose of their study was to determine the effect of bone marrow shielding on wound contracture following irradiation. One group of rats received whole body irradiation while a second group of rats had their hind limbs and pelvis shielded from

the irradiation source. Four days following irradiation one half of the animals in each of the various groups were wounded by means of a full thickness excision of a circular piece of dorsal skin. The degree of wound contracture was then recorded. Initially there was little difference between the degree of wound contracture of the shielded and non-shielded animals. Later however, the rate of wound contracture of the shielded animals approached that of the sham irradiated group, while non-shielded animals had a severe delay in contracture and their wounds never developed normal appearing granulation beds. The authors could not state the exact mechanism involved whereby bone marrow shielding allowed for wound contracture that approximated that of control animals. They did speculate that following irradiation there is apparently a delay in adherence of the wound edge to the underlying granulation tissue bed which may be a result of dysfibroblastogenesis. These results are difficult to evaluate because undoubtedly whole body irradiation has a deleterious action on the normal cellular and biochemical processes of acute inflammation which are necessary for wound healing.

Greenlee (78) utilized the light and electron microscope to study the early events in repair after division of the deep flexor digital tendon of the hind foot of the rat. At 18 hours after tendon division, a single layer of cells, the majority being PMN leucocytes, was seen adhering to the stump of the tendon. By 24 hours, the

predominant cell type was a mononuclear cell that had actively surrounded individual collagen fibrils. By 36 hours these cells had proceeded down the tendon stump and assumed the morphological appearance of immature fibroblasts. At 10 days new collagen fibrils were noted which were smaller than the usual mature tendon fibrils. The interpretation of the author was:

> Mononuclear cells with phagocytic potential invade the cut end of the tendon stump, transform into actively phagocytizing macrophages, as time progresses begin to acquire more rough endoplasmic reticulum for the synthesis of collagen, and ultimately take part in the repair of the injured tendon.

The author concluded:

> Unfortunately morphologic studies such as the one just described cannot definitely prove this hypothesis, and additional studies using tracer techniques are required.

Proponents of the Tissue Origin of the Fibroblast

A number of investigators have been adamant in their contentions that blood cells do not have the capacity to transform into connective tissue fiber-forming cells. Many of these workers have duplicated the studies performed by proponents of the hypothesis that the hematogenous leucocyte serves as a precursor for the fibroblast in healing wounds. Negative results in these repeated experiments have been interpreted as strong evidence that blood cells do not serve as progenitors for fibroblasts. However, negative experimental results involving *in vitro* tissue culture procedures may represent a

deficiency in the methods employed.

In 1938 Hall and Furth (79) described experiments that did not support the opinion of Bloom (65, 66) and other workers, that lymphocytes of the thoracic duct are transformed in vitro into monocytes. Hall and Furth made a series of cultures obtained by cannulation of dog and rabbit thoracic ducts. Immediately following explantation the lymphocyte cultures contained approximately one percent monocytes. The fate of the monocytes was followed in cultures by the addition of neutral red and/or trypan blue. The number of monocytes present at the end of the culture period never exceeded the number at the time of explantation. To eliminate the criticism that the culture medium used in these experiments would not support fibroblastic growth, splenic tissue from the same lymph donor animals was cultured in identical medium and proved suitable for the growth of fibroblasts. The authors stated:

> Evidence is wanting that in tissue cultures lymphocytes are transformed into monocytes and monocytes into fibroblasts.

The cultivation of mouse bone marrow in vivo has been studied by Berman and Kaplan (80). Diffusion chambers containing murine bone marrow were implanted into the peritoneal cavity of the host animals. All stages of erythroid maturation were observed through 20 days of cultivation. Mitotic proliferation and differentiation of myeloid elements were found in the chambers at much later times.

Lymphoid elements were present in 30 day cultures; however, the predominant cell type was myeloid. By two and one half months the majority of cells found were of the macrophage series. Why all cell forms of the marrow do not persist indefinitely in the chambers could not be explained by the authors. They did note that no evidence was obtained that implanted bone marrow cells transformed into "fibroblast-like" or "epithelial-like" cells.

The work of Petrakis et al. (70) was repeated by Ross and Lilly-white (81) in a study of the fate of buffy coat cells grown in subcutaneously implanted diffusion chambers. Blood was obtained by carotid artery cannulation to avoid possible contamination of the buffy coat with extraneous cells during venipuncture or cardiac puncture. The authors demonstrated that 91 percent of the chambers containing cardiac puncture blood contained collagen and connective tissue-forming cells, while only six percent of the carotid cannulation chambers contained collagen. They concluded,

> ...positive results in similar systems previously reported, represent contamination by extraneous connective tissue cells and that further investigations must be pursued before blood leucocytes can be shown to be precursors of fibroblasts.

These findings are in marked contrast to those of Allgöwer and colleagues (67, 68) who also attempted to control contamination of blood by using carotid artery cannulation and who found no significant difference in fibroblast proliferation between cannulated and cardiac

puncture derived buffy coat cultures. More recent work reported by Stirling and Kakkar (72) was not in agreement with the data of Ross and Lillywhite.

The development of fibroblastic colonies in chicken buffy coat macrophage cultures obtained from cardiac and venous blood and peritoneal exudate has been studied by Rangan (82). The author noted that fibroblastic colonies developed only in macrophage cultures obtained from cardiac blood or peritoneal exudate and not in venous blood cultures. This investigator concluded that a strong possibility of connective tissue fragment contamination of blood exists when it is obtained by the cardiac route.

A recent report by Rasmussen and Hjortdal (83) utilized a unique approach for obtaining blood via cardiac puncture for buffy coat cultures. Cardiac blood samples were withdrawn by first inserting a canula through the heart wall to the lumen of the left ventricle. The canula was sealed at the tip with a wax plug. Then a metal rod was introduced into the canula and inserted until the wax plug was pushed out and the rod was then withdrawn. Next a second or inner canula was inserted through the first and passed into the left ventricle. Blood was then aspirated by a syringe connected to the inner canula. The authors felt this procedure would greatly reduce the contamination of the blood with connective tissue cells during sampling. The blood obtained by this new method was treated exactly the same way

as in a previous experiment in which blood was obtained by the conventional cardiac puncture method (84). In contrast to the results reported in their first experiment, microscopic examination revealed that no fibroblasts or connective tissue fibers developed in the chambers when precautions were taken to avoid contamination of the blood with extraneous connective tissue cells. Thus, the authors felt, earlier data which indicated development of fibroblasts in blood and buffy coat cultures was due to contamination of the blood with connective tissue cells during sampling of the blood.

MacDonald (73) was one of the first investigators to use ^{3}H-Tdr as an experimental tool to study the origin of fibroblasts. Since ^{3}H-Tdr is incorporated only into those cells which are actively synthesizing deoxyribonucleic acid (DNA), any cells which are stimulated to undergo mitosis following tissue injury are likely recipients of the isotopically labeled Tdr. Those cells which have taken up the label can be later identified by radioautographic techniques. MacDonald theorized that since blood elements penetrated the wound area prior to the appearance of recognizable fibroblasts, it should be possible to follow any differentiation of these blood cells into fibroblasts by proper selection of the interval between injection and wounding. Rats were wounded by scalpel incisions of the dorsal skin, and were given a single intraperitoneal injection of ^{3}H-Tdr at intervals from zero to six days after the wounds had been made. The wounds

were biopsied at varying intervals from two hours to fourteen days after the wounding and labeling. MacDonald found that labeled lymphocytes were present in the incisions and in adjacent dermis and subcutaneous tissues almost immediately after wounding, and two to four hours after the lymphocytes appeared, labeled macrophages were identified. During the third postoperative day the labeled lymphocytes and mononuclear cells reached their peak and then declined in quantity. Near the end of the second day, label was seen in the fixed undifferentiated connective tissue cells along the adventitia of veins and arteries, in similar cells in hair follicles, and along injured muscle cells. During the third day, the fibroblasts migrated into the wound site and commenced dividing. No label was seen in the fat and mononuclear cells of the dermis. Thus, he concluded, these cells do not appear to take part in proliferative activity during wound repair, and lymphocytes, monocytes and macrophages play no part in the formation of fibroblasts.

Grillo and Potsaid (85) reported the variable inhibitory effect of a single application of local X-irradiation on proliferation of new connective tissue depending upon time of application of irradiation in relation to time of wounding. The maximum effect was observed following irradiation between 24 and 48 hours after wounding, while only a slight effect was noted following irradiation at five day or later. It is a well established principle that rapidly dividing cells are more

radiosensitive than less active cells. The authors speculated that the increased radiation effect seen between 24 and 48 hours after wounding was due to local cells undergoing mitosis and differentiation into fibroblasts. Histological observations indicated there was a depression of capillary proliferation into the wound site following irradiation. Grillo and Potsaid concluded that hematogenous cells were not present in sufficient numbers at the time of irradiation to be responsible for the subsequent reduction in the rate of wound repair. The possibility that the depression of capillary proliferation into the wound following irradiation would in turn reduce the availability of hematogenous elements for fibroblastic formation was not even discussed by the authors.

In a later report Grillo (86) incorporated X-irradiation to study the question of the origin of fibroblasts in healing wounds. Guinea pigs received X-irradiation in moderate doses to the wound site before and after they had been wounded. The rate of fibroblastic proliferation was studied five days after wounding by detecting the presence of labeled nuclei by use of autoradiography. Labeled Tdr uptake was most prominent in the active area of the wound margin while few fibroblasts were labeled in the loose connective tissue a short distance from the edge of the wound. The highest concentration of labeled cells was generally seen surrounding capillaries in the newly forming tissue. The exudate overlying the granulation tissue as well as

the dermal cells in areas close to the wound were inactive. When irradiation was given 20 minutes prior to wounding only a slight depression in cellular proliferation was noted. Irradiation of the wound at 20 minutes after injury resulted in a slight but significant depression of cellular proliferation. However, there was a marked reduction of cellular proliferation when irradiation was given 28 hours postwounding. Grillo stated that inflammatory cell infiltration from the blood stream was not markedly affected by the irradiation. The author made the assumption that by using moderate irradiation dosages, only those cells about to divide would be damaged. Thus, the depression in fibroblastic proliferation observed when irradiation was applied 20 minutes after wounding indicated to the author that only local cells undergoing mitosis were damaged by the irradiation. It was further assumed that at this time period no hematogenously derived cells were actively dividing in the wound and therefore they were not damaged by the irradiation. If in fact the inflammatory cells did not receive irradiation damage they could not then be responsible for the later depression of fibroblastic proliferation. Grillo concluded:

> The present experiments did not deny the possibility that some cell arriving from the vascular system either had or could develop the ability to function as fibroblasts, and might contribute to fibroplasia after injury, but rather indicated that the major source of fibroblasts in repair was from pre-existent, local, previously resting cells.

Working with transected tendon Dodd, Sigel and Dunn (87)

found evidence that fibroblasts in tendon regeneration were derived from perivascular sheaths surrounding the blood vessels at the wound site. These data are similar to the findings of Hadfield (75) who studied the healing process in rat dermis. Dodd's conclusions that fibroblasts originate from within the healing tendon is in complete opposition to the results reported by the later work of Greenlee (78) who found the macrophage as the source of newly forming fibroblasts in the injured tendon.

Observations by Glücksmann (88) in studies using colchicine to arrest cell mitosis, supported the notion that the repair of dermal tissue losses was largely due to the activity of perivascular fibroblasts.

Ross et al. (58) studied the origin of the wound fibroblast in parabiotic animals. Inbred rats were joined in pairs via a flap of skin in which later cross-circulation was established. Following a two week healing process, both animals of each pair were given 800 r of irradiation from a cobalt - 60 source. The femurs of one of the animals were shielded during the irradiation procedure so that their marrows could serve as hematopoietic tissue for both animals. Three days after the irradiation, both animals of each pair were wounded by making a series of linear incisions in the dorsal skin. An intraperitoneal injection of ^{3}H-Tdr was given daily to the animal in each pair that had active bone marrow, while the other animal mate was

given a large dose of unlabeled Tdr. At the time of Tdr injection cross-circulation in the tissue flap between animals was arrested with the use of a rubber clamp, and 20 minutes later the clamp was removed. Animals were killed and wound biopsies were prepared for autoradiography 1, 2, and 6 days after wounding. Tritiated Tdr was observed in epidermis, endothelium, leucocytes, fibroblasts, and mast cells in the wounds of the shielded animal. In the wounds of the nonshielded parabiont, only PMN leucocytes, monocytes, and lymphocytes were labeled. No labeled fibroblasts were seen in the wounds of the nonshielded parabionts through the 6-day period studied. The authors concluded that their

> ...observations provide further evidence that wound fibroblasts do not arise from hematogenous precursors and, therefore, must arise from adjacent connective tissue cells.

It is apparent from the foregoing literature survey that the origin of the fibroblast in the healing wound is still a matter of debate. The varied experimental approaches that have been reviewed have provided many pertinent observations; yet, they have not furnished a definitive answer to the origin of the newly formed fibroblast in connective tissue repair. The statement by Grillo (86) seems to summarize the status of the controversy concerning the inception of newly proliferated fibroblasts:

> After a century of experimentation and discussion, the origins of fibroblasts in wound repair has remained unclear.

Statement of the Thesis Problem

Weimar (17) recognized and utilized the fact that cells can be easily observed migrating through the corneal stroma following a centrally placed corneal wound. The author cited the work of Stearns (89) who reported that the fastest rate of migration observed for fibroblasts *in vivo* was 0.29 mm per day. At this rate of migration it would require approximately 10 days for fibroblasts to traverse the distance between the limbus and the center of the adult rat cornea, a distance of approximately 3 mm. This evidence would suggest that the perivascular connective tissue cells at the edge of the cornea or limbus are not contributing fibroblasts in response to a centrally placed corneal wound. Weimar therefore concluded that the fibroblasts found in central corneal wounds must arise from cells already present in the cornea or from cells which migrate at a more rapid rate and transform into fibroblasts. An example of a cell capable of this rapid migration is the monocyte. Studies (90, 91) have indicated that the monocyte has the capacity to migrate in tissue at a rate of 0.35 mm per hour and could reach the center of the cornea in eight to nine hours. Investigations of the morphology of corneal wound healing by Weimar has shown that most of the corneal stromal cells originally present at the wound edge were transformed into fibroblasts during the first 24 postoperative hours. However, by

inspection alone as the healing progressed, Weimar found it difficult to account for the increased numbers of fibroblasts at the wound edge based solely on the original number of stromal cells present at the time of injury. A study was then undertaken by Weimar (15) to determine quantitatively to what extent mitosis and/or transformation of the stromal cells contributed to the formation of fibroblasts during the corneal wound healing process. Colchicine was employed to aid in determining the degree of mitosis at the wound site. Counts of the cell types present at the wound edge through all depths of the corneal stroma were carried out on whole mounts. A 2 mm knife wound was made as near the center of the cornea as possible. Wounds were then analyzed at different time intervals following the injury. Cell counts from the colchicine treated animals indicated that no detectable cell division occurs during the first 24 postoperative hours. During this period no cells resembling fibroblasts could be seen migrating between the limbus and the wound. Cell division was not seen until the 30th hour after wounding. However, it was calculated that only 12.3 percent of the fibroblasts present at the wound edge 60 hours after injury could have been derived by cell division from transformed corneal stromal cells originally present at the wound edge. Monocytes were seen in these studies at various stages of transformation from ordinary monocytes to forms which could be differentiated from fibroblasts only with difficulty, beginning with the 12th

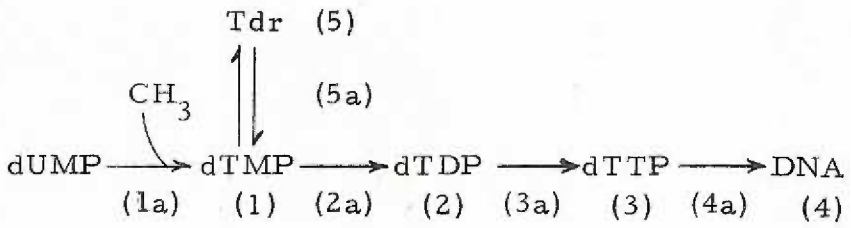
postoperative hour. From the rate of appearance of these transforming monocytes at the wound edge and the rate of their maturation, Weimar (15) estimated that these cells accounted for about two-thirds of the fibroblasts found at the wound edge at 60 hours. Although the studies by Weimar have elegantly documented the transformation of the monocyte to a fibroblast in corneal wound repair, this evidence has not been accepted by some investigators. Limitations in methodology available at the time of the work of Weimar largely precluded the possibility of definitively determining other than by morphological studies, whether or not a fibroblast can be derived from a circulating white blood cell. With the relatively recent introduction of radioisotope tracer techniques a new and valuable tool became available to verify and extend the experimental work of Weimar.

If the assumption is made that blood leucocytes can transform into new stromal connective tissue cells during wound healing, then it should be possible to label the native stromal cell population with one radioisotope and the infiltrating blood leucocytes with a second radioisotope and later differentiate the origin of the new stromal cells based on their specific radioisotopic labeling. With the proper application of this double radioisotopic tracer technique, it is anticipated that results will reveal the role the blood mononuclear cells, and the monocyte in particular, play in the formation of new fibroblasts following injury to the cornea.

Thymidine as a Radioactive Tracer Molecule

The DNA base Tdr was selected as the tracer molecule for this double isotope procedure and was labeled with carbon 14 or tritium. The unique physical and chemical properties possessed by thymidine are responsible for the experimental approach selected in this study to determine the origin of corneal wound fibroblasts. Since an understanding of these properties of thymidine are essential for the proper evaluation of subsequent experimental data, a brief discussion of thymidine is included here.

Thymidine does not occur naturally in the main intracellular pathways that lead to DNA synthesis, but is introduced into them by a single phosphorylation step to thymidine monophosphate (TMP). Although Tdr is incorporated rapidly into DNA in most organisms, it is essential in only a few organisms which cannot make their own TMP (92). Even within the same mammalian family there are species that lack the enzyme Tdr Kinase which is necessary for direct utilization of thymidine for DNA synthesis. For example in rodents, the woodchuck, the ground squirrel, and the chipmunk do not utilize injected ^{3}H-Tdr, whereas the mouse, rat, and hamster incorporate ^{3}H-Tdr readily (93).



1. Deoxyuridine-monophosphate
1a. Thymidylate synthetase
2. Deoxythymidine-monophosphate
2a. Thymidine monophosphate kinase
3. Deoxythymidine-diphosphate
3a. Thymidine diphosphate kinase
4. Deoxythymidine-triphosphate
4a. DNA polymerase
5. Thymidine
5a. Thymidine kinase

In addition to the Tdr substrate, the Tdr Kinase reaction has an absolute requirement for ATP and magnesium ions in mM concentrations. The ATP apparently acts as an activator as well as a phosphate donor.

The existence of Tdr Kinase has been an enigma, because it is not normally found in the pathways that lead to DNA sythesis. The most probably explanation for the existence of Tdr kinase is that the enzyme functions in a DNA reutilization cycle. For example, it has been determined that within the bone marrow about 35 percent of the thymine bases in the DNA of the erythroid precursors are used for local DNA synthesis after the nuclei are extruded at the orthochromatic stage (94). The presence of Tdr kinase appears to enable cells in the body to synthesize DNA more economically by salvaging extruded DNA from dying or necrotic cells (95).

Since Tdr is the nucleoside of thymine and deoxyribose and is incorporated exclusively into DNA, Tdr in its radioisotopically labeled form is the molecule of choice in studying the movement and maturation of different cell populations by autoradiography. The labeled DNA is not a water-soluble substance and is not removed by conventional fixation and embedding procedures, if the solvents used are in the neutral to alkaline pH range (92).

In animals an intravascular injection of ^{3}H-Tdr was removed from the circulating blood within minutes and generally no trace of the labeled compound could be found after 40 - 60 minutes (96). It has been demonstrated that ^{3}H-Tdr was detectable in mouse bone marrow DNA 15 seconds after labeling by intravenous injection (plus 90 additional seconds to isolate the marrow from the killed animals and to prepare the cytological specimens for autoradiography) (97). Thus the ^{3}H-Tdr passed through the blood stream, the vascular barrier, the cell wall, and the steps of phosphorylation, and became incorporated into DNA all within 15 seconds. The probability that an individual cell will incorporate ^{3}H-Tdr is dependent on at least four major factors: the distance from the cell to its blood supply; the rate of cellular replication; the availability of Tdr kinase; and the natural pool of TMP, TDP, and TTP (92).

Following a single injection of ^{3}H-Tdr into the anterior chamber of the eye the concentration of ^{3}H-Tdr falls very rapidly and only

about 10 percent of the original inoculum remains after 60 minutes (98).

When ^{3}H-Tdr is not incorporated into DNA following a in vivo pulse, or is not reutilized following the death of labeled cells, the ^{3}H-Tdr is catabolized. The major pathway for degradation is through thymine, dihydrothymine, β-ureidoisobutyric acid, and β-amino-isobutyric acid which is then excreted in the urine (99). DNA turnover and/or fibrocyte renewal is relatively low in the corneal stroma; therefore, once the fibrocyte DNA has incorporated labeled Tdr, the radioactive marker can persist for many months (100, 101).

Monocytes and Macrophages

Following an injury to the rat cornea an acute inflammatory reaction ensues. During this period of inflammation only two groups of non-corneal cells can be observed at the wound site prior to the appearance of the first fibroblast. These two groups of cells are the PMN leucocytes and the blood borne monocytes. As stated earlier in the thesis introduction there has never been any strong evidence to include the PMN leucocyte among those cells capable of undergoing metaplasia into fibroblasts (59). Thus, if the premise is true that a hematogenous leucocyte serves as a precursor for the fibroblast in the healing cornea, the monocyte must fill this role.

It perhaps would be helpful to have an exact definition of a

macrophage and a monocyte before embarking on a description of their structure and properties. Monocytes and macrophages are commonly referred to collectively as mononuclear phagocytic cells. They are found in a variety of mammalian tissues and fluids. Although these cells differ widely in their structural forms and have been assigned a variety of names they all exhibit certain physiological properties: adherence to glass, membrane ruffling, and ingestion of fluid droplets (pinocytosis) or of particles (phagocytosis) from the environment (102). Present data supports the concept that these mononuclear phagocytes arise from stem cells in the bone marrow in immature form, enter the blood as young adult forms (monocytes), and a short time later emigrate into tissues. The monocytes undergo further maturation in the tissues, which varies depending on the particular tissue environment, to become macrophages (103, 104, 105). Nelson (106) considered as typical mammalian macrophages those cells found free in the peritoneal fluid and alveoli; but includes as probably macrophages a number of "fixed" cells: Kupffer cells lining hepatic sinusoids; mononuclear phagocytes of the spleen, lymph nodes, and thymus; and the "resting wandering cells" or histiocytes of connective tissue.

Studies have been conducted for more than 50 years in an attempt to determine the origin of peripheral blood monocytes and tissue macrophages (107). Until the introduction of radioisotope

labeling techniques, investigators were restricted primarily to the use of vital dyes and carbon particles. These cell markers were not sufficiently specific and stable to permit identification of the phagocytic cells. The high concentration of vital dyes needed to stain the phagocytic cell may have led other cells to take up the dye by the process of pinocytosis, rather than by phagocytosis, and thus yielded unreliable results. The major drawback to the use of carbon particles as phagocytic cell markers was that they could be reingested after the death of the initially labeled cells.

Two conflicting theories did evolve from earlier literature to explain the origin of the macrophage in an inflammatory lesion. Proponents of the first theory stated that the macrophages were derived from lymphocytes (108). While advocates of the second theory argued that macrophages in the inflammatory exudate were derived from monocytes of the peripheral blood. This second view was not generally accepted even though Ebert and Florey (109) demonstrated as early as 1939 that macrophages in an inflammatory lesion were derived from monocytes of the peripheral blood. With the application of a stable label, ^{3}H-Tdr, it was possible to establish more conclusive data from investigations concerning the origin and kinetics of mononuclear phagocytes.

Volkman and Gowans (104) used the subcutaneous coverslip technique in paired parabiotic rats to examine the cellular response

in a non-immunological inflammatory reaction. Leucocytes of one member of a parabiotic pair, the donor, were labeled with ^{3}H-Tdr while cross circulation of the label to the recipient was prevented. Subcutaneous coverslips were surgically inserted into the donor and recipient 24 hours after the ^{3}H-Tdr injection, and the coverslips were removed 24 hours later. Autoradiographs prepared from the coverslips showed

> ...that the coverslips from both donor rats had a normal proportion of labeled macrophages and it is clear that the labeled macrophages were also present on the recipients' coverslips must have been derived from the donor rats because autoradiographs of intestinal epithelium show that no ^{3}H-Tdr could have leaked from donor to recipient.

The authors demonstrated that monocytes circulating in the blood and subsequently found attached to the coverslip were derived from a relatively small, rapidly dividing pool of precursors. Volkman and Gowans (103) further determined that these cells were derived from bone marrow, rather than lymphoid tissues, by injecting ^{3}H-Tdr labeled bone marrow cells or lymphoid cells and observing that only the marrow cells could be seen to appear as macrophages in touch prints of damaged skin. Similar results were obtained when lymphoid tissues were X-irradiated while the bone marrow was shielded during the procedure. The destruction of the lymphoid tissues by the X-irradiation did not prevent the appearance of macrophages at the wound site. In those animals where the bone marrow was not shielded during

the irradiation procedure, macrophages were not observed subsequent to tissue injury. These important investigations by Volkman and Gowans demonstrated that the principle mononuclear phagocytic cell in the healing wound is not of lymphoid tissue origin, but rather derived from the bone marrow and arrives via the blood. The participation of the resident tissue cells (histiocytes) appears to be minimal in these studies.

Van Furth (107) has discussed the differences in the life history phases of the PMN leucocytes and mononuclear phagocytes. Radioisotope studies have shown that almost immediately following a single injection of ^{3}H-Tdr, labeled monocytes appear in the peripheral blood, indicating a small maturation pool. This short stay in the bone marrow for the maturating monocytes is in contrast to the granulocytes which do not appear in the peripheral blood as labeled cells for some days. Van Furth noted that experimental data has demonstrated the average transit time in the circulation of 32 hours for the monocytes which is much longer than for PMN leucocytes. The total lifespan of the mononuclear phagocytes, upon reaching the tissues, may be in the order of months (108), while the granulocytes remain in the tissues for only a few days.

Fedorko and Hirsch (102) have reviewed the structural properties of the monocytes and macrophages. The authors described the structural features of bone marrow promonocytes, of blood

monocytes and of normal and stimulated macrophages as seen in various tissue and in vitro environments. A suspension of bone marrow cells was allowed to settle on glass. During the first two hours monocytes and granulocytes and their precursors were found adherent to the glass. At six hours the preparations contained predominately monocytic cells. The in vitro conditions are not suitable for granulocyte survival for more than a few hours. Light and electron microscopic studies on 6-hour old cultures revealed a cell population containing a spectrum of cells of varying degrees of maturity. An occasional blast cell was seen with a large nucleus, little evidence of nuclear indentation, poorly developed rough endoplasmic reticulum, prominent nucleoli, and loosely dispersed chromatin granules. Numerous mitochondria were present and a few vesicles of pinocytic origin could be demonstrated in electron micrographs. More mature cells referred to as promonocytes were seen. These cells were somewhat larger than the blast cells and had elongated nuclei. The cytoplasm contained many aggregated ribosomes and a few strips of endoplasmic reticulum. The Golgi zone had become well developed. Mature monocytes were in abundance in these preparations and the author found these cells identical to the peripheral blood monocytic structure. Mouse blood monocytes that had been attached to glass appear in electron micorscopy to have elongated nuclei with peripherally distributed clumped chromatin. They contain numerous small

circular or rod-shaped mitochondria and a more extensive network of rough endoplasmic reticulum than the promonocytes. The cytoplasm contains a few dense granules of varying size and occasional large pinocytic vacuoles are present near the prominent Golgi zone.

The mouse peritoneal cell is considered a representative of a tissue macrophage. Ultrastructural studies on mouse peritoneal cavity cells show a variety of maturing cellular stages ranging from small cells resembling blood monocytes through relatively large cells with extensive and complex cytoplasm. These large cells are usually characterized by an elongated nucleus, sparsely aggregated ribosomes, and the presence of strips of partially rough and smooth endoplasmic reticulum located at the periphery of the cell or arranged in stacks. Numerous granules are found at the periphery of the Golgi zone of these mature cells. These granules have been identified biochemically as containing acid phosphatase and other specific hydrolytic enzymes which are characteristic of lysosomes (111).

MATERIALS AND METHODS

Experimental Protocol

Experimental data from these studies were derived from two experiments that were run consecutively. Rats were used exclusively in these studies. In the first experiment the rats were divided into four groups. The first group of rats had their corneal stromal cells labeled with ^{14}C-Tdr and their mononuclear blood cells labeled with ^{3}H-Tdr followed by a standardized freeze injury to their corneas. The second group of rats had only their corneal stromal cells labeled with ^{14}C-Tdr and one half of these animals had their corneas frozen. The third group of rats had their mononuclear blood cells labeled with ^{3}H-Tdr and one half of these animals had their corneas frozen. The fourth group of rats were maintained as controls.

Corneal stromal cells were labeled by curette scraping of the epithelium followed by two ^{14}C-Tdr injections into the anterior chamber, and two weeks later the mononuclear blood cells were labeled by two intravenous injections of ^{3}H-Tdr via the lingual vein. Corneal freeze injuries were administered by a temperature-controlled cryostatic probe. Upon completion of the labeling and freezing procedures the corneas were allowed to undergo complete wound repair for a period of three weeks. The rats were killed and their eyes were

enucleated and placed in tissue fixative. The corneas were then embedded in epoxy resin and 1 μ sections were made for double emulsion autoradiographic analysis. The second experiment was similar to the first experiment with the exception that fewer animals were used and a change was made in the amounts of radioisotope administered. Tables 1 and 2 schematically illustrate how the two experiments were conducted. The number of animals listed for the various groups in tables 1 and 2 represent those animals used in the first experiment. In the second experiment groups I, II, III and IV contained 5, 4, 4, and 2 animals respectively.

Animals

Sprague-Dawley rats of either sex were used for all experiments. At the start of the experiments the animals were approximately six weeks old and weighed 140 - 160 g. The animals were fed a standard diet and water ad libitum.

Animals were marked as to experimental group and individual membership within each group, by placing various combinations of colored dye bands on their tails. This marking procedure at first glance may not appear worthy of comment; however, the more conventional marking procedures such as ear punching and toe cutting do inflict a competing wound that could alter experimental results.

TABLE-1

Time	GROUP-I 14C-Tdr + 3H-Tdr 6 animals	GROUP-II 14C-Tdr only 12 animals	GROUP-III 3H-Tdr only 12 animals
Day 0	Corneal epithelium removed	Same procedure as in GROUP-I	Same procedure as in GROUP-I
Day 0 +12 hrs.	Animals received anterior chamber injection of 14C-Tdr	Same procedure as in GROUP-I	Sham procedure
Day 1	Animals received second anterior chamber injection of 14C-Tdr	Same procedure as in GROUP-I	Sham procedure
	Eyes were allowed to undergo repair		
Day 14	Animals received 1 uc of 3H-Tdr/gram of body wt. via the lingual vein	Same procedure as in Group-I	Sham procedure
Day 14 + 10 hrs.	Animals received second injection of 1 uc of 3H-Tdr/gram of body wt. via the lingual vein	Sham procedure	Same procedure as in GROUP-I
		GROUP-II-A 6 animals / GROUP-II-B 6 animals	GROUP-III-A 6 animals / GROUP-III-B 6 animals
Day 14 +18 hrs.	Center of cornea was frozen with cryostatic probe set at -20°C.	Center of the cornea was frozen as in Group-I / Cornea was not frozen	Center of the cornea was frozen as in Group-I / Cornea was not frozen
	Eyes were allowed to undergo repair		
Day 36	All animals were killed and their corneas were processed for autoradiography		

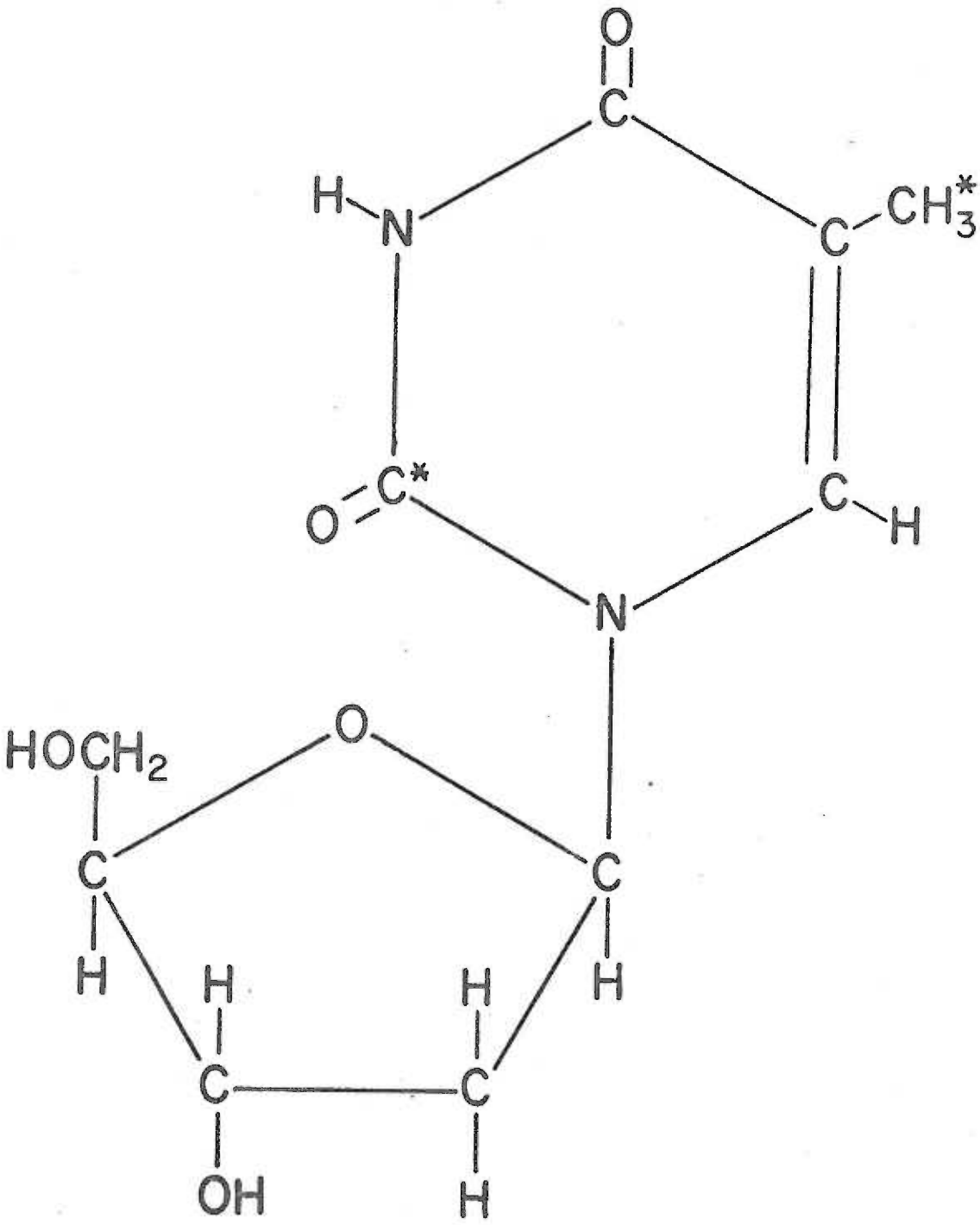
TABLE-2

Time	GROUP-IV No radioactive isotopes given 12 animals	6 animals	GROUP-V Controls 6 animals
Day 0	Corneal epithelium removed		
Day 0 +12 hrs.	Sham procedure		
Day 1	Sham procedure		
	Eyes were allowed to undergo repair		
Day 14	Sham procedure		
Day 14 +10 hrs.	Sham procedure		
	GROUP-IV-A 6 animals	GROUP-IV-B 6 animals	GROUP-IV-C
Day 14 +18 hrs.	Center of cornea was frozen as in GROUP-1	Cornea was not frozen	Center of cornea was frozen as in GROUP-1
Day 36	—Eyes were allowed to undergo repair		
	All animals were killed and their cornea's processed for autoradiography		

Radioactive Isotopes

Thymidine-2-^{14}C and thymidine-methyl-^{3}H were the two radioactive molecules used in these studies. Figure 3 graphically illustrates the position of the two radioactive atoms in the pyrimidine ring of the molecule. The labeled compounds were purchased from New England Nuclear Corporation of Boston, Mass., and were packaged in sterile aqueous solutions in multidose vials which were stored at 5°C. Radiochemical purity was determined by the supplier to be greater than 98.5 percent following paper chromatographic analysis and ultra-violet spectrophotometry. Reported contaminates in the ^{14}C-Tdr samples were thymine-2-^{14}C, less than 0.5 percent, and thymine-2-^{14}C riboside less than 0.1 percent, while the contaminates found in the ^{3}H-Tdr sample were thymine-methyl-^{3}H, less than 0.3 percent, and thymine-methyl-^{3}H-riboside, less than 0.1 percent. Further radiochemical purity checks were performed on samples taken from the ^{3}H-Tdr and ^{14}C-Tdr vials following delivery, to be certain that they did contain the proper compounds. These analyses were determined by two dimensional paper chromatography using n-butanol:acetic acid:water (4:1:5; v:v:v) as the solvent system. Areas on the chromatography paper visible under ultra-violet were cut out and were analyzed for ^{14}C and ^{3}H activity by liquid scintillation counting. Results indicated that the samples were ^{14}C-Tdr and

Figure 3. Thymidine molecule showing locations (*) of labeling positions in specifically labeled Tdr (2-^{14}C, 5-methyl-^{3}H).



THYMIDINE

^{3}H-Tdr. The specific activities of the ^{3}H-Tdr and ^{14}C-Tdr used in the first experiment were 2.0 curies/millimole and 5.38 millicuries/millimole respectively. In the second experiment the specific activity of the ^{3}H-Tdr was increased to 18.9 curies/millimole while the specific activity of the ^{14}C-Tdr was not changed.

Methoxyflurane

All surgical procedures in these studies were performed with the aid of the inhalation anesthetic methoxyflurane. Methoxyflurane is marketed under the trade name Penthrane by Abbott Laboratories, North Chicago, Illinois. Successful surgical procedures on the rat eye proved to be extremely difficult with the two commonly employed small animal anesthetics, ethyl ether and pentobarbital sodium. These agents have distinct disadvantages when compared with the newer anesthetic agent, methoxyflurane (112).

Ethyl ether is a highly volatile explosive inhalation anesthetic and as a tissue irritant it may interfere with respiration because of increased flow of saliva and mucus. To maintain the desired level of anesthesia for prolonged surgical procedures the strict attention of the operator is required to prevent respiratory depression.

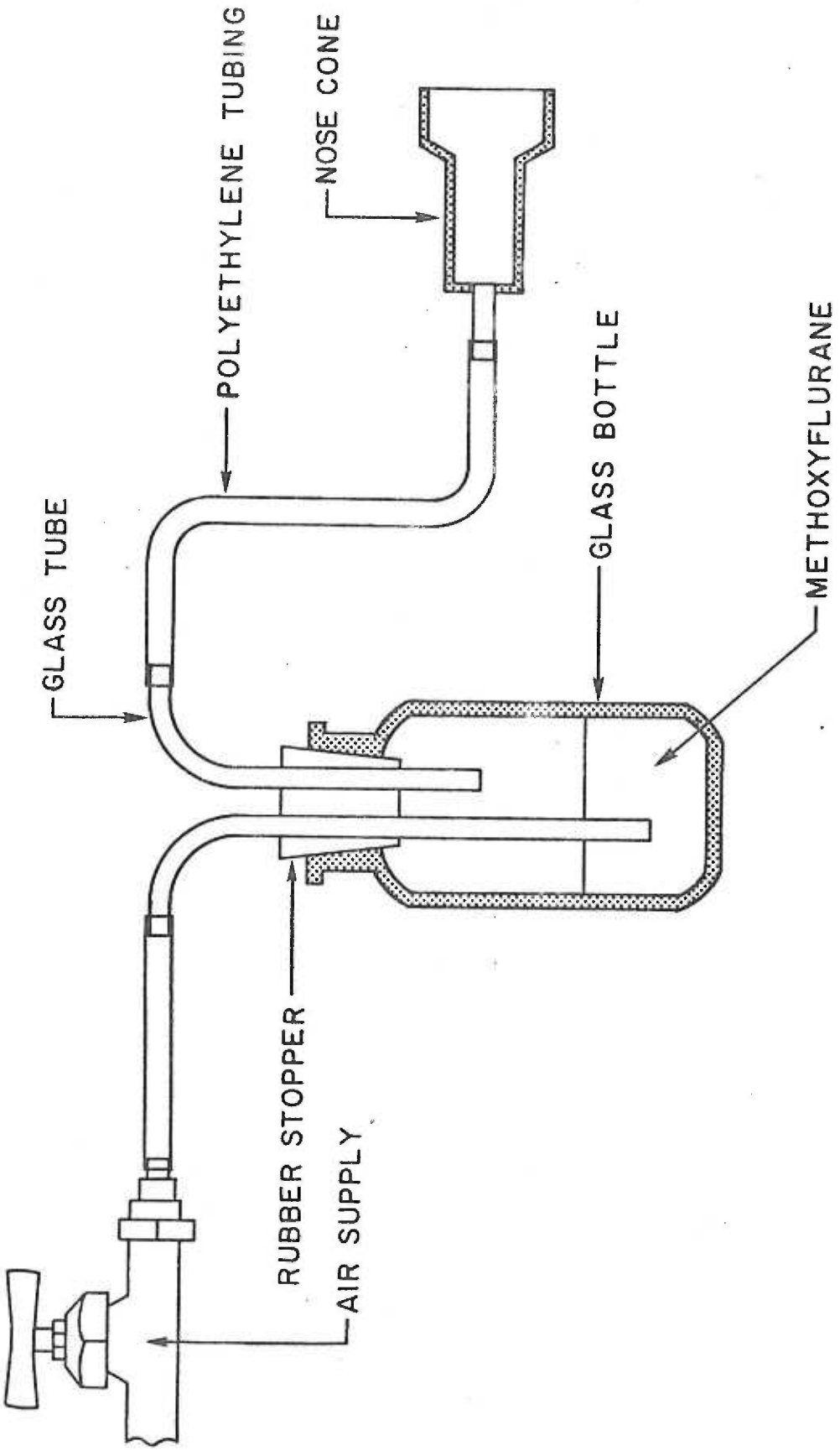
The disadvantages of the injectable agent pentobarbital sodium are the wide variation in dose response, low therapeutic index, and the irreversibility of an overdoseage once administered. Complete

recovery from surgical anesthesia often takes hours and it is extremely hazardous to return the animal to the anesthetic state during this period. The mortality with this anesthetic in mice averages 20 percent (113) and has approached this figure with rats used in preliminary work in these studies.

Methoxyflurane (2,2-dichloro-1,1-difluroethylmethyl ether) is a liquid, nonflammable inhalation anesthetic with a pleasant fruity odor, used primarily in human surgery. At relatively light planes of anesthesia methoxyflurane provides substantial muscular relaxation (114). Although Hagan and Hagen (110) have reported the satisfactory use of methoxyflurane as an anesthetic agent in mice, it has not received wide acceptance in experimental animal surgery.

Anaesthesia was produced by placing the rat in a small bell jar containing a pledget of methoxyflurane-soaked gauze. When the animal became unconscious, it was removed from the bell jar and transferred to a simple inhalation apparatus in order to maintain anesthesia (figure 4). Air was bubbled through methoxyflurane contained in a small bottle. The air-methoxyflurane mixture was administered via tubing to a plastic nose cone. Depth of anesthesia was assessed by response to pain, character of respiration, and absence of corneal reflexes. If anesthesia became too deep the air flow was decreased or the nose cone temporarily removed. Duration of the recovery period was directly related to length of anesthetic exposure, but has

Figure 4. Shown is the inhalation apparatus used to maintain methoxyflurane anesthesia during experimental surgical procedures. Air was bubbled through the anesthetic agent and the air-anesthetic mixture was then administered to the animal via tubing to a plastic nose cone.


POLYETHYLENE TUBING
NOSE CONE
GLASS TUBE
GLASS BOTTLE
METHOXYFLURANE
RUBBER STOPPER
AIR SUPPLY

④

been no greater than 15 minutes with the surgical procedures which lasted 20 - 30 minutes in these studies. Methoxyflurane anesthesia was employed for over 300 surgical procedures without one anesthetic death, even when some animals were anesthetized six times. The primary advantage of methoxyflurane is that this agent rarely produces irreversible respiratory failure or circulatory collapse. However, prolonged exposure is reported to cause respiratory acidosis (115). The use of methoxyflurane during surgical procedures on the cornea, in contrast to pentobarbital sodium, eliminated the need for a local topical anesthetic such as benoxinate hydrochloride (Dorsacaine). Since local topical anesthetics may retard the normal physiological response to tissue injury, the elimination of their use would appear to be advantageous.

Labeling of Corneal Stromal Cells

Weimar (116) noted that severe injury produced by crushing the corneal epithelial cells, leads to activation within one hour of the underlying stromal cells to transport the vital dye neutral red. It was also observed that following this crushing injury to the epithelium the growth of epithelium over the denuded stroma caused a pronounced transformation of corneal stromal cells into fibroblastic cells throughout the cornea (40).

Preliminary experiments were conducted to determine whether

the activated stromal cells would incorporate radioactive Tdr. The procedure was successful and resulted in a high percentage of labeled stromal cells. Why these cells so readily incorporate thymidine is not fully understood by this writer. The procedure appears to kill a few stromal cells immediately beneath the epithelium and only an occasional mitotic figure was seen in the stroma 24 hours postoperatively. Perhaps these activated stromal cells incorporate ^{3}H-Tdr as they undergo DNA repair following injury.

Corneal labeling was accomplished by anesthetizing the rat and then conducting the surgical procedure under a binocular dissecting microscope (at approximately 15X) while maintaining the level of surgical anesthesia with methoxyflurane (figure 5). The eye that was to be labeled was grasped by the sclera with the aid of curved iris tissue forceps and held firmly while the entire cornea was scraped to remove the epithelium.

The scraping was performed with a stainless steel 2.5 mm chalazion curette, while care was taken not to penetrate the stroma proper with the curette. The animal was then returned to its cage and allowed to regain consciousness. Twleve hours later this procedure was repeated on the same animal through the step of grasping the sclera with the tissue forceps. At this point a small incision was made at the extreme periphery of the cornea with a Swan needle knive (117). The knife was inserted into, but not through, the cornea

Figure 5. The surgical procedure in which the corneal stromal cells were labeled by injection of the isotope into the anterior chamber is shown. The rat was positioned under a binocular dissecting microscope for injection procedure while maintaining the level of surgical anesthesia with methoxyflurane.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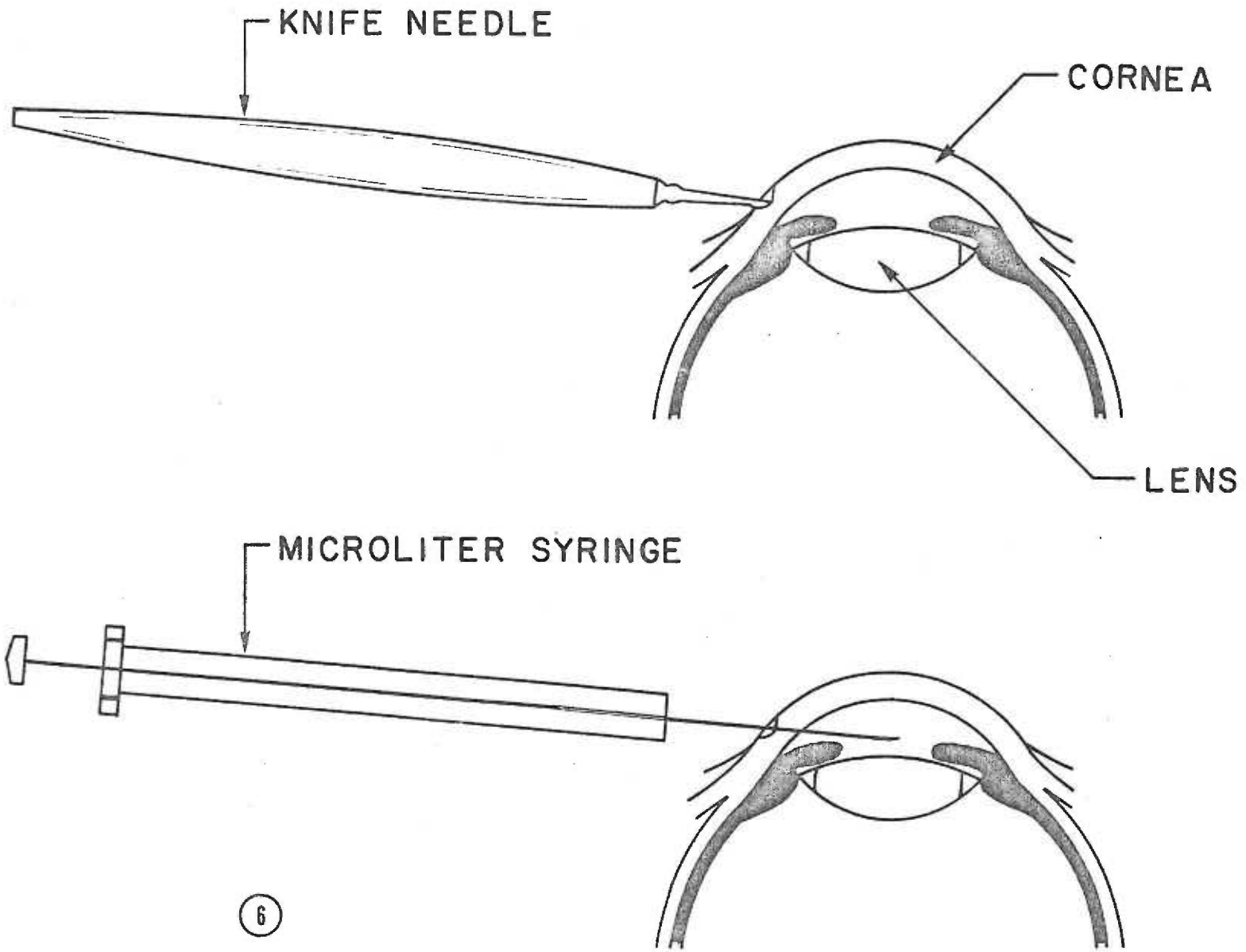
(figure 6). In the next step the needle tip of a microliter syringe was placed into the previously made incision and the needle was pushed through the cornea into the center of the anterior chamber, as illustrated in figure 6. The microliter syringe used was purchased from Hamilton Company, Inc., Whittier, California, and had a total capacity of 10 μl with the syringe barrel marked in 0.1 μl divisions. The incision was made to reduce resistance to the needle penetration and to lessen the possibility of a hyphema. A 5 μl solution containing 5 μC of ^{14}C-Tdr was slowly injected into the anterior chamber. Because of a slight loss of aqueous humor when the syringe needle was withdrawn, the needle was held in place for two minutes following the injection, thus allowing thorough mixing of the ^{14}C-Tdr and aqueous humor. In the second experiment the dosage of ^{14}C-Tdr was reduced to 1.3 μC/injection. The labeling procedure was repeated 12 hours after the animals received their first anterior chamber injection of ^{14}C-Tdr. Both eyes of each animal in Group I and Group II were labeled.

Intravenous Labeling of Peripheral Mononuclear Blood Cells

Following a modification of the method of Gillman and Wright (63, 118) ^{3}H-Tdr was injected intravenously to label developing blood mononuclear cells at the bone marrow level. This procedure was performed two weeks after the animals in Group I received their last

Figure 6. The surgical procedure for labeling the corneal stromal cells is graphically illustrated in this figure. A small incision was made at the extreme periphery of the cornea with a Swan needle knife followed by the injection of labeled Tdr with the aid of a microliter syringe through the incision. Because of a slight loss of aqueous humor when the syringe needle was withdrawn, the needle was held in place for two minutes following the injection, thus allowing thorough mixing of the Tdr and aqueous humor.



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anterior chamber injection of ^{14}C-Tdr, and the animals of Group III underwent a sham anterior chamber injection.

Individual animals were anesthetized and then placed in the supine position on a surgical retaining board. The tongue of the rat was drawn out to one side of the incisor teeth by grasping the tip of the tongue with a curved mosquito hemostatic forceps. The animals lower lip was retracted with hemostatic forceps to allow better visibility of the injection site. Traction was applied so that the pair of veins on the under surface were exposed near the root of the tongue. Methoxyflurane anesthesia was maintained by inserting the rat's upper jaw and nostrils into the anesthetic nose cone. Using a 1 ml tuberculin syringe with a sharp 27 gauge needle and viewing the injection site under a binocular dissecting microscope, the operator inserted the needle into one of the small lingual veins and approximately 0.2 ml of physiological saline containing 1 μC of ^{3}H-Tdr/g. of body weight was slowly injected into the vein. The distinct advantage of this injection method was that the point of the needle and the entering injection fluid could be seen readily through the thin wall of the vein (119). For this reason the lingual veins was selected as the route of injection as opposed to the lateral tail vein where it is difficult to assess whether the needle has reached the lumen of the vein. Pressure was applied over the puncture with a cotton tipped applicator as the needle was withdrawn to prevent the formation of a perivenous hematoma.

The ^{3}H-Tdr labeling procedure was repeated 10 hours later.

Standardized Corneal Freeze Injury

Freezing was selected as the method to inflict the experimental corneal wound for two principle reasons: 1. A standard area of injury can be achieved. 2. By adjusting the duration and temperature of the freeze the degree of tissue damage can be controlled. In a series of preliminary experiments it was determined that a corneal freeze of 6 sec. duration at -20°C. would kill virtually all stromal cells in the area of the freeze without severely disrupting the normal stromal lamellar architecture. A precise time-temperature regime was found necessary to achieve the desired level of tissue damage with any reproducibility. Corneal freezes of 6 sec. duration conducted at a temperature slightly below -20°C. produced vascularization in a number of the wounds. On the other hand if the freeze was conducted a few degrees above -20°C, not all stromal cells were destroyed. Since both corneal vascularization and the retention of viable stromal cells following the freezing procedure were deemed undesirable in these studies, accurate and reliable cryogenic equipment was necessary.

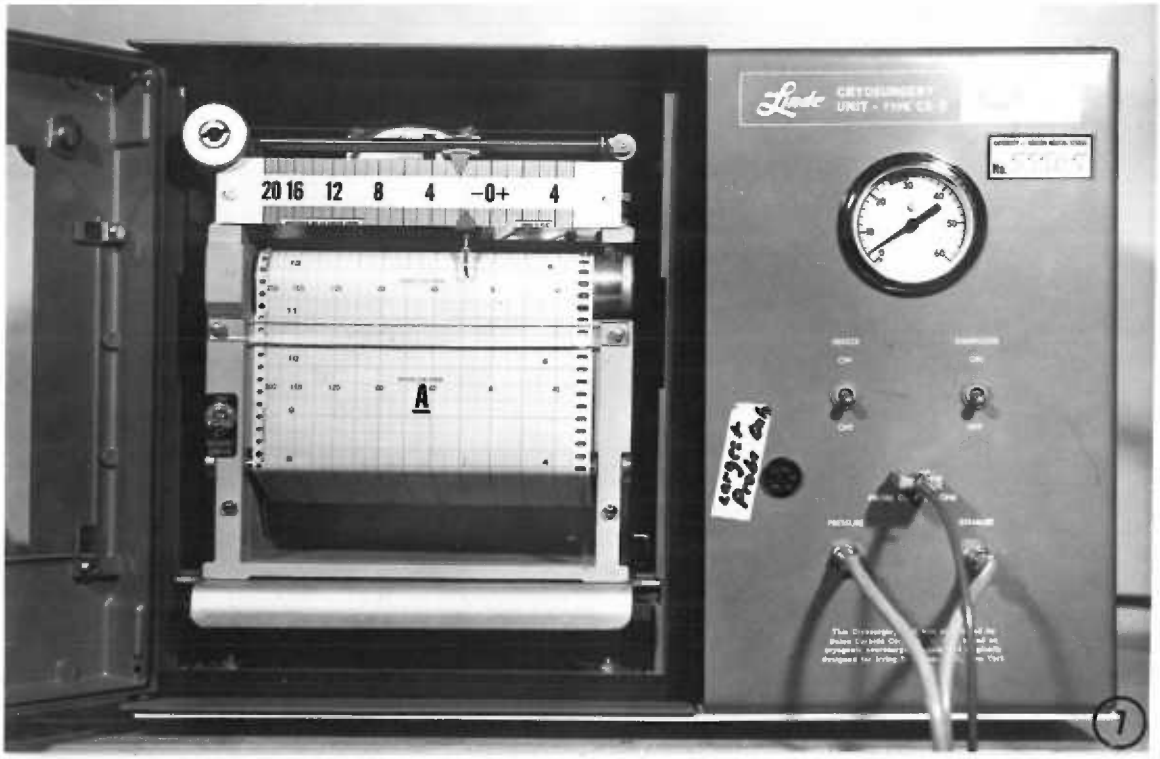
The Linde CE-3 Cryosurgery Unit, manufactured by the Union Carbide Corporation (Linde Division, Cryogenic Product Department, 2770 Leonis Blvd., Los Angeles, Calif.) fulfilled these criteria.

This cryosurgery unit (figure 7) provides precise control of temperature between 37° and -160°C., by utilizing liquid nitrogen as the cooling agent. The refrigerant is stored in the handle of the cryoprobe used to administer the freeze (figure 8). The tip of the cryoprobe has a surface area of 3.24 mm^2 and incorporates an electric heater and thermocouple. The probe is filled with liquid nitrogen which is absorbed in a porous material in an inner container. The thermocouple and heater are attached to the control console by an electric connector. Pressure and exhaust hose are connected between the probe and the console. The unit is equipped with a potentiometric probe temperature indicator which is coupled to a strip chart recorder which provides a permanent record of the duration and temperature of each freeze. The unit is designed to allow the operator to preselect the probe tip temperature in accordance with the surgical objective. Temperature is regulated by controlling the rate of flow of cold gas through the probe tip. An outside dry nitrogen gaseous source forces liquid nitrogen from the absorbing material in the probe to the probe tip on its way to the exhaust port. The operator depresses a foot switch for freezing and releases it for warming the tip to body temperature by the incorporated heater.

Those animals scheduled to have their corneas frozen (tables 1 and 2) were anesthetized and the freezing procedure was carried out by placing the probe tip on the center of the corneal surface with

Figure 7. Shown is the Linde CE-3 Cryosurgery Unit used to produce a standardized corneal freeze injury. This cryosurgery unit provides precision control of temperature between 37°C and -160°C by utilizing liquid nitrogen as the cooling agent. The unit is equipped with a potentiometric probe temperature indicator which is coupled to a strip chart recorder (A) which provides a permanent record of the duration and temperature of each freeze.

Figure 8. Shown is the hand-held cryoprobe that was used to administer the corneal freeze injury. The probe has a freezing tip (A) with a surface area of 3.24 mm^2 and incorporates an electric heater and thermocouple. The thermocouple and heater are attached to the control console by an electric connector (B). Dry gaseous nitrogen supplied via a pressure hose (C) forces liquid nitrogen from the probe handle (D) through the probe tip and out the exhaust hose (C).



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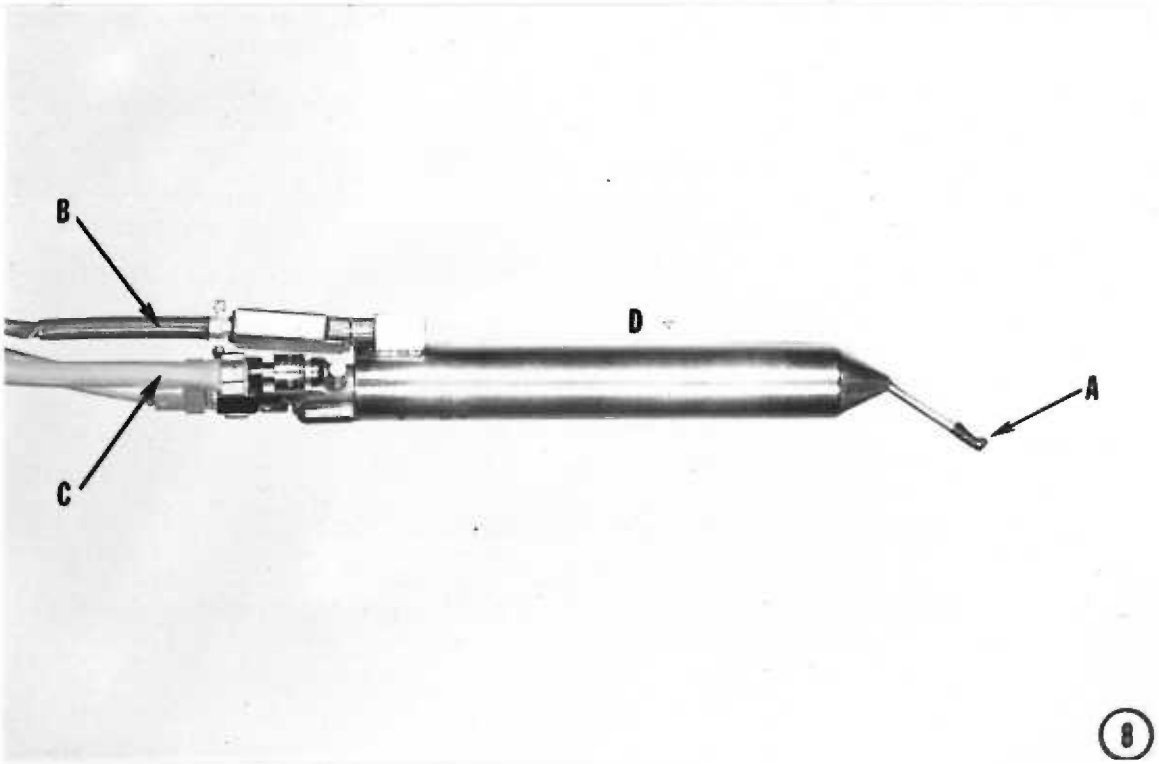

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light pressure as illustrated in figure 9. The foot switch was depressed and following a six second freeze at -20°C., the switch was released and the cornea was allowed to thaw before removing the probe.

Figure 10 illustrates a representative temperature curve recorded while freezing the right and left corneas of one animal. The corneal freezing procedure was performed upon the animals eight hours following their last intravenous injection of ^{3}H-Tdr.

Fixation and Embedding of Corneal Tissue

Three weeks following the freezing procedure all rats in the experiment were killed with ether and their eyes were immediately enucleated and fixed in formalin-alcohol solution (33) (2 parts 36.3 percent neutral formalin, 1 part 88 percent alcohol) for not less than three, and not more than six hours. The choice of the histological fixative was dictated by the necessity for maintaining the normal corneal curvature during the fixation process so that the corneas would be oriented for embedding. Glutaraldehyde proved to be an unsatisfactory fixative in these studies because the fixation process produced marked contortion of the corneas, and they could not be oriented properly for embedding. On the other hand, fixation with formalin-alcohol resulted in corneas that retained their symmetry when dissected from the eye globe, and were firm enough to allow handling

Figure 9. Shown is a graphic representation of the manner in which the cornea was frozen. The probe tip was placed centrally on the surface of the cornea and while maintaining light probe pressure, the injury was produced by a 6 second freeze at -20°C. The probe tip and the cornea were allowed to thaw before removing the probe.

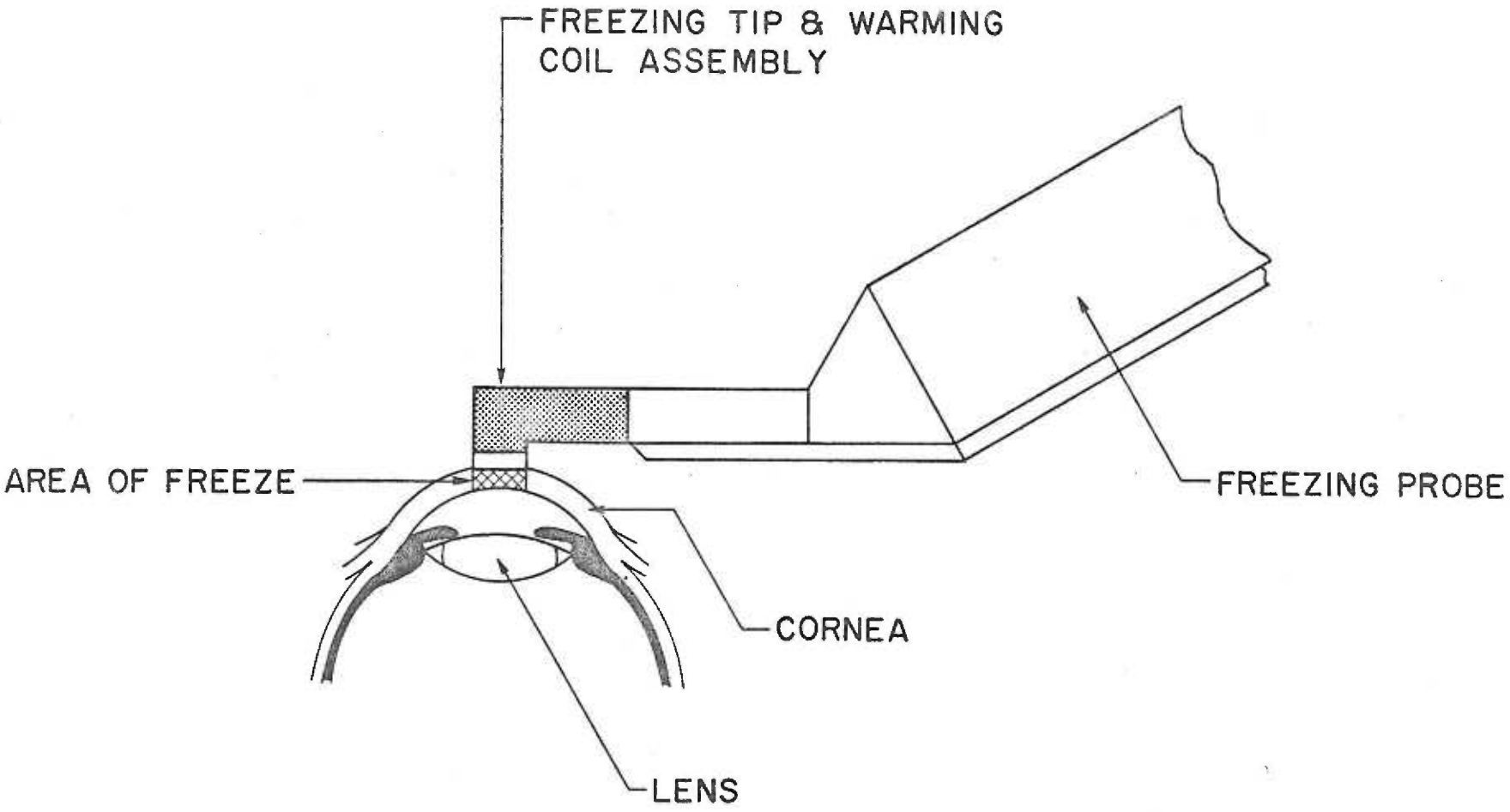
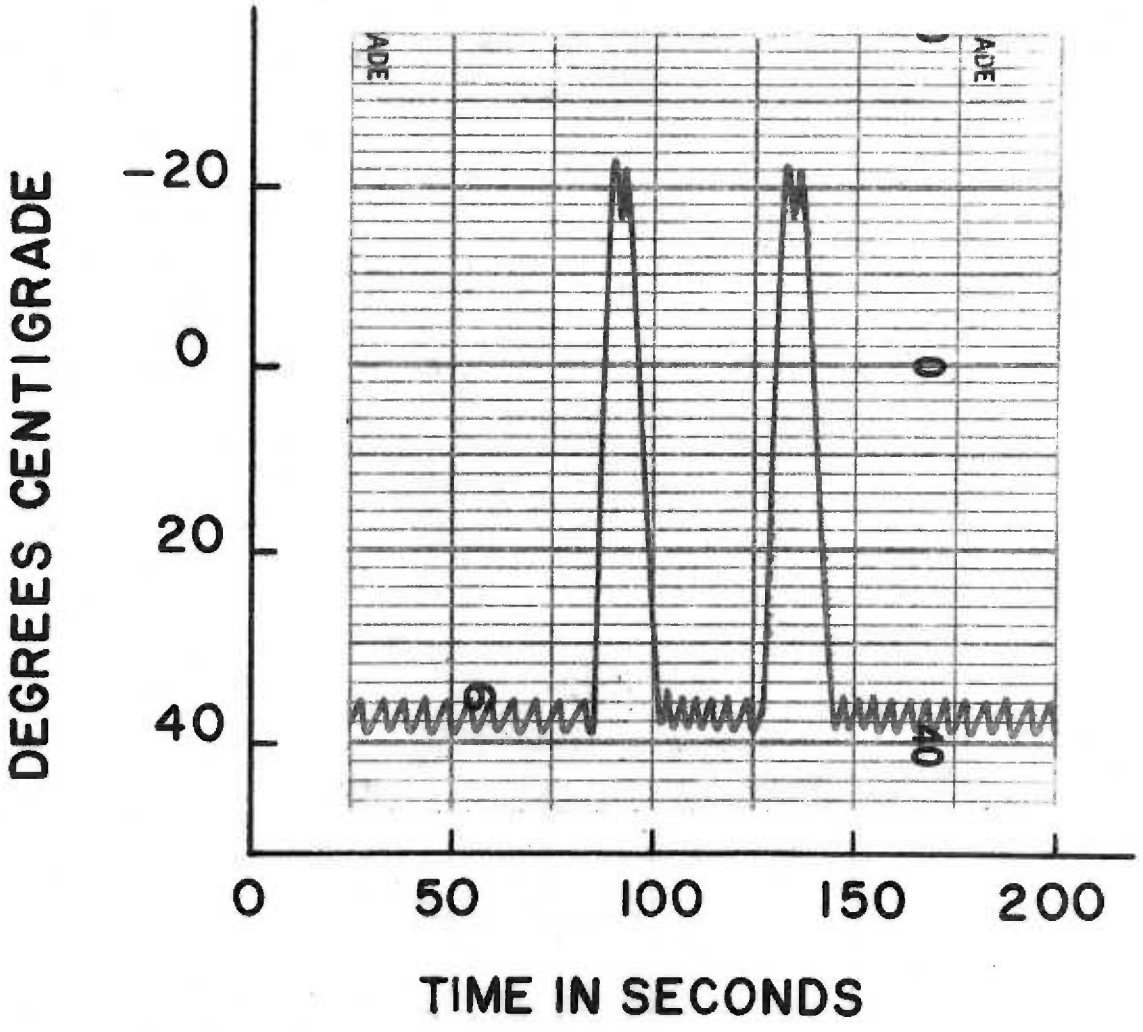

FREEZING TIP & WARMING
COIL ASSEMBLY
AREA OF FREEZE
FREEZING PROBE
CORNEA
LENS

Figure 10. A representative probe tip temperature curve recorded while freezing the right and left corneas of one animal is shown. The injury was produced by a 6 second freeze at -20°C.



(10)

during the embedding process. In addition, the formalin-alcohol fixative gave good cytological results. After the fixation period the entire cornea, with a 1 to 2 mm scleral flap, was dissected and placed in a stainless steel tissue cassette. The tissue was then washed over night in running tap water to remove excess fixative. This step is essential to prevent the formalin from later affecting the autoradiographic emulsion and giving rise to chemography (120). Since autoradiograms register only such label that is bound to molecules which are not removed from the specimen by the process of fixation, pilot experiments had to be conducted to be certain that the labeled DNA was not leached out by the formalin-alcohol fixative. Results from these experiments indicated that there was sufficient retention of the label for autoradiography following fixation in formalin-alcohol solution.

The rinsed tissue was then dehydrated in a graded series of ethyl alcohol as follows:

1.	50 percent ethyl alcohol	- 20 min.
2.	70 percent ethyl alcohol	- 15 min.
3.	95 percent ethyl alcohol	- 15 min.
4.	Absolute ethyl alcohol	- 15 min.
5.	Absolute ethyl alcohol	- 15 min.

Tissues were not removed from one bath to another, but rather the alcohol was poured out slowly from the vial allowing the tissue to remain behind and the next alcohol bath immediately added.

The epoxy resin infiltration procedure was initiated by

submerging the tissue in propylene oxide for 15 min. followed by a second 15 min. propylene oxide bath. The tissues were next placed in complete resin-propylene oxide (1: 1, v: v) where they were allowed to remain overnight. Complete resin is defined as a combination of resin mixtures A and B plus the accelerator. This embedding formulation will be discussed in detail later.

The resin was purchased from Ladd Research Industries, Inc., Burlington, Vermont and is marketed as Epon 812. The amount of epoxy resin can vary from batch to batch of Epon 812. Therefore, the manufacturer analyzes each batch of Epon 812 for its epoxy equivalent (W. P. E. = weight of epoxy resin containing one equivalent weight of epoxide). The results of these analyses allow adjustments to be made in the embedding formulations to obtain reproducible blocks for sectioning when more than one batch of Epon 812 is employed. Prior to encapsulation the tissues were transferred from the complete resin-propylene bath into 10 - 15 ml of complete resin. In this step most of the solvent-diluted resin surrounding the tissues will be removed. The hardness of the blocks for sectioning can be controlled over a wide range by using different blends of the two mixtures. The two resin mixtures were made as follows:

Mixture A
Epon 812 (W. P. E. = 160) 80 g.
DDSA (dodecenyl succinic anyhydride) 94 g.

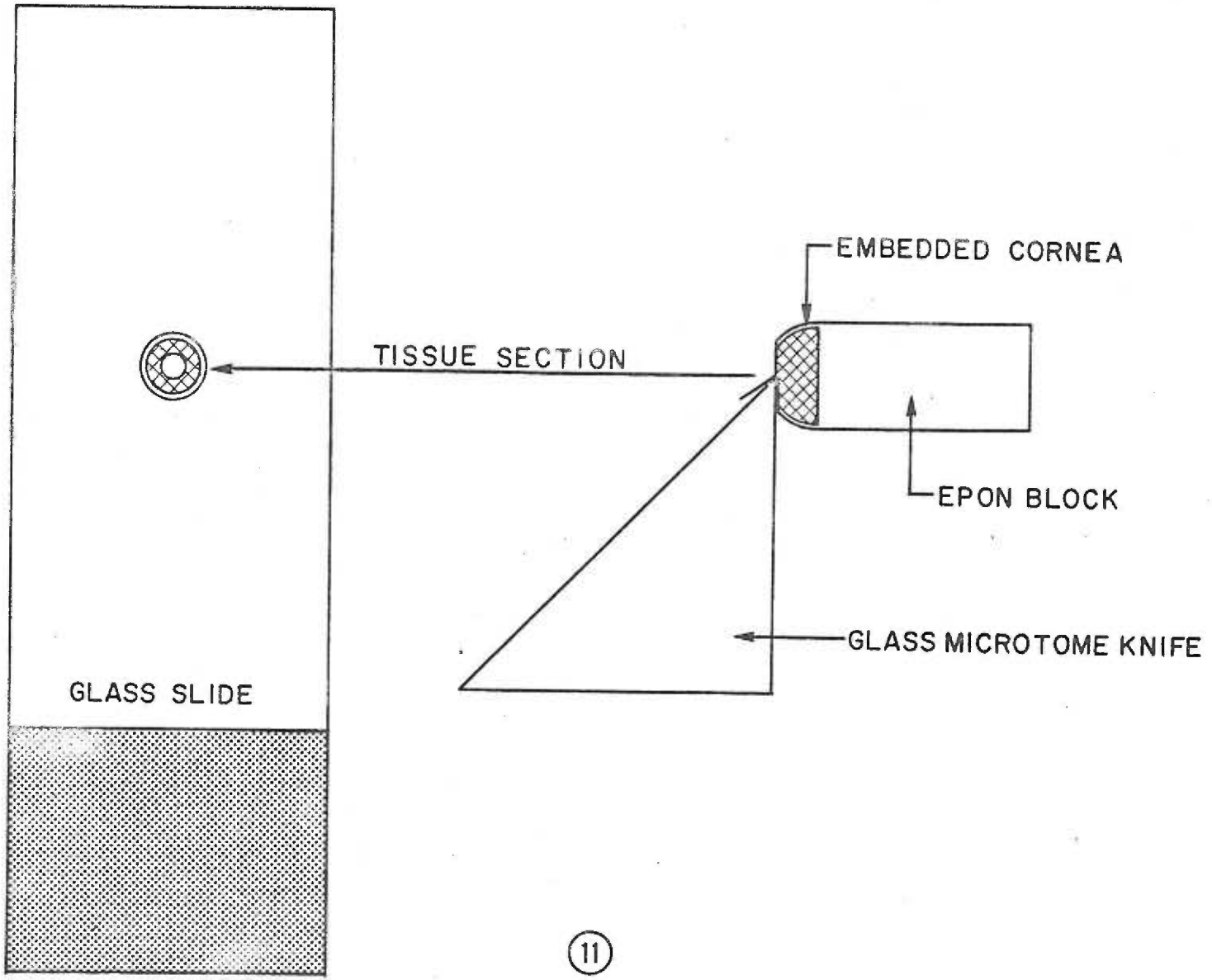
Mixture B

Epon 812 (W. P. E. = 160) 100 g.
MNA (methyl nadic anhydride) 78 g.

The desired degree of block hardness was determined to be one part of mixture A to one part of mixture B, and this resulted in an equivalent hardness to 15 - 20 percent methyl methacrylate in N-butyl alcohol. For each 10 grams of combined resin mixtures A and B, 0.14 ml of DMP - 30 (2, 4, 6-tridimethylaminomethylphenol) accelerator was added and the resin mixture was vigorously stirred with the aid of a glass rod for five minutes to assure that the accelerator was uniformly distributed throughout.

Completing the embedding procedure involved filling No. 00 gelatin capsules with complete resin and transferring the corneas to the capsules. The corneas were allowed to settle to the bottom of the capsules and then positioned with their anterior, or epithelial, surfaces downward as is illustrated in figure 11. Care was taken not to allow gas bubbles to adhere to the corneas because the bubbles increased the buoyancy of the corneas and during the polymerization period the corneas would then float upward and lose their proper orientation. The gelatin capsules employed were found to contain deleterious quantities of water that would lead to a "wrinkling" of the surface of the block during the curing process. To prevent this condition, the capsules were baked at 37°C. overnight before use. The loaded gelatin capsules were labeled by writing the block number on

Figure 11. The entire cornea is Epon-embedded in a gelatin capsule with the cornea positioned with its anterior, or epithelial surface, against the bottom surface of the capsule. One μ coronal sections were cut with a glass knife and transferred to a glass microscope slide in preparation for the autoradiographic procedure.


EMBEDDED CORNEA
TISSUE SECTION
EPON BLOCK
GLASS MICROTOME KNIFE
GLASS SLIDE

paper and including the paper within the capsule so that it became embedded in the transparent resin. Polymerization of the capsules was induced by allowing them to remain at room temperature for six hours followed by a series of three heat incubations: 1. incubated overnight at 37°C., 2. incubated next day at 45°C., 3. incubated next night at 60°C.

The classical embedding medium, parafin wax, will not produce acceptable 1 - 2 μ corneal tissue sections for autoradiography. The dense fibrous nature of the cornea allows the penetration of the wax into the tissue only with difficulty. An additional drawback of wax embedding is the need to remove the wax completely from the sections before autoradiography because any trace of wax left on the slide can prevent the even spreading of the liquid emulsion over the slide, which in turn may result in poor adhesion between slide and emulsion. In contrast, epoxy resin penetrates the corneal tissue well and yields 1 - 2 μ sections having a uniform, flat upper surface which is easier to cover with a smooth layer of emulsion.

Tissue Sectioning

A Porter-Blum microtome equipped with a binocular dissecting microscope was employed for tissue sectioning. Glass knives were used for cutting the sections and were prepared with an LKB knife maker. A trough filled with distilled water which wetted the

knife edge allowed the cut sections to float freely. Strips of 10 mm wide plastic electrical tape were used to make the troughs in the conventional manner as described by Pease (121). Dental wax was employed to fill and seal the heel of the trough to the irregular back of the knife.

Prior to mounting the block in the microtome chuck, the gelatin capsules were dissolved away from the resin by soaking the block in warm water. The blocks were then thoroughly dried at 37°C. before sectioning could proceed. Fortuitously the bottom curvature of the gelatin capsule coincided almost exactly with the curvature of the cornea. This relationship eliminated the necessity of trimming the block face as a prerequisite to sectioning. Sections of 1 μ thickness were cut and allowed to float in the trough fluid. Individual sections were then transfered from the trough with the aid of a small wooden spatula to a glass microscope slide. This pre-treatment of the slides altered their surface properties so that a convex miniscus could not be achieved by merely placing a drop of water on their surfaces. To solve this problem a small rubber "donut" was placed on the slide and filled with distilled water. The tip of the spatula with a section upon it was dipped into the ring of water and the section was allowed to float free. The sections were settled on the slide by evaporating the water with a slide warmer at 50°C.

Pre-Treatment of Glass Microscopic Slides

Speirs, Jansen, Speirs, Osada and Dienes (122) has stressed the importance of pre-cleaning the autoradiographic slides, not only for removal of dirt which invariably accumulates on the slides, but for reduction of background fog. Apparently an exact explanation of why pre-cleaning the slides is effective is lacking, but empirically it has been shown that autoclaving glass slides in a resin and caustic soda solution greatly reduces the background grain and fog. Using the procedure outlined by Speirs et al (122), the slides were first wiped off with a cloth saturated with alcohol to remove particulate matter. The slides were then placed in stainless steel racks, and autoclaved for 15 minutes in a solution consisting of:

100 ml 0.01 M ECTA (Ethylenediaminetetracete; versene)
1 ml 1 percent NaOH

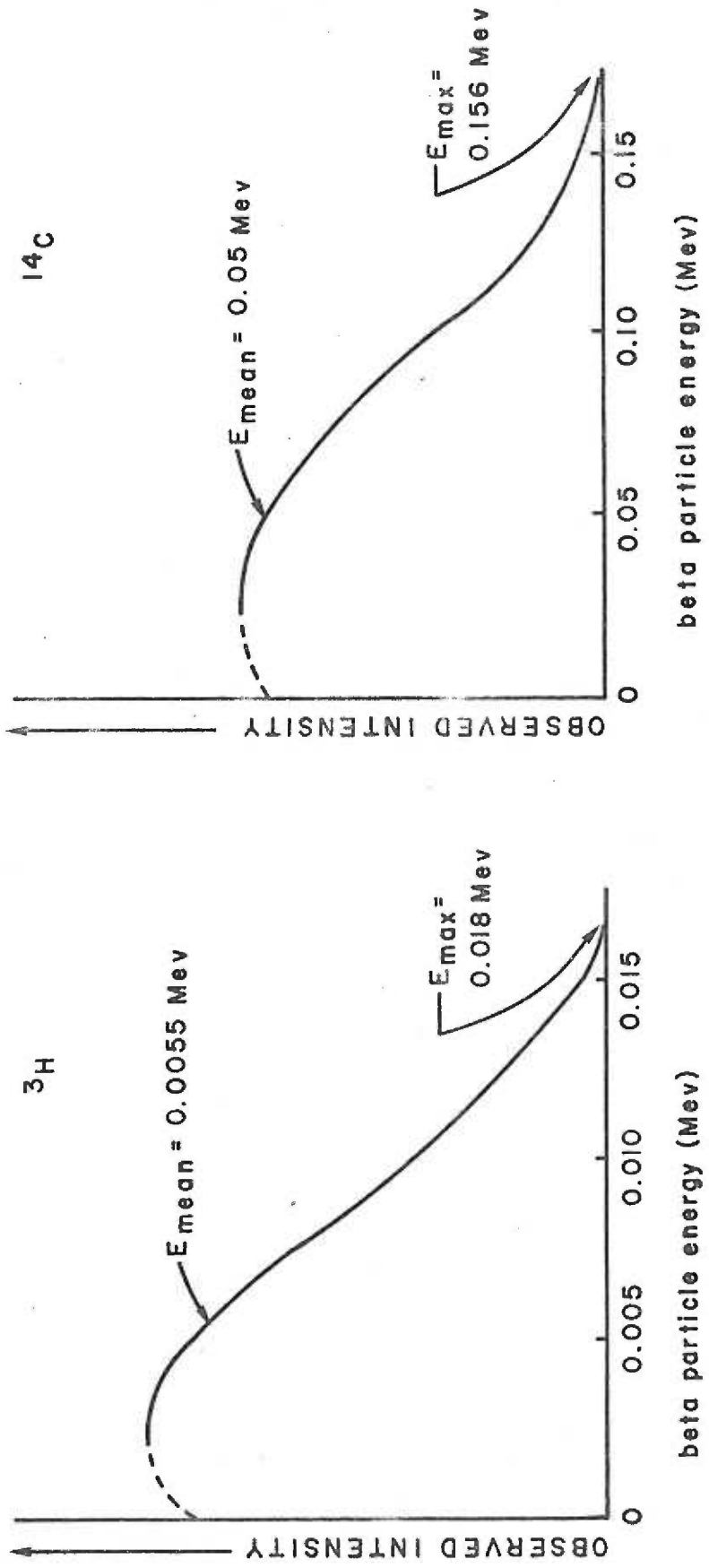
The slides were then removed, rinsed thoroughly in double glass distilled water, and re-autoclaved for 15 minutes in distilled water to produce a hard surface coating. Wrapping the slides in aluminum foil afforded protection for their storage. Glass slides, size 75 x 25 mm with frosted ends, were used because the frosted portion of the slide simplifies labeling and also aids in determining the emulsion side of the slide when working in the reduced lighting conditions of the darkroom.

Double-Layer Autoradiography

The double-layer autoradiographic technique allows the registering of two different isotopes contained in the same specimen. The technique is based upon the fact that the average range of beta particles (a charged electron) from ^{3}H is considerably shorter than that of ^{14}C particles in autoradiographic emulsion. "Range" denotes the distance penetrated by the beta particle and is a measure of the linear distance from the point of origin of a charged particle to its extinction. Lajtha and Oliver (123) determined that the average range of beta particles from ^{14}C and ^{3}H in emulsion were 60 μ and 1.5 μ, respectively. There is a positive correlation between the energy of emission of the beta particle and its range. The higher the energy the greater the distance penetrated (124). During radioactive decay particles emitted from the same radionuclide will vary in energy and therefore in range. The graphs shown in figure 12 represent the beta energy spectrum of ^{3}H and ^{14}C. Note that the mean energy (E_{mean}) of ^{14}C is larger by a factor of 10 when compared to the E_{mean} of ^{3}H. Although the range of the beta particles depends on the value of maximum energy (E_{max}), the actual radiation dose from a beta emitter is calculated using the E_{mean} value (125).

Buckaloo and Cohn in 1956 (126) reported a method that allows the detection of two different isotopes contained in the same specimen

Figure 12. Energy distribution curve of β-particles for ^{3}H and ^{14}C. The dashed line at the low-energy portion of each spectrum indicates that this portion is theoretically calculated, since experimental determination is not readily feasible. (Taken from Wang and Willis (125,p. 47).


^{14}C
E_{mean} = 0.05 Mev
E_{max} = 0.156 Mev
OBSERVED INTENSITY
0
0.05
0.10
0.15
beta particle energy (Mev)
^{3}H
E_{mean} = 0.0055 Mev
E_{max} = 0.018 Mev
OBSERVED INTENSITY
0
0.005
0.010
0.015
beta particle energy (Mev)

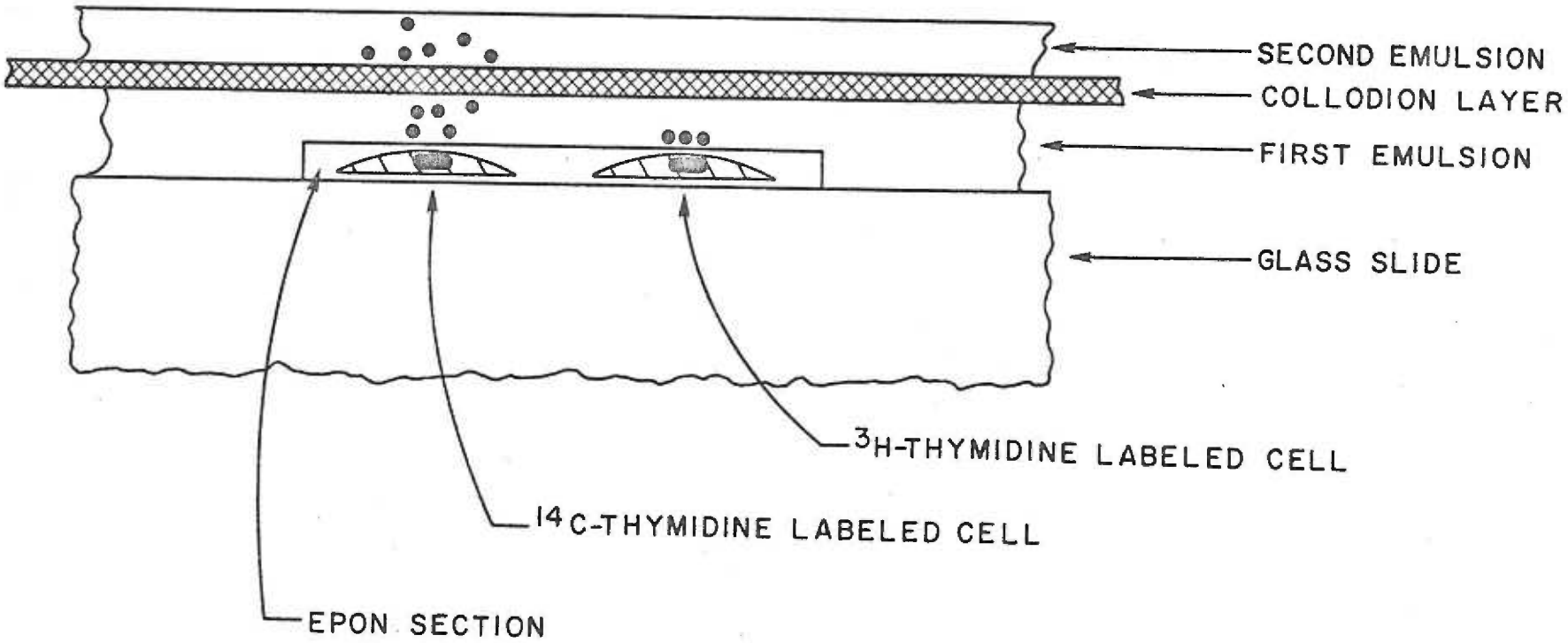
(12)

by employing multilayer color film. This technique has not gained wide acceptance possibly due to the relatively complex photographic processing required.

Perhaps the simplest method to distinguish the beta particles of ^{14}C and ^{3}H was described by Ficq (127). In this method the specimen section was coated with a single thick emulsion which registered the higher energy beta particles of ^{14}C as scattered tracks of grains and the lower energy beta particles of ^{3}H as single grains closer to the tissue emulsion interface. This single emulsion method, however, provided only a coarse distinction between the two isotopes. A modification of the thick emulsion technique was introduced by Krause and Plaut (128) who placed two layers of stripping film over the tissue sections. The first film detected both ^{3}H and ^{14}C while the second film detected only ^{14}C. Because of the greater ease of application, liquid emulsions are more popular now than stripping film for double-layer autoradiography. The stripping film had a tendency to shift on the specimen or even to become lost during photographic processing and subsequent staining. Also, frequently air locks appeared between the stripping film and the tissue sections.

Baserga (129, 130) further refined the double emulsion approach by coating the tissue section with two layers of liquid emulsion separated by a layer of collodion. In this autoradiographic technique (figure 13) the emulsion layer closer to the specimen will register

Figure 13. Schematic cross section of double-layer autoradiogram, with two layers of liquid emulsion separated by a layer of collodion. Black dots located over the cell surfaces represent processed metallic silver grains.


SECOND EMULSION
COLLODION LAYER
FIRST EMULSION
GLASS SLIDE
^{3}H-THYMIDINE LABELED CELL
^{14}C-THYMIDINE LABELED CELL
EPON SECTION

(13)

beta particles both from ^{3}H and from ^{14}C, while the first emulsion layer and the collodion prevent particles from ^{3}H from registering in the second layer, but will have little effect on higher energy particles from ^{14}C. This technique was selected for use in this thesis project.

Other double emulsion methods have appeared in the literature, including the use of a dye-coupling reaction to distinguish by color the grains produced by ^{3}H and ^{14}C (131, 132), and also a method whereby an emulsion which is relatively more sensitive to ^{3}H is applies to one side of the tissue section, and the other side coated with celloidin and a ^{14}C-sensitive emulsion (133). These methods were deemed relatively too complex for practical application in working with the cornea.

Nuclear Track Emulsions

Emulsion types NTB-2 and NTB-3 were employed in these studies. The emulsions were purchased from Eastman Kodak Co., Eastern Region, Rochester, New York. Type NTB-3 is an emulsion with uniform, yet fine grain, and has high sensitivity which is capable of recording all charged particles. Type NTB-2 is an emulsion with slightly finer grain, lower sensitivity, and lower inherent background than type NTB-3. NTB emulsions can be stored for up to two months, preferably at $4^{\circ}C$. The length of time the emulsion can be

stored is limited by the amount of background that can be tolerated for the specific use for which it is intended.

Application of the First Emulsion Layer

Slides were run in triplicate with the addition of test slides that were used to determine the optimum exposure time for the experimental material. Tissue sections of the first experiment were coated with full strength type NTB-3 emulsion. In the second experiment the tissue sections were divided into four duplicate groups and were coated with emulsion in the following manner:

Group a.	full strength type NTB-3 emulsion
Group b.	type NTB-3 emulsion - distilled water (1: 1;v: v)
Group c.	full strength type NTB-2 emulsion
Group d.	type NTB-2 emulsion - distilled water (1: 1; v: v)

The application of the emulsion was carried out in a darkroom with a red safelight. The temperature of the darkroom was 23^{o}C. with the relative humidity in the range of 45 to 50 percent. Unprocessed type NTB emulsions must be handled no less than four feet from a Kodak safelight filter (Wratten series No. 2) with a 15 watt bulb. The emulsion which was contained in its light-tight shipping box was removed from the refrigerator and allowed to equilibrate to room temperature. The emulsion was gelatinous at this temperature and must be liquified prior to use. This was done by unpacking the four ounce plastic bottle containing the emulsion from its shipping box in

the darkroom and placing the bottle in a constant temperature water bath at 43°C. for approximately 45 minutes. The liquid emulsion was then poured from the stock bottle into a smaller glass vessel. This vessel with dimensions of 77 x 32 x 18 mm was especially designed for liquid emulsion slide dipping and is more conservative in the amount of emulsion required than the commonly employed Coplin Jar. A porcelain spoon was used to stir the emulsion gently to help release trapped air bubbles. A period of 5 - 10 min. was then allowed for the air bubbles to rise and dissipate from the emulsion surface before dipping the first slides. Two slides carrying sections were placed back to back (frosted end up) and dipped for approximately 10 sec. in the melted emulsion. The slides were withdrawn carefully and drained quickly of superfluous emulsion before they were placed vertically on filter paper for 5 - 10 sec. The two slides were next separated and suspended individually from plastic clothes pins that were attached to a horizontal wire. The slides were allowed to dry with the darkroom safelight off for about 1 hour. The coated slides, approximately 12 - 15 slides per box, were then placed in 20 slot black slide boxes (Bakelite) in which there was a small amount of drying agent anhydrous $CaSO_4$ (Drierite), packed in bags made of lens paper. The boxes were then closed and sealed with black plastic electrical tape, followed by wrapping in aluminum foil. The slide boxes were then placed in a refrigerator for exposure at 4°C.

Photographic Processing of First Emulsion Layer

At the end of the proper exposure time, as determined by the test slides, the slide boxes were removed from the refrigerator and allowed to equilibrate with room temperature before the slides were transferred to plastic slide holders (Peel-a-way plastic Slide Grip, Peel-A-Way Scientific, South El Monte, Calif.)which were designed to aid in transferring the slides from one photographic bath to another. The entire photographic process was carried out without interruption at 18°C. using Coplin jars. The emulsions were developed with Eastman Kodak Fixing Bath. The subsequent photographic processing steps were:

1. Develop slides for five min. in D-19 with occasional agitation.
2. Rinse briefly (30 sec.) in distilled water.
3. Fix for ten min. in diluted fixer (fixer - distilled water (1:1; v:v) with occasional agitation.
4. Rinse in two or more changes of distilled water, for five or more min. each.
5. Air dry at room temperature.

Staining of Tissue Sections

Richardson's stain (134) was found to penetrate the epon well and to satisfactorily bring out the histological details in the corneal tissue. The staining was accomplished by placing a drop of Mallory's Azur II - Methylene Blue solution (made by taking equal volume of

1 percent Methylene Blue in 1 percent borax solution and 1 percent Azur II in distilled water) directly over the section. After about five sec. excess stain was rinsed off the section with distilled water and the slide was dried at room temperature. In the event the section and/or the emulsion gelatin was over-stained, an effective destaining procedure was carried out by placing the slide in distilled water and allowing the excess stain to disperse into the fluid. It was noted empirically that the second emulsion of the destained slides generally had less background contamination than that of the slides not requiring destaining. The apparent explanation for this phenomenon was that destaining reduced the amount of stain that could diffuse from the first to the second emulsion, which would in turn minimize the degree of chemographic contamination. Following the recognition of the relationship between the stain and chemographic contamination, all sections were intentially over stained and then destained.

Application of the Collodion Layer

Prior to coating the slides with collodion, photomicrographs were taken to record the autoradiographic results of the first emulsion. The dry autoradiograms were then coated by dipping them in a solution of collodion (Parlodion, Mallinckrodt Chemical Works, St Louis, Mo.). Parlodion is described as a highly purified form of non-explosive cellulose nitrate, specially prepared for embedding

tissue for section cutting and for preparing semipermeable membranes (135). A 7.0 percent Parlodion stock solution was first prepared and subsequently diluted for slide dipping. The stock Parlodion solution was formulated by adding 93 ml of absolute ethyl alcohol - anhydrous ethyl ether (1:1; v:v) to 7.0 gm. of parlodion. Pilot studies were conducted to determine the proper collodion thickness necessary to absorb the ^{3}H beta-particles while still allowing the more energetic ^{14}C beta-particles to enter the second emulsion. Slides carrying ^{3}H-labeled corneal tissue sections were dipped into a graded series of Parlodion solution. Results indicated that a 1.5 - 2.0 percent Parlodion solution satisfactorily blocked the ^{3}H energy. Micrometer readings revealed that at this Parlodion concentration the layer formed was 2.5 μ in thickness.

Application of the Second Emulsion Layer

The procedure for coating the slides with the second emulsion layer was identical to that of the first emulsion. Double emulsion slides were also prepared to use in determining the proper length of exposure for the experimental slides.

Photographic Processing of the Second Emulsion

The developing and fixing of the second emulsion was performed using the identical steps that were followed with the first emulsion.

Care was taken in handling the slides during the photographic processing to reduce the probability of the second emulsion separating from the collodion layer. The slides were allowed to dry overnight and then photomicrographs were taken of the autoradiograms.

Assessment of the Bone Marrow Labeling Procedure

It was important to know the efficiency of the intravenous labeling procedure used in these studies for specifically isotopically tagging the mononuclear blood cells. It was not practical to use the experimental animals from the previously described groups because any attempt to secure bone marrow or blood samples would inflict a competing wound. Therefore, a separate group of rats were labeled with ^{3}H-Tdr. The labeling protocol was identical to that of the previously described animals receiving ^{3}H-Tdr. Samples were obtained from two groups of animals (three animals/group) at 8 and 32 hrs. following the last intravenous injection of ^{3}H-Tdr respectively. The samples consisted of smears of bone marrow, peripheral blood, and blood buffy coat, and imprints of liver and spleen tissue. The processed autoradiograms were stained with Wright's stain (136) and then evaluated by microscopic observation.

Frozen Corneal Sections for Microscopic Examination of the Corneal Freeze Injury

Frozen tissue sections were prepared to study the histopathology of the corneal freeze injury and the subsequent wound healing process at specific time intervals. The corneas were mounted on an aqueous paste of gum tragacanth, frozen and sectioned with a freezing microtome in a cryostat (Harris Refrigeration Co., Cambridge, Mass.). The corneas were sectioned coronally through the full thickness of the cornea with the wound centered as much as possible. In such sections, the cells appear flat, spread out, and the cells are considerably easier to identify than in sagittal sections. The sections (15 μ) were air dried, fixed for 10 minutes in absolute methanol, and stained in a dilute buffered Giemsa stain for 10 minutes. After staining they were rinsed in distilled water, dried on a warming plate at approximately 45°C., rinsed in acetone and mounted in Permount.

The Giemsa stain was made up as follows: 50 ml distilled water, 5 ml of 10X phosphate buffer (0.18 M in anhydrous dibasic sodium phosphate and 0.488 M in monobasic potassium phosphate), pH 6.4 (137) and 1.5 ml of stock Giemsa solution (Allied Chemical, Morristown, N.J., U.S.A.).

Counting of Labeled Stromal Cells

Labeled stromal cells were counted with the aid of a rectangular graduated grid disc that was placed in the microscope eyepiece. One quarter of a corneal section was counted and the number of labeled cells was recorded in terms of percentage. In the event there were less than 100 total cells present in the first count, an additional one quarter of the area of the section was counted.

RESULTS

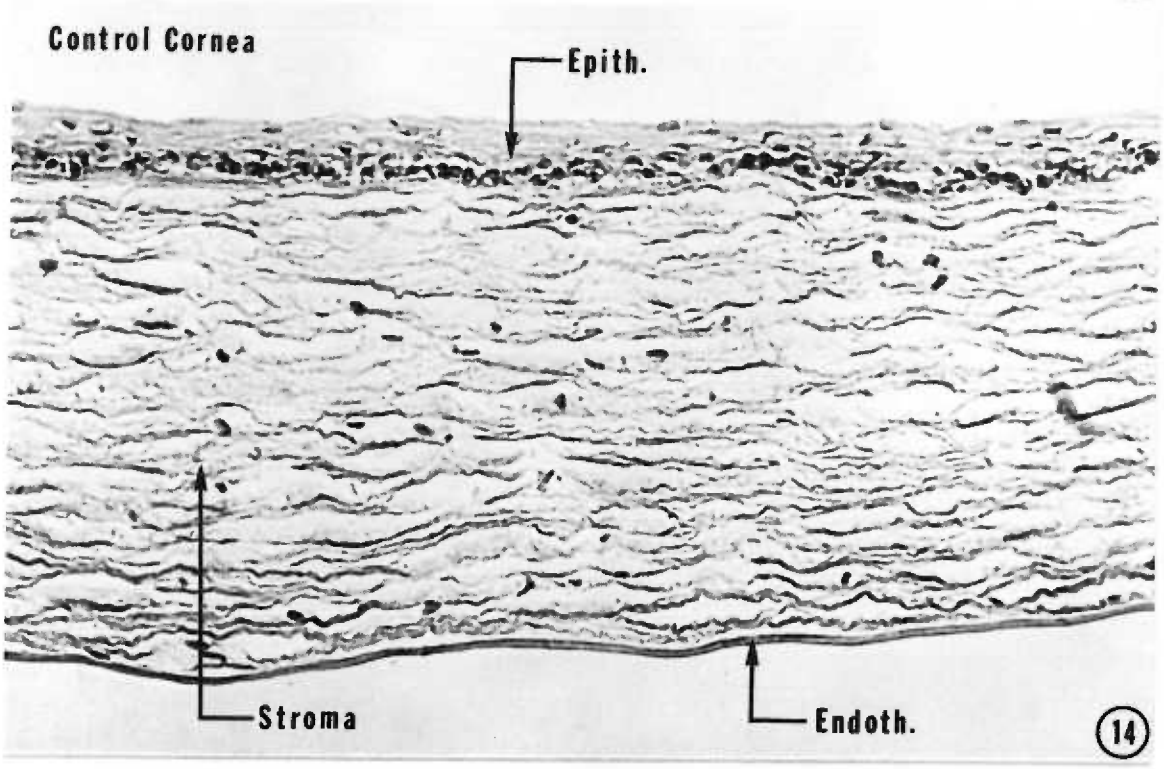
Histopathology of the Corneal Freeze Injury

Twelve Hour Postoperative Wound

Twelve hours after a 6 sec. application of the cryoprobe (cooled to -20°C) to the rat cornea the stromal connective tissue cells have disappeared in the region of the injury (compare figure 14, normal cornea, with figure 15, frozen cornea, and figure 16, normal cornea, with figure 17, frozen cornea). At this time a number of PMN leucocytes were seen under the epithelium of the frozen zone. There was a definite fragmentation of the nuclei of the endothelial and epithelial cells and marked loss of these tissues by the process of detachment. Due to edema the anterior-posterior thickness of the wound had become two and one-half to three times the thickness of the normal cornea (compare figure 14 and 15). With increased magnification, the area immediately adjacent to the frozen-killed zone revealed the presence of a mixed cell population (figure 17). In addition to the PMN leucocytes there remained a few stromal cells that were swollen and showed fragmentation of their nuclei. Apparently these stromal cells were severely damaged because they had disappeared by 24 hours after injury.

Figure 14. A transverse paraffin section of a control cornea. The stromal cells are seen in profile between the lamellae of the collagen and are less easily identified than in coronally cut sections (compare with figure 16). (X 400)

Figure 15. Twelve hours following the freeze injury to the cornea, the stromal connective tissue cells have disappeared in the region of the injury. At this time a number of PMN leucocytes were seen under the epithelium of the frozen zone. Due to edema the anterior-posterior thickness of the wound had become two and one-half to three times the thickness of the normal cornea. (X 250)


Control Cornea
Epith.
Stroma
Endoth.
14

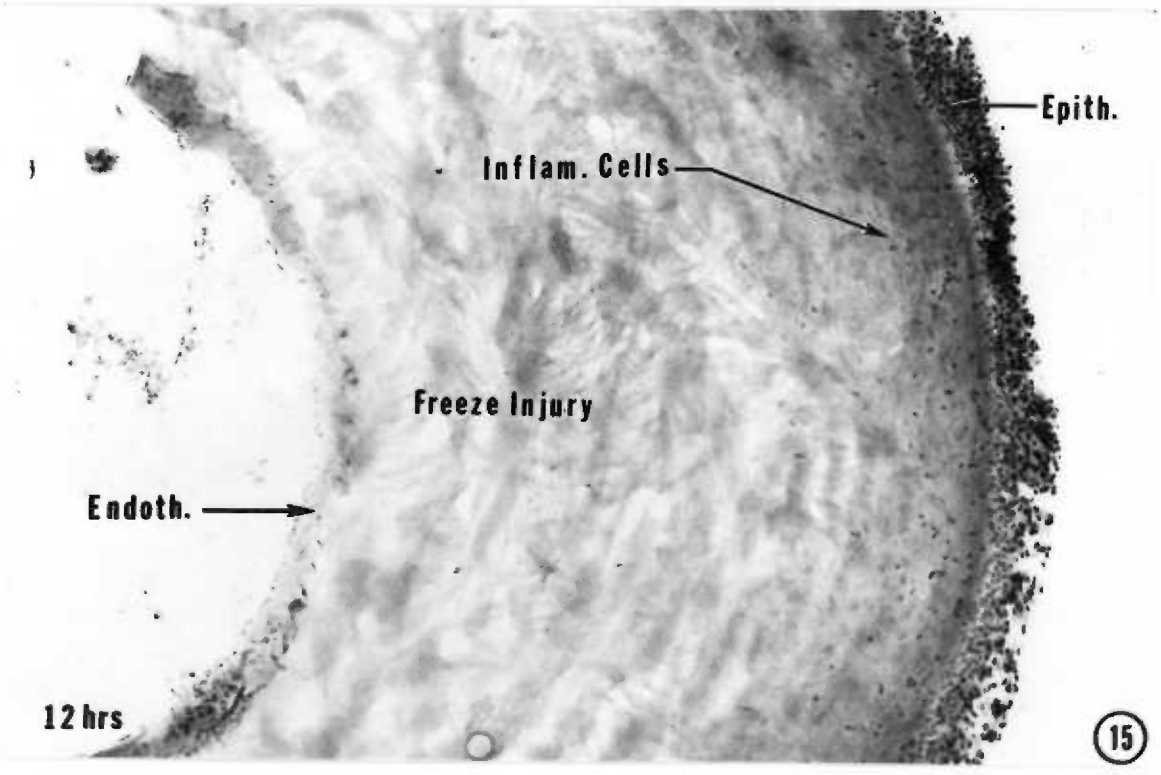
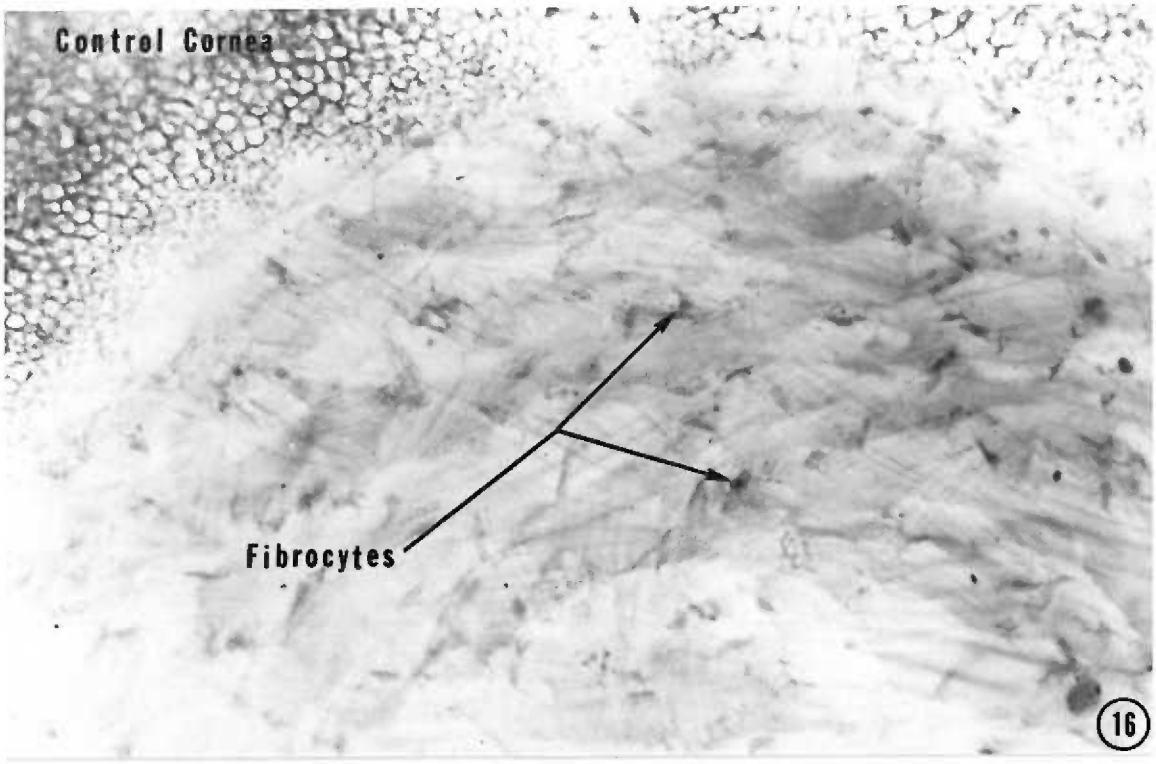

Epith.
Inflam. Cells
Freeze Injury
Endoth.
12 hrs
15

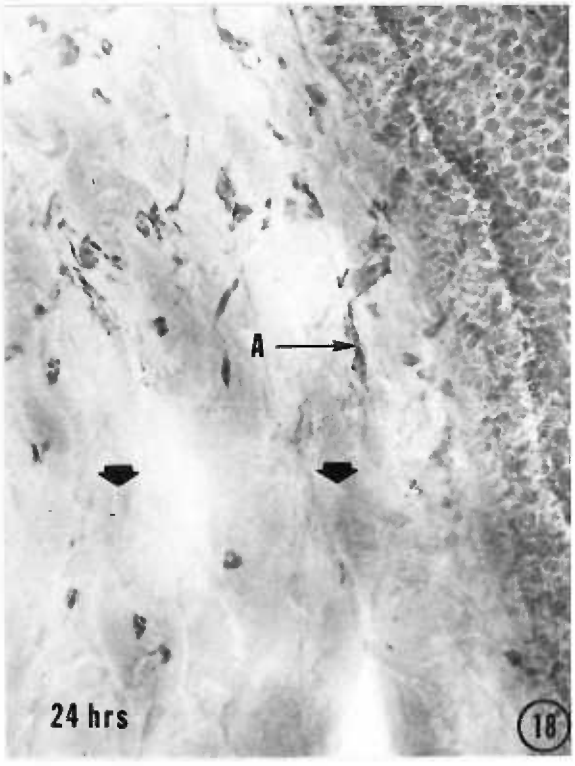
Figure 16. Control cryostatic corneal section showing the histology of the stroma prior to the freeze-injury procedure. The relatively quiescent fibrocytes can be seen lying spread out between the stromal lamellae. (X 400)

Figure 17. Upon increased magnification the area immediately adjacent to the frozen-killed zone revealed the presence of a mixed cell population. In addition to the PMN leucocytes (arrow) there remained a few stromal cells that were swollen and showed fragmentation of their nuclei. (X 400)

Figure 18. Between the twelfth and the twenty-fourth postoperative hours, the surviving corneal stromal cells (A) adjacent to the frozen-killed zone had undergone profound changes in their morphological appearance, including the development of two or three large nucleoli, an increase in basophilic staining and were enlarged in size when contrasted to the uninjured stromal fibrocyte. These activated stromal cells also become more spindle in shape with an accompanying increase in their protoplasmic processes.


Control Cornea
Fibrocytes
16


12 hrs
17

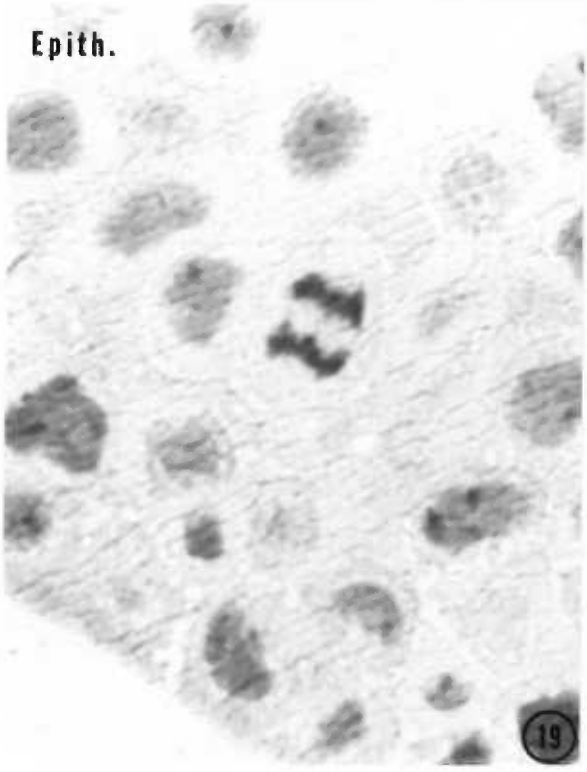

A
24 hrs
18

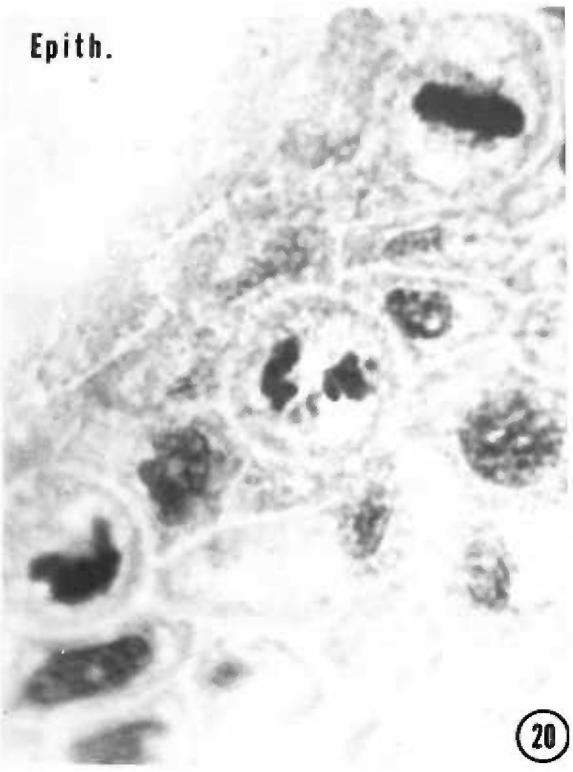
Twenty-four Hour Postoperative Wound

Between 12 and 24 hours after wounding the surviving corneal stromal cells adjacent to the frozen-killed zone had undergone profound changes in their morphological appearance, including the development of two or three large nucleoli, an increase in basophilic staining, and hypertrophy (figure 18) when contrasted to the uninjured stromal fibrocyte (compare figure 16 and 18). These activated stromal cells had also become more spindle shaped with an accompanying increase in their protoplasmic processes. Few, if any mitotic figures were seen in the stroma at this time. There was also a steady increase in the number of invading PMN leucocytes. Occasional monocytes could be found near the edge of the injured tissue. The epithelium had completely covered the damaged area at this stage of repair and numerous mitotic figures were observed in its basal cell layers as shown in figures 19 and 20. Mitotic activity was also seen in the endothelium. Neighboring endothelial cells appeared to be migrating to cover over the freeze-induced denuded area. The edema was still present in the 24 hour wound as indicated by the increased anterior-posterior corneal thickness and the presence of lamellae separation. Grossly the cornea had developed moderate opacity as result of the edema.

Figure 19. Numerous mitotic figures were observed in the basal cellular layers of the epithelium by the twenty-fourth postoperative hour. This photomicrograph is included to illustrate that the histological preparation of the corneal tissue does allow the observation of cells undergoing division. (X 1000)

Figure 20. An example of mitosis in the corneal epithelium subsequent to the freeze injury. (X 1000)


Epith.
19

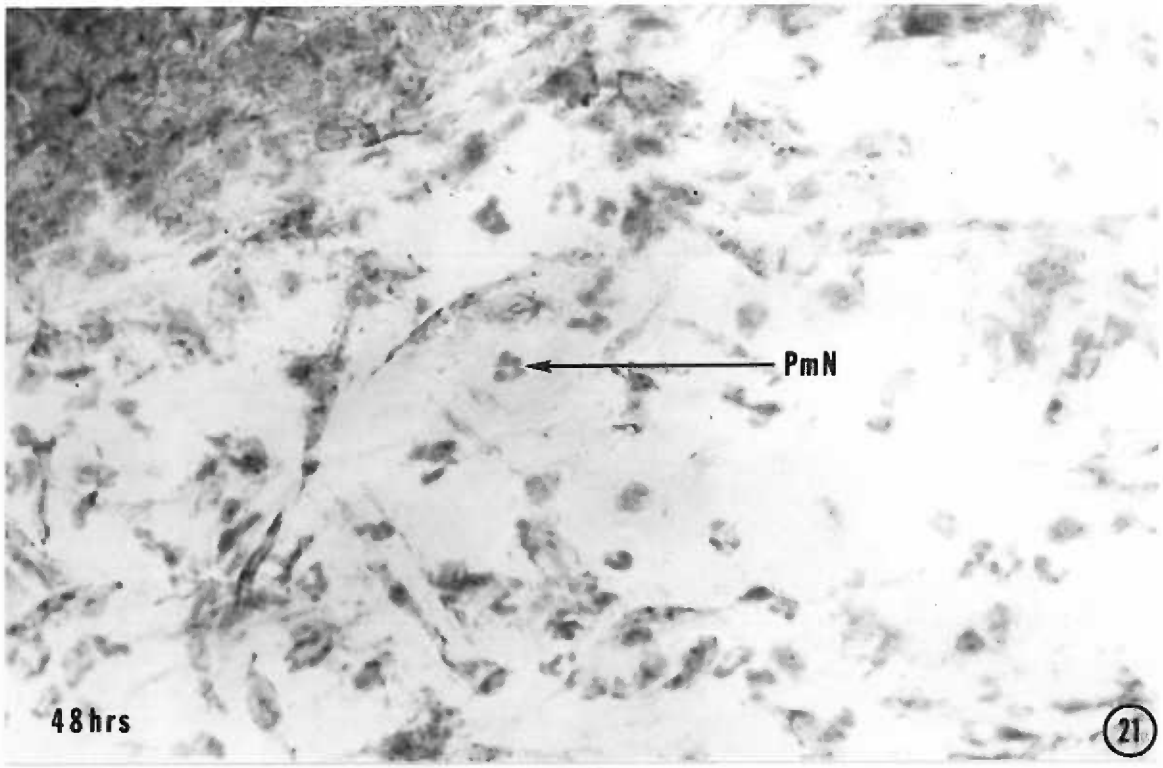

Epith.
20

Forty-eight Hour Postoperative Wound

By 48 hours after injury extensive invasion of PMN leucocytes appeared to be subsiding and many of the PMN leucocytes present could be seen in the various stages of cellular death (figure 21). The number of monocytes had increased at the wound edge during the past 24 hours. At this time the monocytes were found to be present in various stages of transformation into fibroblast-like cells as shown in figure 22. The morphological characteristics of these transforming cells were similar to those described by Weimar (15) in transforming monocytes found following a central corneal incision. The author classified the monocyte transformation into six consecutive stages: Stage I. Horseshoe nucleus containing one or more developing nucleoli and only dust-like granular cytoplasm; Stage II. Horseshoe nucleus containing very large nucleoli and the first appearance of a lacy cytoplasm; Stage III. Horseshoe nucleus containing even larger nucleoli and surrounded by a developing lacy cytoplasm. The nucleus was about twice as large as it was in stage II (figure 23-cell B); Stage IV. Horseshoe nucleus containing very large, well-developed nucleoli and long tails of very basophilic cytoplasm. The nucleus was larger than it was in Stage III cells (figure 23 - cell C); Stage V. Similar to Stage IV, except long tails of cytoplasm have developed; Stage VI. The nucleus has begun to elongate and

Figure 21. By the forty-eighth postoperative hour the extensive invasion of PMN leucocytes appeared to be subsiding and many of the PMN leucocytes present could be seen in the various discernible stages of cellular death. The number of monocytes had increased at the wound edge during the past 24 hr. (X 1000)

Figure 22. At the forty-eighth postoperative hour the monocytes were found to be present at the wound site in various stages of transformation into fibroblast-like cells. The morphological characteristics of these transforming cells are labeled in consecutive stages commencing with cell (A) and ending with cell (D). (X 400)


PmN
48hrs
21

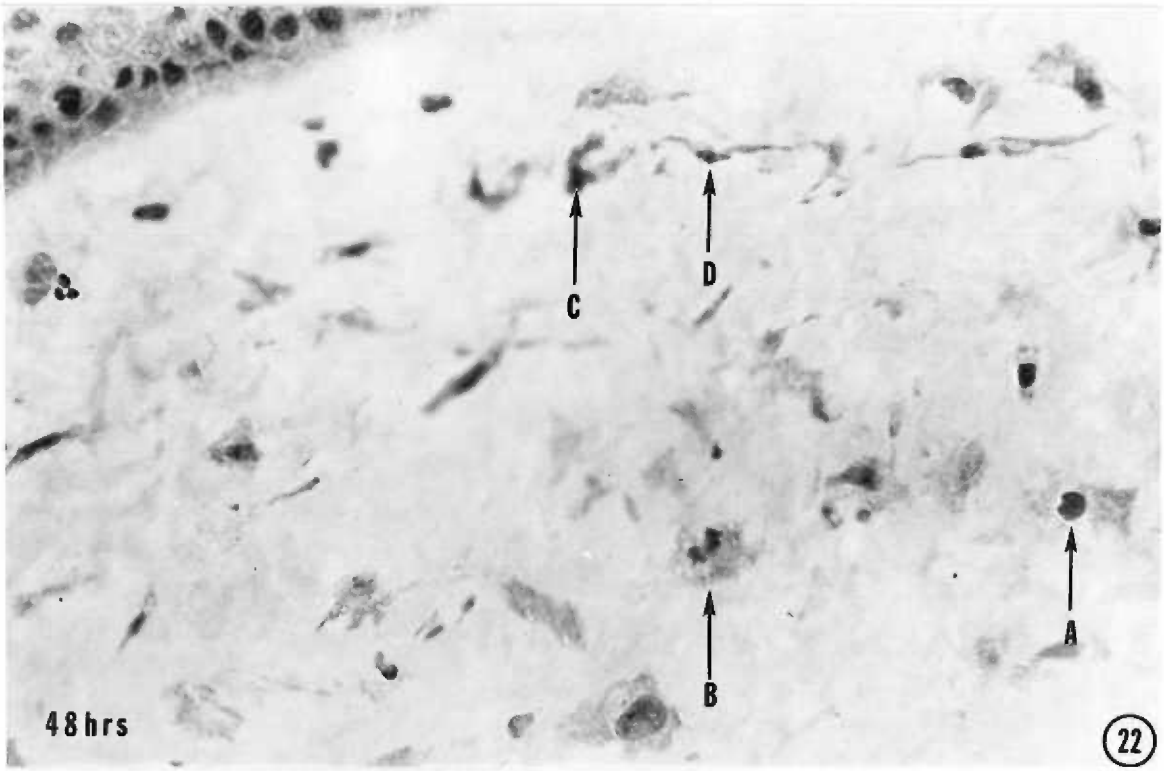
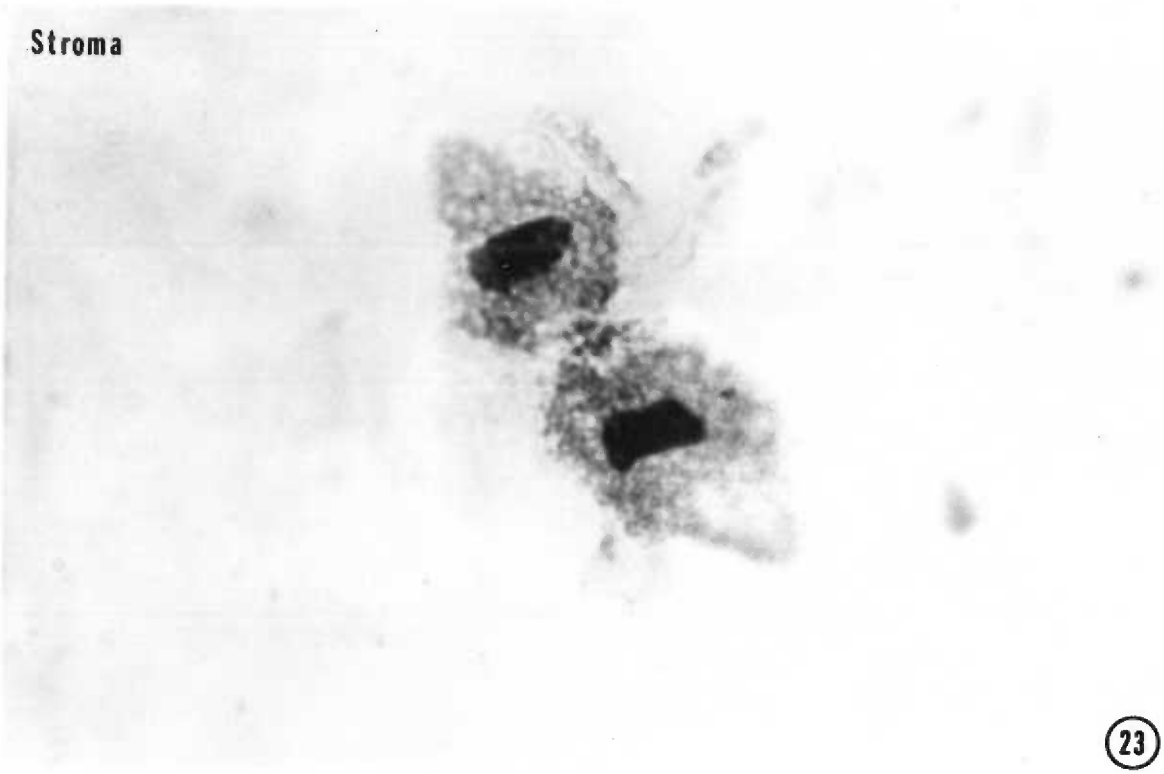

C
D
A
B
48hrs
22

Figure 23. This photomicrograph depicts an example of a stromal connective tissue cell in mitosis and illustrates the point that the lack of mitosis is not attributable to the inability to visualize mitotic activity in the experimental tissue sections. (X 1000)

Stroma



(23)

straighten, but some trace of the horseshoe shape still remains. At this stage the cell has taken a fusiform or spindle shape (figure 23-cell D).

Mitotic figures were rarely seen in the stroma suggesting that the observed increase in cell population was the result of cellular emigration and not due to mitosis of existing stromal cells. Figure 21, depicts an example of a rare stromal connective tissue cell in mitosis and illustrates the point that the apparent lack of mitosis is not attributable solely to the inability to visualize mitotic activity in these tissue sections.

The endothelial cells had continued to cover the denuded area in the 48 hour wound. The fibroblasts located at the edge of the wound were elongated with prominent nucleoli and there was an indication that the cells were organizing and aligning their longitudinal axis toward the acellular frozen-killed zone in preparation for cellular migration.

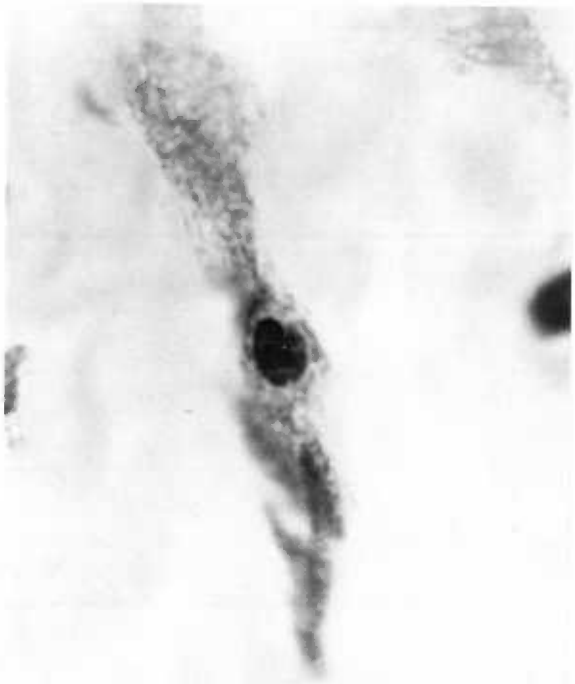
Figure 24 is an example of a typical fibroblast-like cell found in a 24 hour wound.

Figures 24, 25, 26 and 27 show the temporal progression of morphological changes of the nucleus of the developing wound fibroblast. The first noticeable morphological changes in the corneal stromal cells were an increase in the size of the nucleoli accompanied by an increase in the number of nucleoli per nucleus (figure 25).

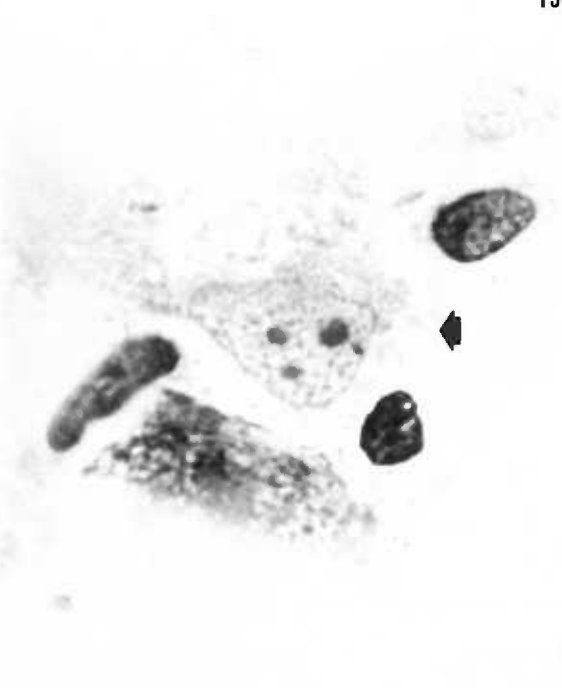
Figure 24. An example of a fibroblast-like cell seen in the twenty-fourth hour postoperative wound. This cell may represent an activated corneal stromal fibrocyte. (X 1000)

Figure 25, 26 and 27.

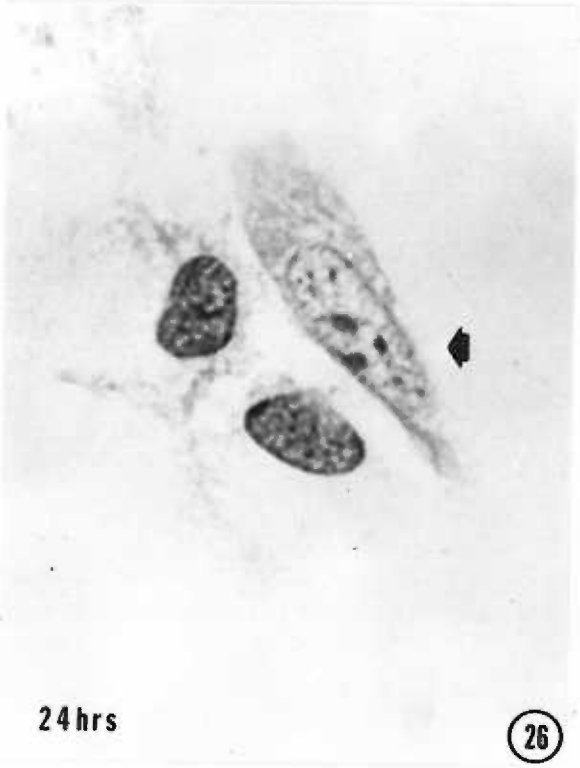
Shown in these photomicrographs are the temporal progression of morphological changes of the nucleus of the developing wound fibroblast-like cell. The first noticeable morphological changes in the corneal stroma cells were an increase in the size of the nucleoli accompanied by an increase in the number of nucleoli per nucleus. (X 1000)



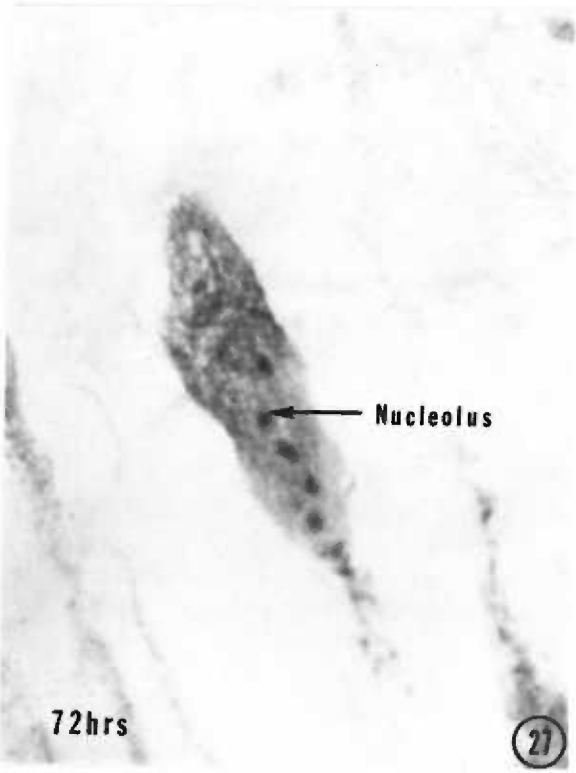
Fibroblastlike Cell (24)



12 hrs (25)



24hrs (26)



72hrs (27)

Weimar (17) reported that following a knife wound to the rat cornea the nucleolar volume per nucleus reached 50 times the original volume by 24 hours after wounding. The author noted that this increase in nucleolar size has been associated with a concurrent increase in cell metabolism, particularly with intracellular protein synthesis. Between the 12th and 48th postoperative hours there was a steady nuclear transition from an oval type (figure 25) to a long, narrow type (figure 27).

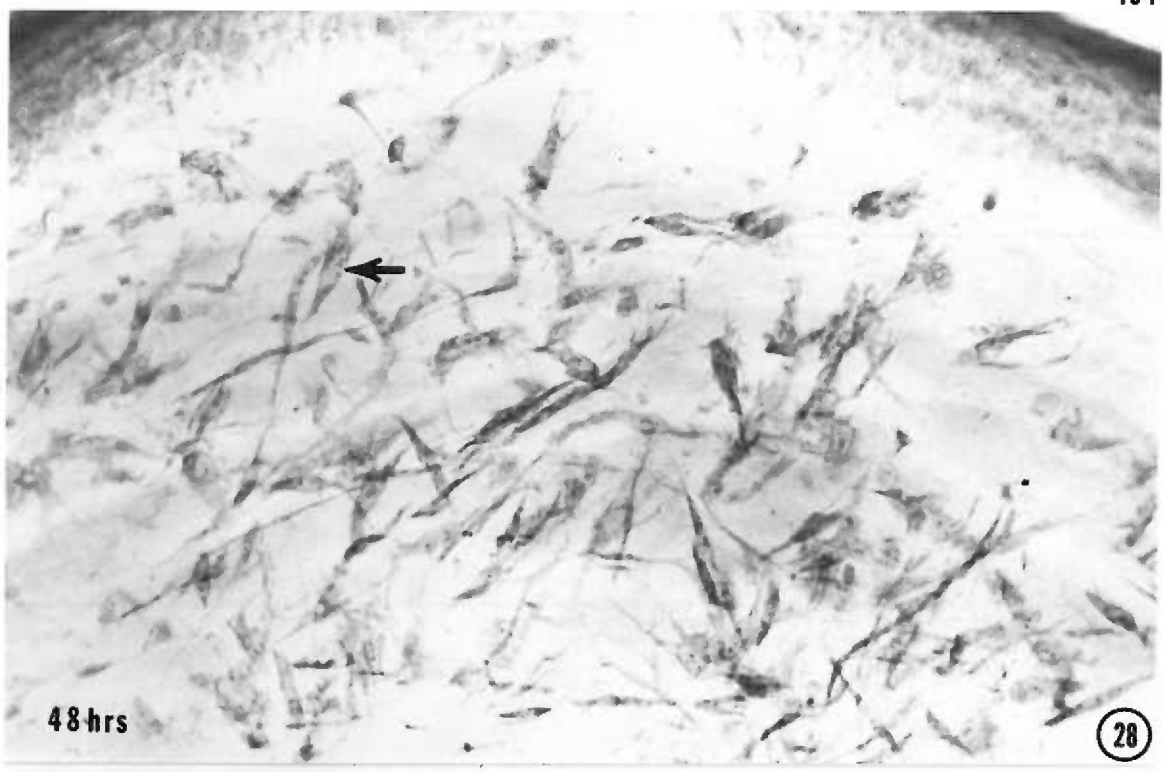
Sections made of corneal tissue immediately adjacent to the margins of the devitalized cell-free zone, disclosed the presence of a relatively large concentration of fibroblasts as is shown in figure 28. If the assumption is made that these fibroblasts were derived solely from activated stromal fibrocytes, then there should be areas of the stroma relatively depleted of cells distal to the wound, as a result of cellular migration. No cellularly deficient areas were found upon examining serial sections of stromal tissue surrounding the wound. Figure 29 shows a tissue section taken from approximately midway between the wound and the limbus. In this corneal section the number and distribution of the stromal cells appears comparable to that of the control cornea (figure 16).

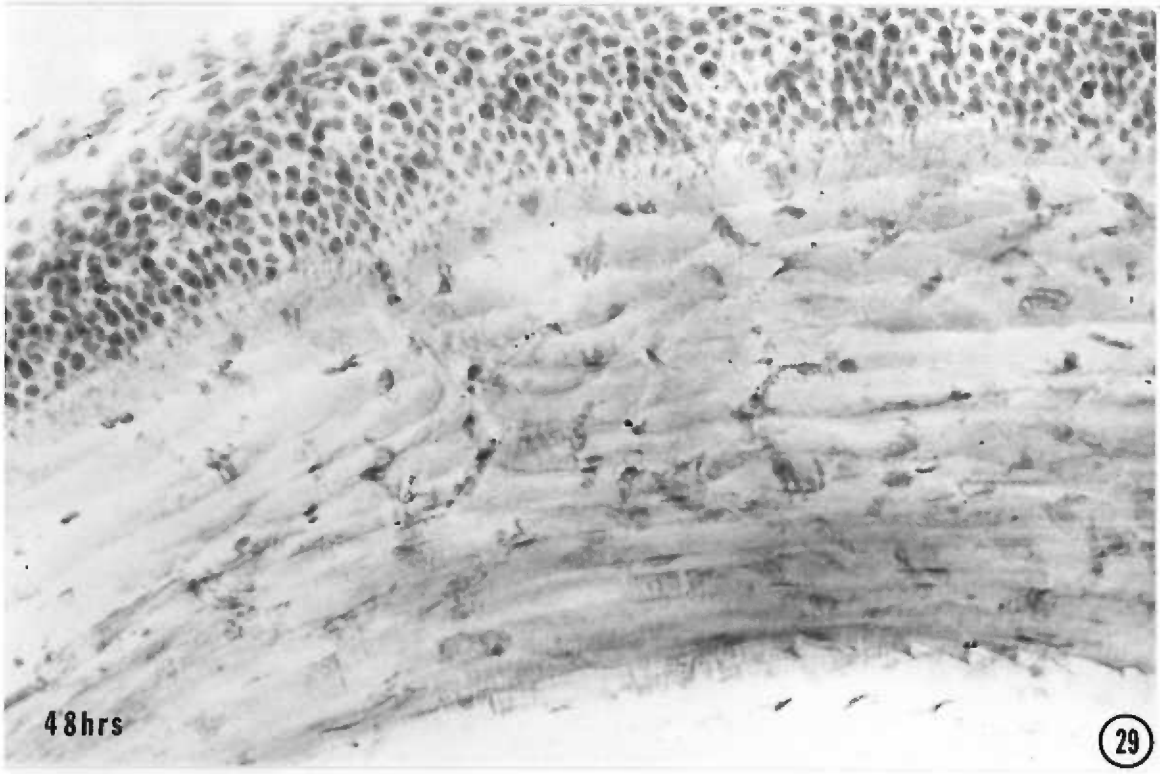
Seventy-two Hour Postoperative Wound

Tissue sections of the 72 hour wound revealed that there had been a steady increase in the number of fibroblasts at the wound edge

Figure 28. In the 48 hour postoperative wound sections made of corneal tissue immediately adjacent to the margins of the devitalized cell-free zone disclosed the presence of a relatively large concentration of fibroblasts. (X 400)

Figure 29. If the assumption is made that those fibroblasts that were seen in figure 28 were derived solely from activated stromal fibrocytes, then there should be areas of the stroma relatively depleted of cells distal to the wound, as a result of cellular migration. The photomicrograph shown represents a tissue section taken from approximately midway between the wound and the limbus. In this corneal section the number and distribution of the stromal cells appears comparable to that of the control cornea. (X 250)


48 hrs
28


48hrs
29

during the preceding 24 hours. These activated cells had begun to move through the area immediately adjacent to the wound (figure 30) and into the previously frozen area (figure 31). Figure 32 shows the striking concentration of closely packed fibroblasts proximal to the wound. A higher magnification of this same area disclosed in greater detail the histological appearance of these fibroblasts (figure 33). Generally speaking, these cells have developed as many as four or five nucleoli in their now fusiform nuclei, and they demonstrated a dust-like granular basophilic cytoplasm. The cytoplasm was often seen streaming behind the nucleus forming what has previously been described by Kitsno and Goldman (138) as "cometary" tails. Mast cells were never seen in the corneas of the normal controls or the wounded eyes in these preparations.

Seven Day Postoperative Wound

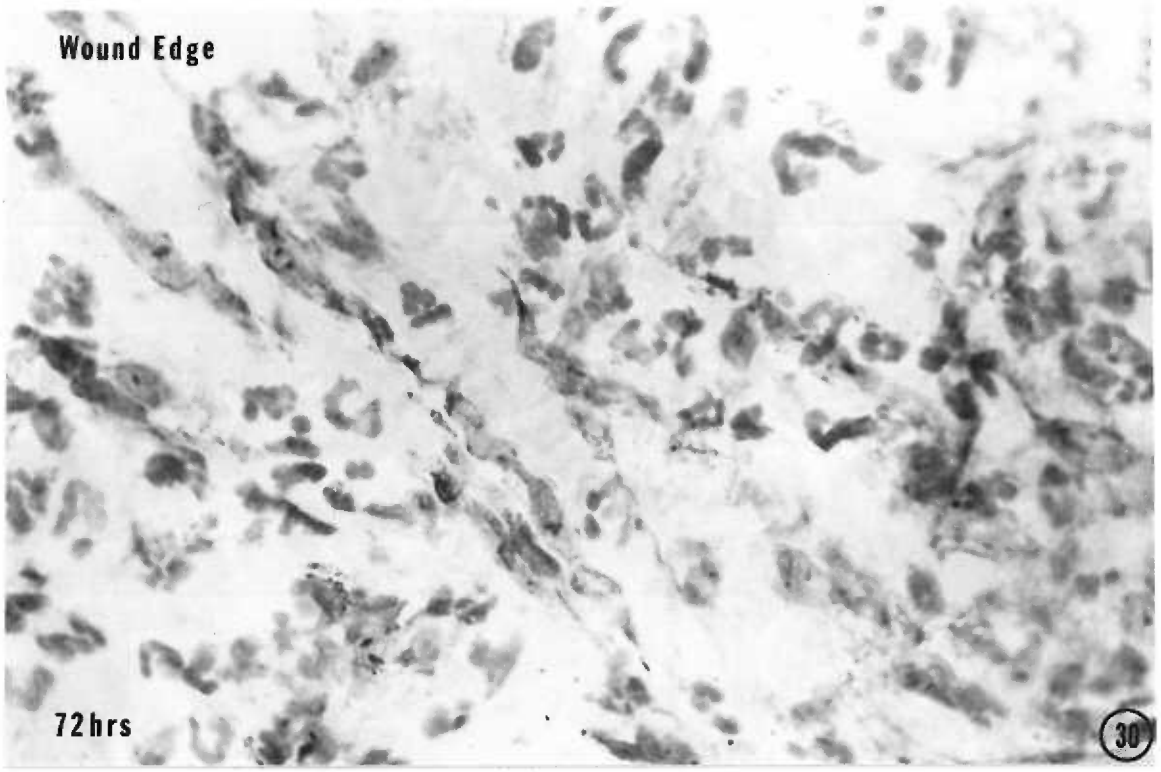
By the seventh day after injury fibroblasts had invaded the acellular wound and had partially repopulated the area (figure 34). The edema of the stroma was nearly absent and the spaces between the corneal lamellae that were seen previously had disappeared at this time.

Fourteen Day Postoperative Wound

Figure 35 shows a corneal section taken from the center of the

Figure 30. In the 72 hour wound there had been a steady increase in the number of fibroblasts at the wound edge during the preceding 24 hours. These activated cells had begun to move through the area immediately adjacent to the wound and into the previously frozen area. (X 400)

Figure 31. Fibroblasts in this photomicrograph are seen in the process of migrating into the acellular freeze wound. (X 400)


Wound Edge
72hrs
30

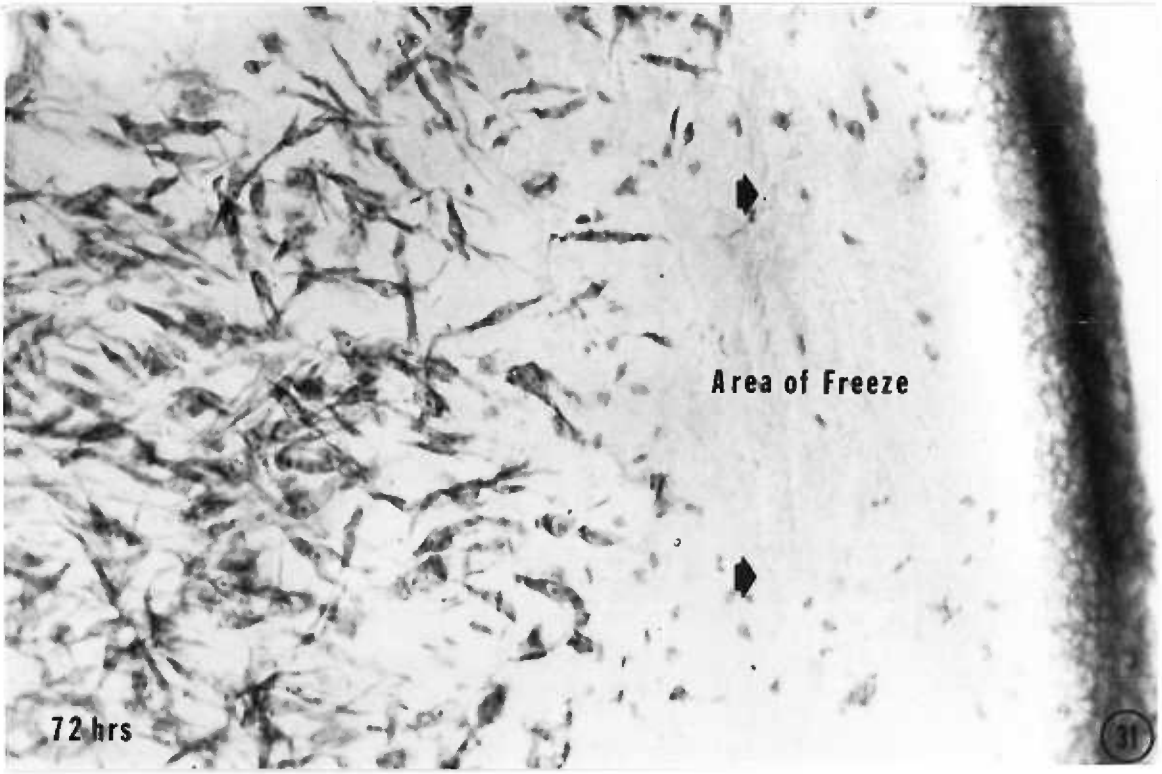

Area of Freeze
72hrs
31

Figure 32. Shown is the striking concentration of closely packed fibroblasts found proximal to the 72 hour wound. (X 400)

Figure 33. A higher magnification of the same microscopic field shown in figure 32 disclosed in greater detail the histological appearance of the wound fibroblasts. The majority of these cells have developed as many as four or five nucleoli in their now fusiform nuclei, and they demonstrate a dust-like granular basophilic cytoplasm. (X 1000)

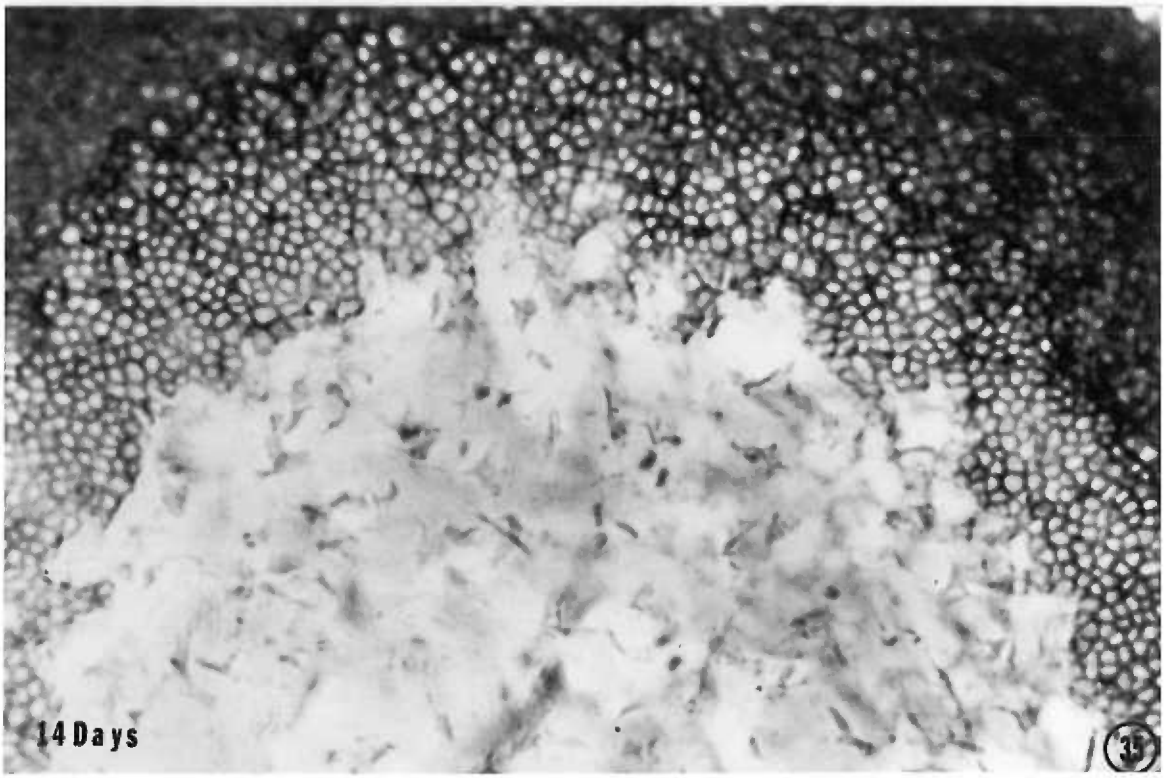

72 hrs
32


72hrs
33

Figure 34. By the seventh day after injury fibroblasts have invaded the acellular wound and have partially re-populated the area. Edema of the stroma was nearly absent and the spaces between the corneal lamellae that were seen previously had disappeared at this time. (X 250)

Figure 35. Shown is a corneal section that was taken from the center of the wound immediately beneath the epithelium and does represent the stromal area most severely damaged by the freeze injury. The cellular density of this area at 14 days following the injury was rapidly approaching that of the control cornea. (X 400)


7 Days
34


14 Days
35

wound immediately beneath the epithelium. This section would represent the stromal area most severely damaged by the freeze injury. The cellular density of this area at 14 days following the injury was rapidly approaching that of the control cornea (figure 16). Many cells appeared morphologically to be reverting back to the more metabolically quiescent stromal fibrocytes.

Twenty-one Day Postoperative Wound

By the 21st day following the freeze injury the cornea had become completely cellular and normal in appearance (figure 36). No corneal opacities were observed upon gross examination, but in a very limited number of repaired corneas some degree of vascularized healing as shown in figures 37 and 38 was found. These corneas that had undergone vascularization were eliminated from the study.

Histopathology of the Corneal Stroma Following Destruction of the Corneal Epithelium

The entire cornea was scraped to remove the epithelium in those animals that were to have their stromal cells labeled with ^{14}C-Tdr. Frozen tissue sections of the cornea prepared from tissue taken at various time intervals following injury were studied. The first event seen postoperatively was an invasion of the cornea by PMN leucocytes which preferentially congregated immediately underneath

Figure 36. By the twenty-first day following the freeze injury, the cornea had become completely cellular and no corneal opacities were observed upon gross examination. (X 400)

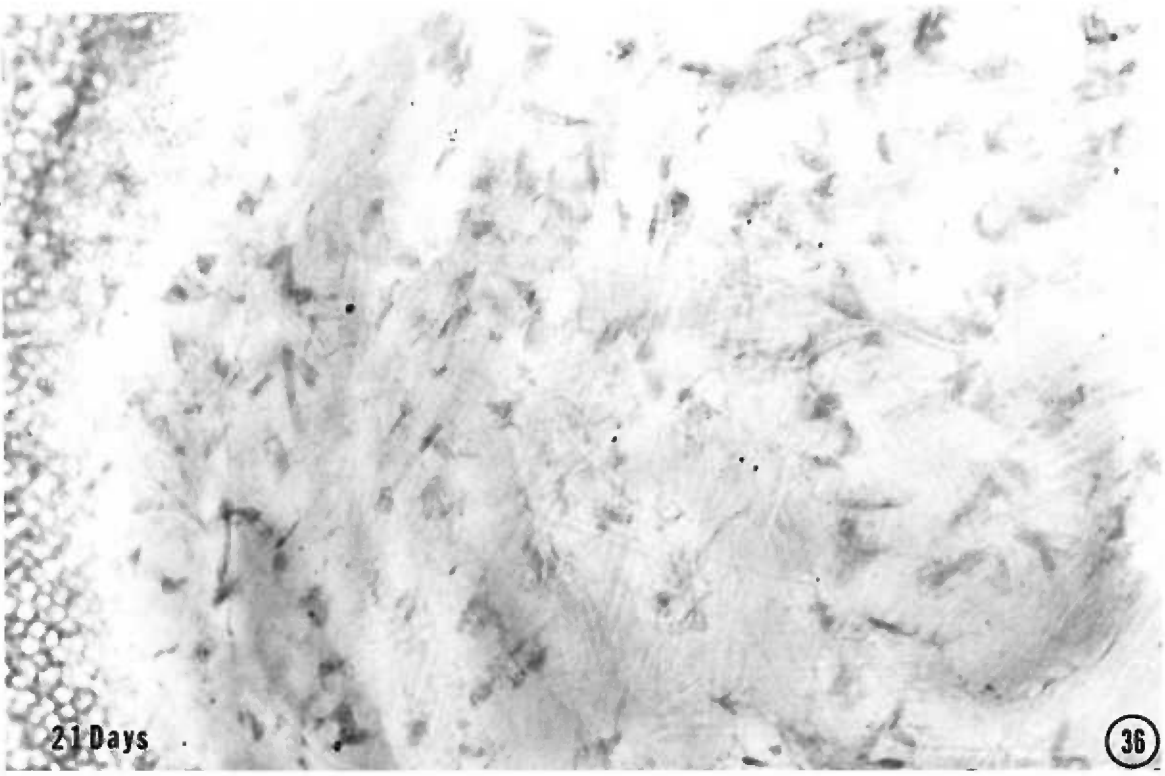
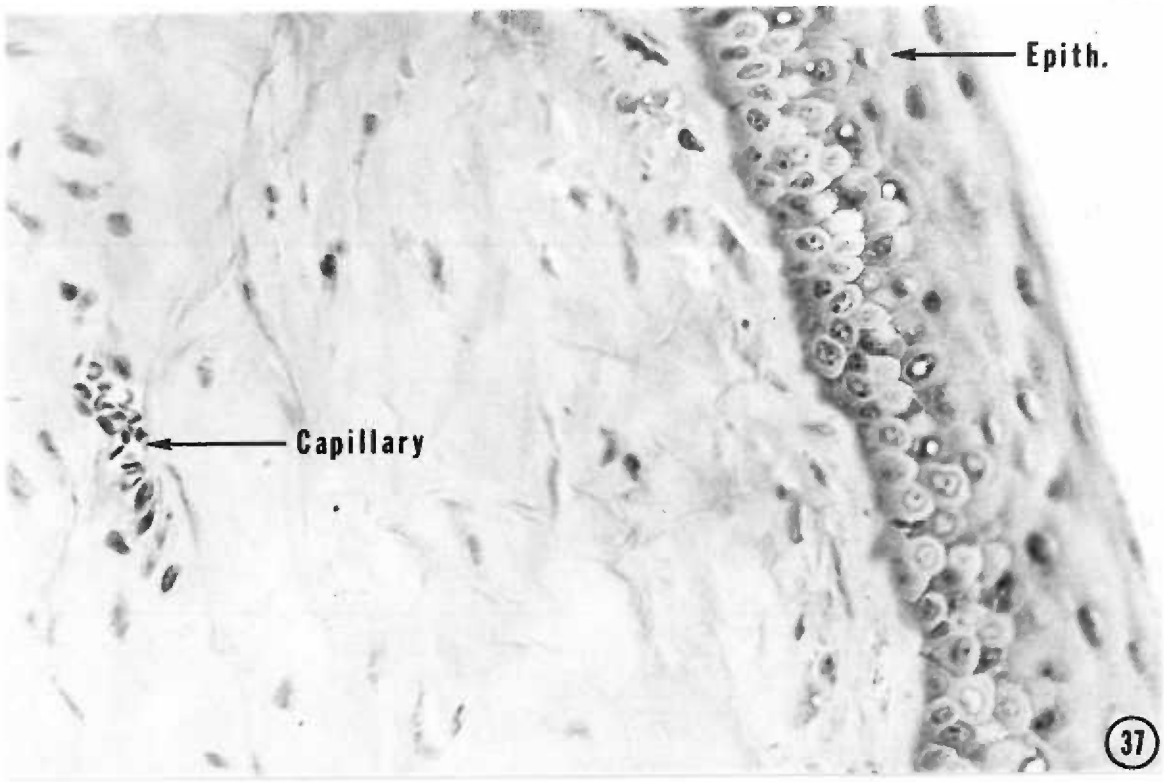
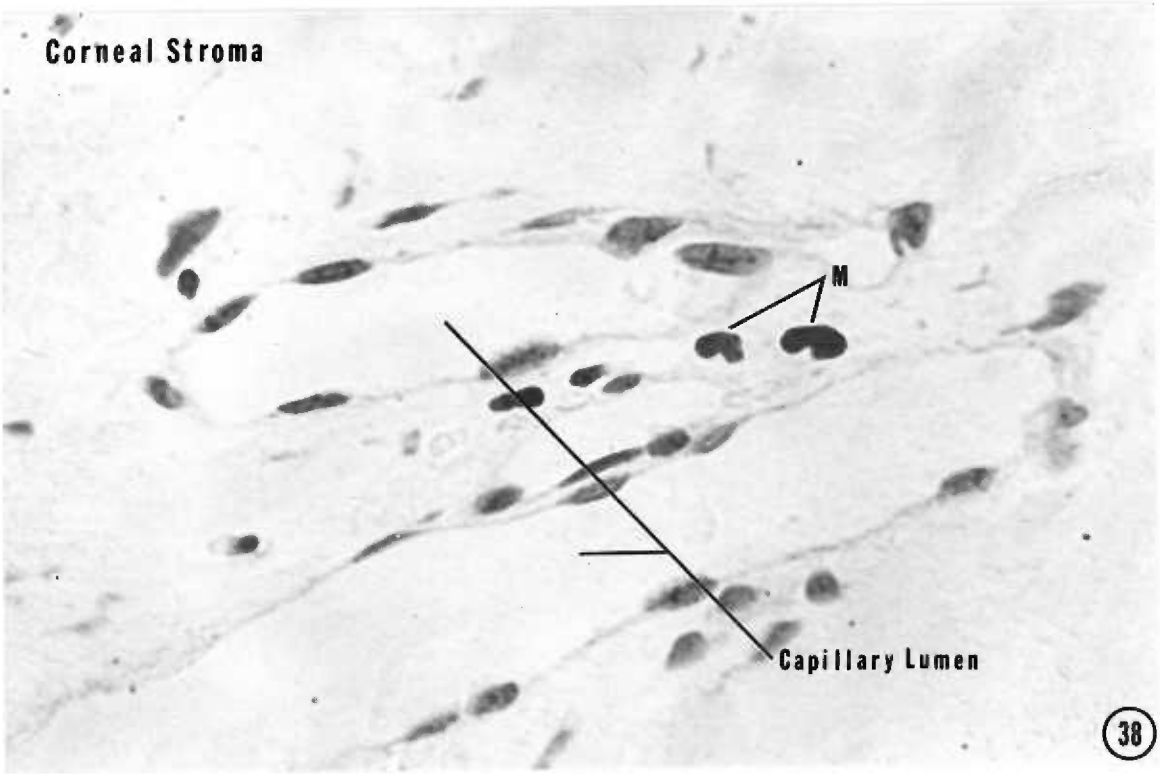

21 Days
36

Figure 37. Vascularized healing did occur in a very limited number of those corneas that were subjected to the freeze injury. Those corneas that had undergone vascularization were eliminated from the study. (X 400)

Figure 38. Vascularized healing in the freeze-injured cornea. Note the presence of two monocytes adjacent to the capillaries. (X 1000)


Epith.
Capillary
37


Corneal Stroma
M
Capillary Lumen
38

the epithelial denuded area. Regeneration of the entire corneal epithelium was achieved in about 6 days. Following the traumatic removal of the epithelium the corneal stromal cells underwent a gradual transition into fibroblasts (figure 39). Few stromal cells apparently were killed in this procedure and there was little evidence of mitotic activity.

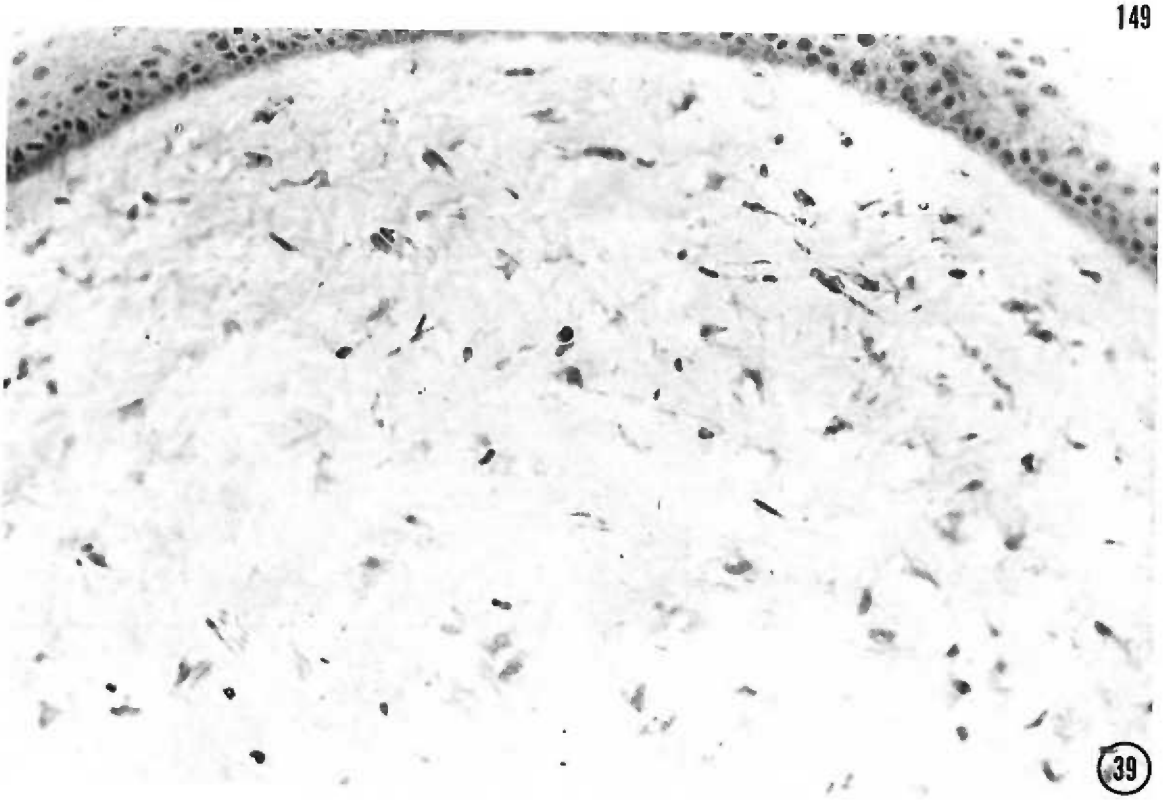
Weimar (40) has presented data which indicates that the crushed epithelium liberates an unknown factor which immediately causes the activation of the underlying corneal stromal cells. Weimar further noted that the growth of epithelium over the denuded stroma caused a pronounced transformation of the activated stromal cells into fibroblast-like cells throughout the cornea. This suggested to the author that a possible cause of the transformation of cells at the wound edge may be substances released by the actively growing epithelium at the wound edge.

Autoradiography

Control Labeling of Corneal Stromal Cells Following Traumatic Removal of the Epithelium

Figures 40 and 41 are representative photomicrographs of autoradiographs of stromal cells labeled with ^{3}H-Tdr. Preliminary experiments were carried out to develop a procedure which would not require relatively large quantities of radioactive Tdr for labeling

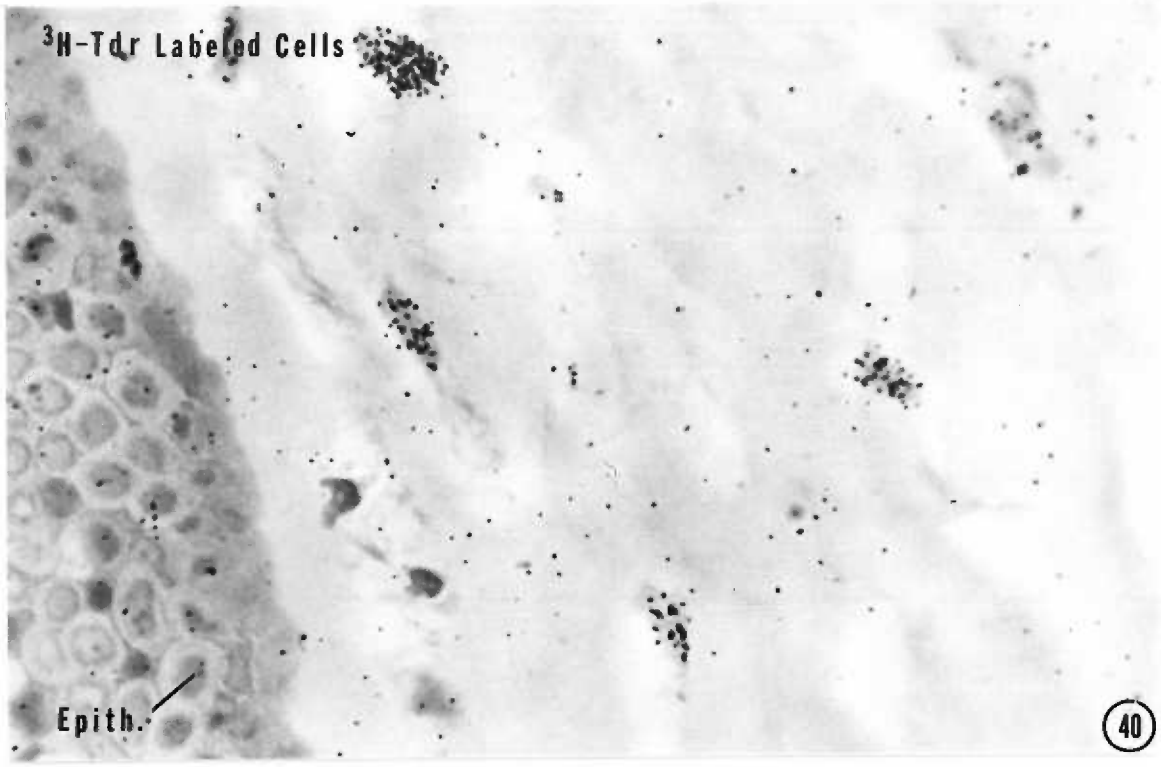
Figure 39. One week following the traumatic removal of the epithelium the corneal stromal cells had undergone a gradual transition into fibroblasts as shown in this photomicrograph. Few stromal cells apparently were killed in this procedure and there was little evidence of mitotic activity. Regeneration of the entire corneal epithelium was achieved in about 6 days. (X 250)

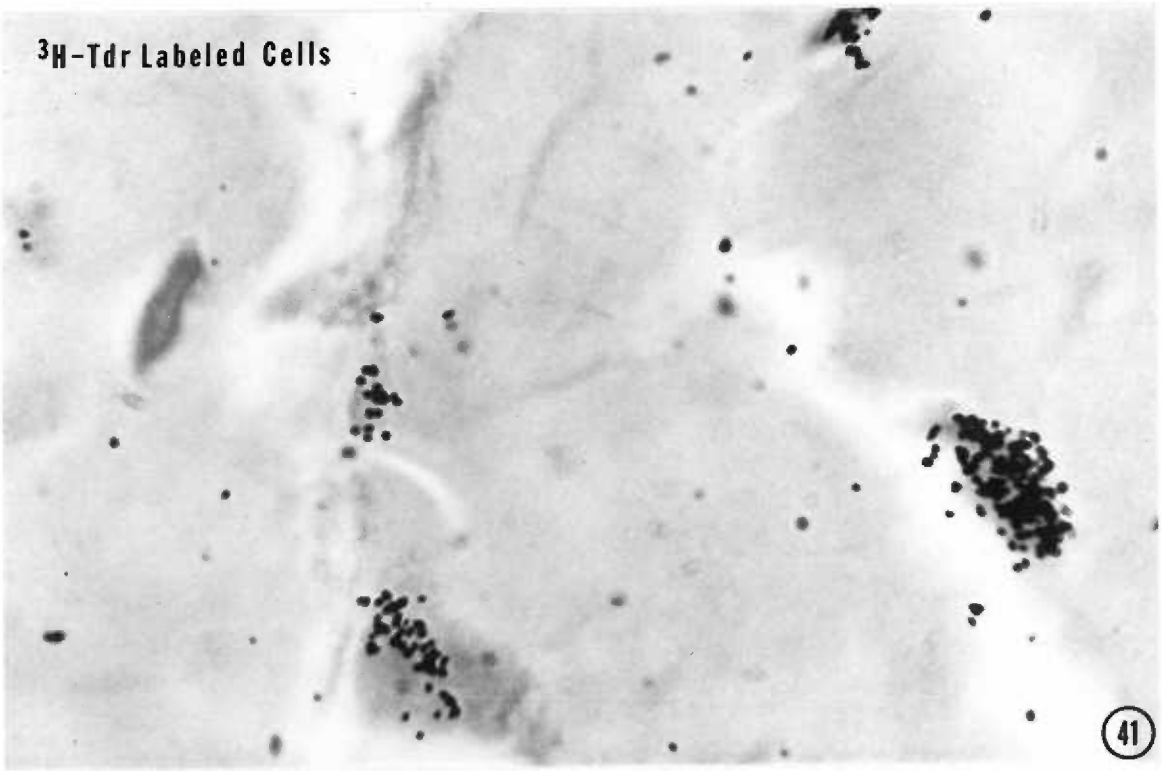


39

Figure 40. Corneal stromal cells labeled via the anterior chamber with ^{3}H-Tdr. The radioactive precursor was able to diffuse from the anterior chamber and traverse the full thickness of the stroma, and subsequently to be incorporated into the DNA of those stromal cells lying immediately beneath the regenerated epithelium. (X 400)

Figure 41. A higher magnification of the area seen in Figure 40 shows the ^{3}H-labeled stromal cells. (X 1000)


^{3}H-Tdr Labeled Cells
Epith.
40


^{3}H-Tdr Labeled Cells
41

the corneal stromal cells. Since the cost of ^{14}C-Tdr is approximately thirty times that of ^{3}H-Tdr for a comparable amount of radioactivity, these preliminary experiments were performed using the less expensive ^{3}H-Tdr. The epithelium has been included in figure 40 to illustrate an important finding. In this labeling technique the epithelium was first scraped off the cornea, and later the injured eye received two separate injections of radioactive Tdr into the anterior chamber. The radioactive precursor was able to diffuse from the anterior chamber and traverse the full thickness of the stroma, and subsequently to be incorporated into the DNA of those stromal cells lying immediately beneath the regenerated epithelium. These results indicate that this labeling technique allows stromal cells undergoing DNA synthesis to incorporate the label regardless of its distance from the anterior chamber. Approximately 65 percent of the stromal cells are labeled in this procedure with the labeled cells appearing to be randomly distributed throughout the stroma.

Intravenous Labeling of the Bone Marrow with Tritiated Thymidine

Autoradiographs were made from bone marrow samples obtained from two groups of animals at 8 hours (figure 42) and 32 hours (figure 43) following the second intravenous injection of ^{3}H-Tdr, respectively. Results from the autoradiographs revealed that the intravenous method selected for labeling the bone marrow

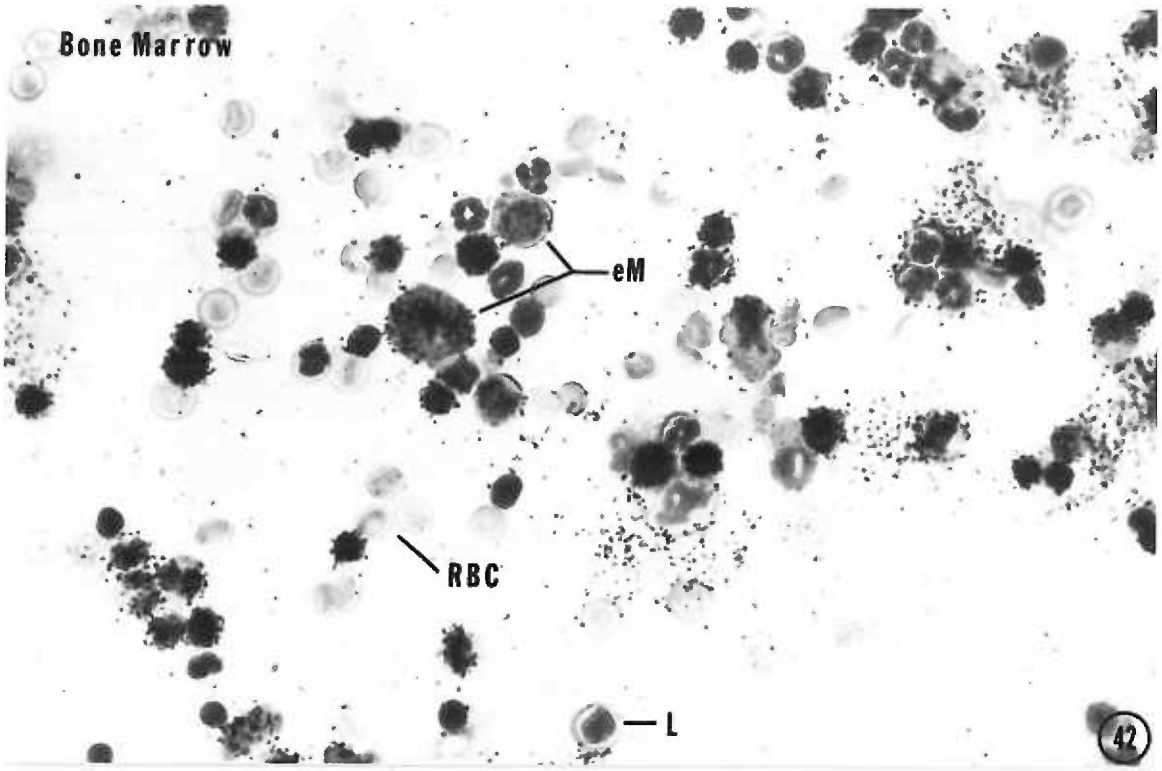
appeared to be quite effective. There was intense labeling (figure 42 and 43) in a large proportion of the developing myeloid elements of the bone marrow. An additional important finding disclosed by the autoradiographs was that the morphologically mature appearing PMN leucocytes were essentially unlabeled. This condition was true for bone marrow samples obtained at both time intervals following the labeling procedure. These results were anticipated on the basis of bone marrow labeling studies conducted by Brant and Kelly (139). These investigators did not see labeled bone marrow PMN leucocytes in mice at one day following an in vivo pulse of ^{3}H-Tdr. Labeled bone marrow neutrophils did appear by the end of the second day, however, these neutrophils were in the band stage of maturation. In mice these band neutrophils are retained in the bone marrow a minimum of one day before they are released to the peripheral circulation as mature segmented neutrophils--i. e., PMN leucocytes (139). Thus, the presence of labeled PMN leucocytes in the peripheral blood is rare until the third day subsequent to the labeling procedure.

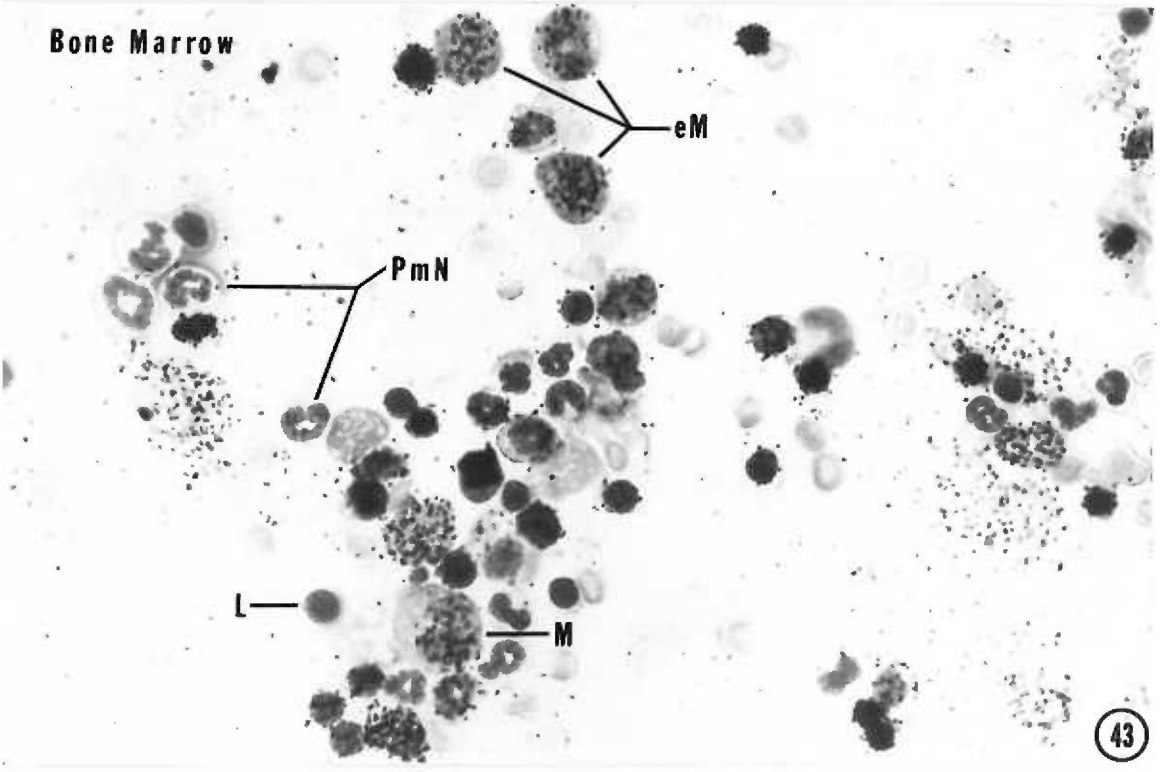
Examination of the Peripheral Blood Following the Intravenous Labeling Procedure

Blood smears prepared from blood samples obtained from two groups of animals at 8 and 32 hours following the second intravenous injection of ^{3}H-Tdr, respectively, revealed the monocyte as the only

Figure 42. Autoradiogram of a bone marrow sample taken 8 hours following the second intravenous injection of ^{3}H-Tdr. There was intense labeling in a large proportion of the developing myeloid elements of the bone marrow. Morphologically mature appearing PMN leucocytes were essentially unlabeled. RBC = red blood cell; eM = early mononuclear cell; L= lymphocyte.

Figure 43. Autoradiogram of a bone marrow sample taken 32 hours following the second intravenous injection of ^{3}H-Tdr. There was little evidence of neutrophil labeling at this time. PmN = polymorphonuclear leucocyte; eM = early mononuclear cell; M = mononuclear cell; L = lymphocyte. (X 400)


Bone Marrow
eM
RBC
L
42


Bone Marrow
eM
PmN
L
M
43

cell prominently labeled. Figure 44 shows the state of the peripheral blood at the time of the administration of the corneal freeze injury. Labeled lymphocytes and PMN leucocytes were not observed at this time. Similar results were seen in blood samples drawn 24 hours later (figure 45). These results strongly suggest that the monocyte would be the only labeled inflammatory cell found in the cornea during the first 48 hours following the freeze injury.

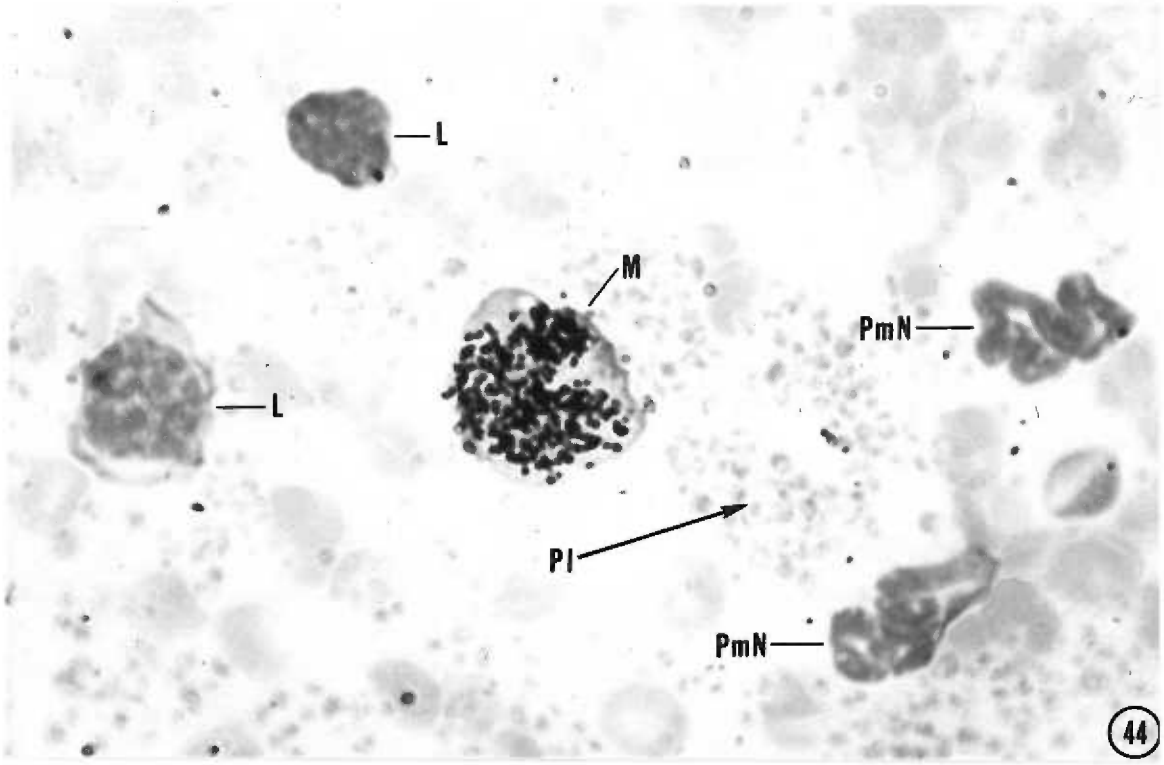
Carbon-14 Thymidine Labeled Corneal Stromal Cells

For reference purposes figure 46 shows the typical histological appearance of the uninjured cornea in a resin section cut at 1 μ as compared with cryostatic sections cut at 15 μ (figure 16).

Figure 47 represents the first emulsion layer autoradiographic results obtained from those animals that had had their stromal cells labeled with ^{14}C-Tdr via the anterior chamber, but had not subsequently received a corneal freeze injury (group II-B). Cell counts made at the completion of the autoradiographic procedure disclosed that approximately 65 percent of the stromal cells had incorporated ^{14}C-Tdr. The degree of isotope uptake by individual cells, as judged by the intensity of the silver grain counts over the cells, indicated that the cells were uniformly labeled. Thus, it seems that the labeling may have been an all or none phenomenon. There appeared to be a random distribution of the labeled cells throughout the full thickness of the stroma. Silver grains produced by ^{14}C-Tdr, owing to the longer range of its

Figure 44. Blood smear autoradiogram prepared from blood obtained from an animal 8 hours following the second intravenous injection of ^{3}H-Tdr. This period corresponds to the time of the administration of the corneal freeze injury in the experimental animals. The monocyte was the only cell prominently labeled. L = lymphocyte; M = monocyte; PmN - polymorphonuclear leucocyte; Pl = platelets. (X 1000)

Figure 45. Blood smear autoradiogram prepared from blood obtained from an animal 32 hours following the second intravenous injection of ^{3}H-Tdr. The monocyte continued to be the cell that was prominently labeled. This finding strongly suggests that the monocyte would be the only labeled inflammatory cell found in the cornea during the first 48 hours following the freeze injury. L = lymphocyte; M = monocyte; PmN = polymorphonuclear leucocyte. (X 1000)


L
M
PmN
L
PI
PmN
44

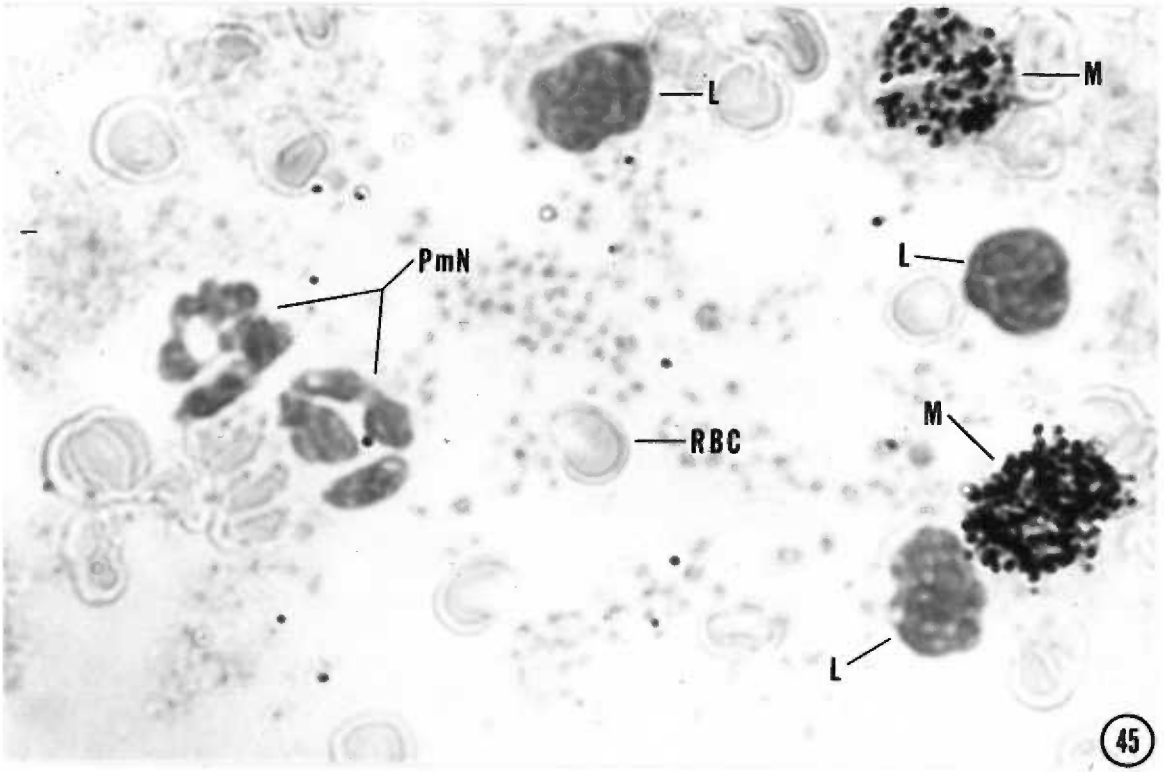
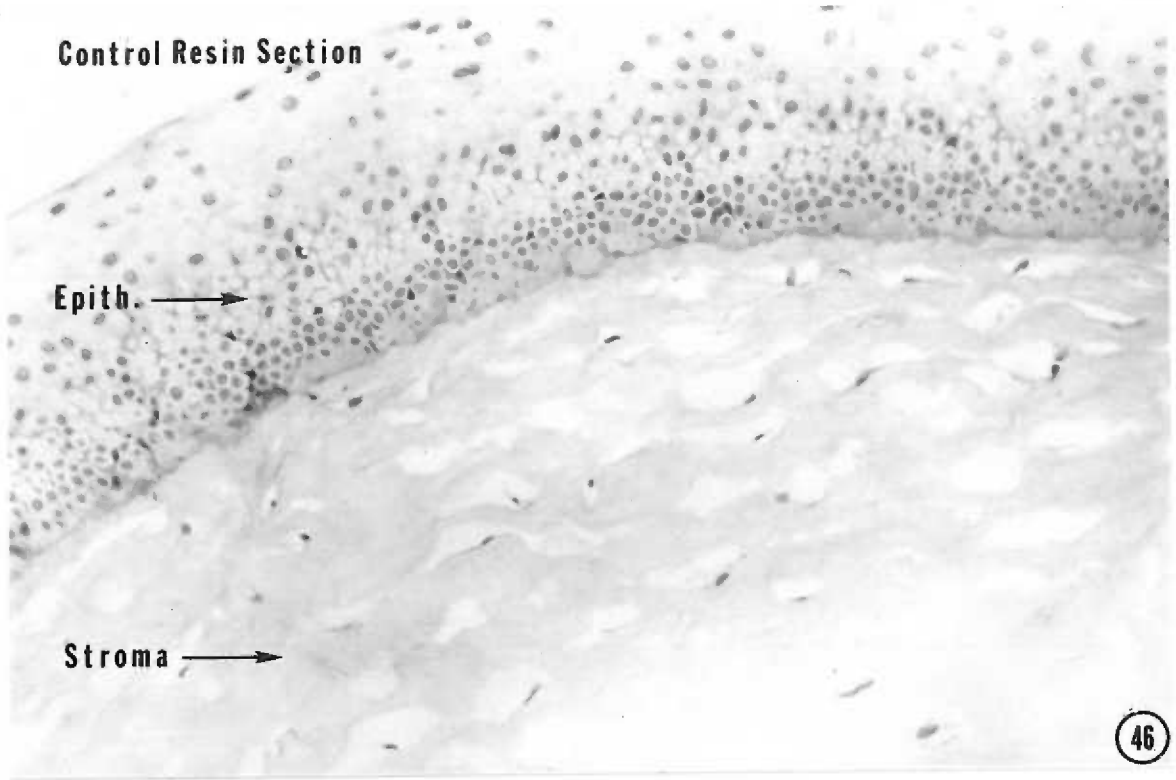
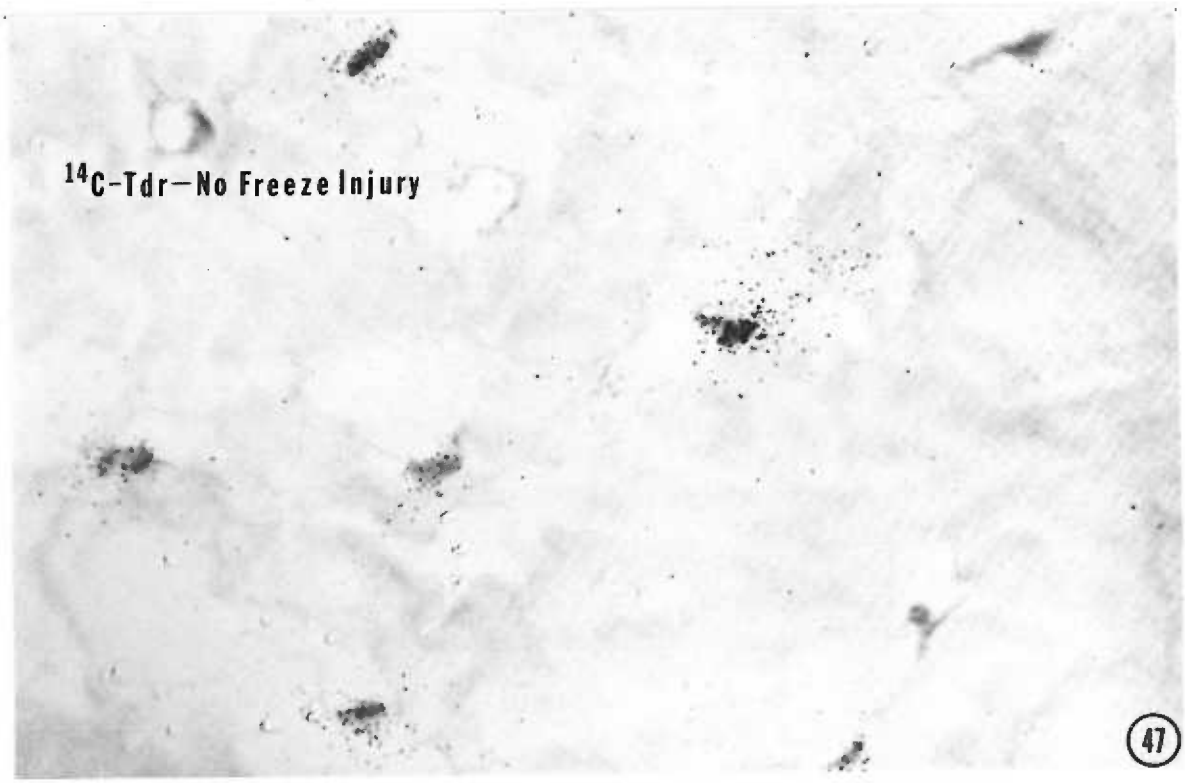

L
M
PmN
L
RBC
M
L
45

Figure 46. Control resin section showing the histological appearance of the uninjured cornea. There are fewer stromal cells in the resin section when contrasted with a comparable cryostatic section. This apparent discrepancy is due to the resin and cryostatic sections being cut at 1 and 15 μ, respectively. (X 250)

Figure 47. A representative autoradiogram of the first emulsion layer autoradiographic results obtained from those animals that had their stromal cells labeled with ^{14}C-Tdr via the anterior chamber, but did not receive a corneal freeze injury. (X 400)


Control Resin Section
Epith.
Stroma
46


^{14}C-Tdr—No Freeze Injury
47

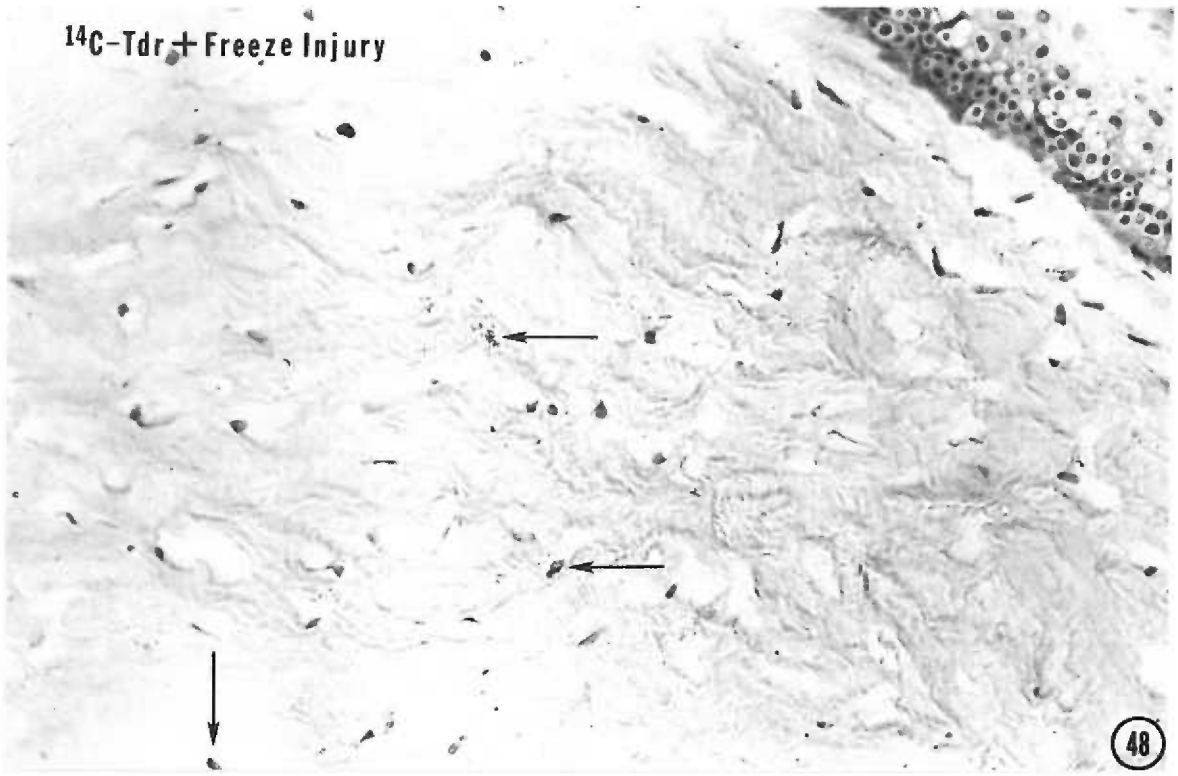
β-particles (E_{mean} = 0.05 Mev), appeared as a halo around the stromal cells (figure 47), while those grains produced by ^{3}H-Tdr, with relatively short range β-particles E_{mean} = 0.0055 Mev), are concentrated within the stromal cell outline (figure 40).

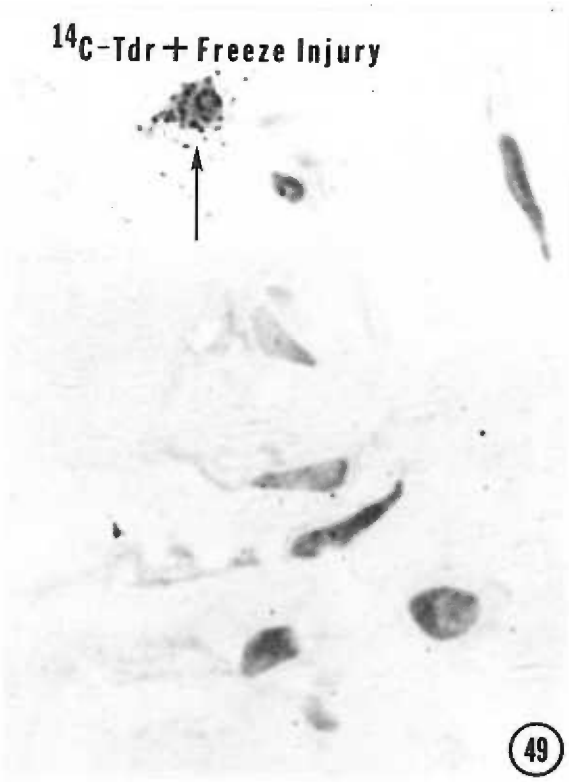
There was a marked decrease in the number of labeled stromal cells observed in those animals that had their stromal cells labeled with ^{14}C-Tdr via the anterior chamber, and had subsequently received a corneal freeze injury (group II-A). Figures 48, 49 and 50 are photomicrographs of autoradiographs of ^{14}C-Tdr labeled corneas that received a freeze injury (group II) and then were allowed to undergo complete wound repair. Since the freeze injury killed all stromal cells beneath the cryoprobe tip, then by necessity those cells present in the repaired wound had to have arisen from infiltrated labeled cells. Nearly 60 percent of these infiltrating cells did not contain ^{14}C as measured by autoradiography. In general the level of ^{14}C activity in those cells that were labeled (figure 49) appeared comparable to the amount of ^{14}C activity found in the labeled stromal, cells of the non-frozen cornea (figure 47). This finding would support the concepts that the ^{14}C labeled cell present in the repaired freeze wound had not undergone mitosis, nor had the cell incorporated the label by isotope reutilization. Had either of these events taken place, then a wide variance in the amount of cellular isotope incorporation

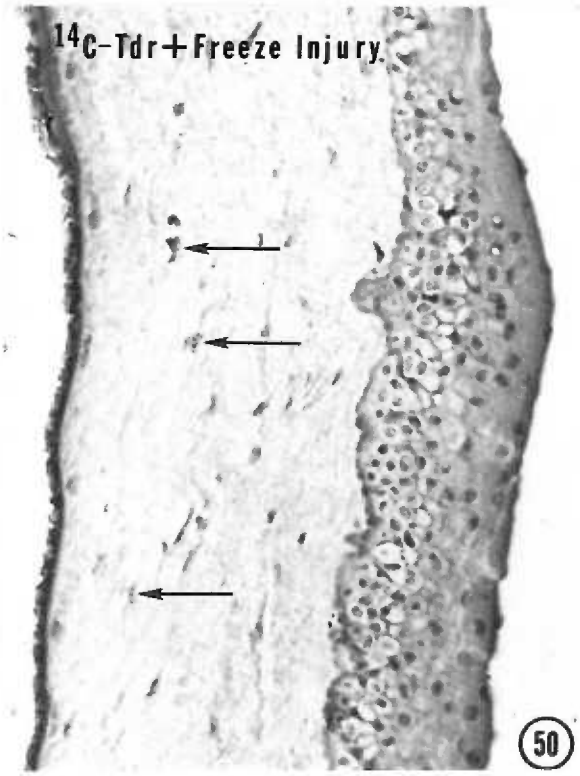
Figure 48. There was a marked decrease in the number of labeled cells observed in those animals that had their stromal cells labeled with ^{14}C-Tdr via the anterior chamber, and subsequently received a corneal-freeze injury. The arrows indicate labeled cells. (X 250)

Figure 49. A representative autoradiogram of a ^{14}C-labeled corneal stroma that had received the freeze injury and was allowed to undergo complete wound repair. The arrow indicates labeled cell. (X 1000)

Figure 50. A cross section of a ^{14}C-labeled cornea that had received the freeze injury and was allowed to undergo complete wound repair. Frequently the ^{14}C-labeled cells were more numerous in the posterior one half of the corneal stroma. The arrows indicate labeled cells. (X 250)


^{14}C-Tdr + Freeze Injury
48


^{14}C-Tdr + Freeze Injury
49


^{14}C-Tdr + Freeze Injury
50

per cell would have been anticipated. Frequently the ^{14}C labeled cells were more numerous in the posterior one half of the corneal stroma.

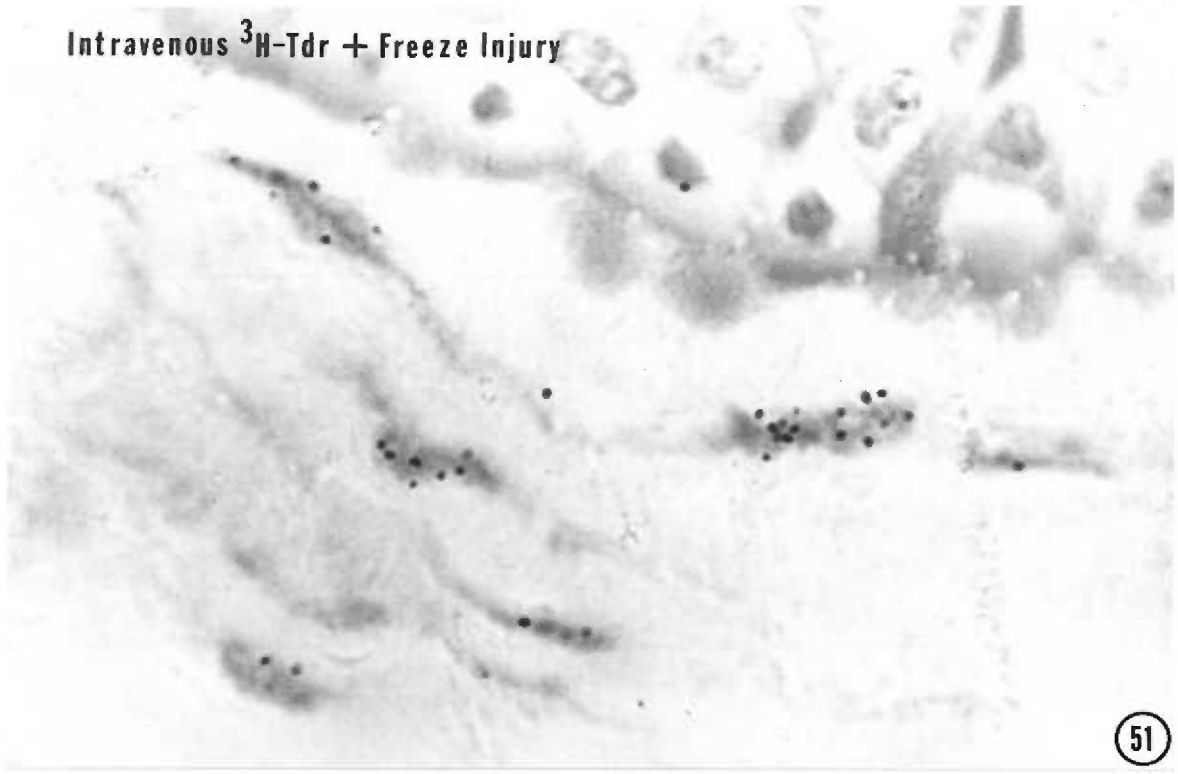
Tritiated Thymidine Labeled Corneal Stromal Cells

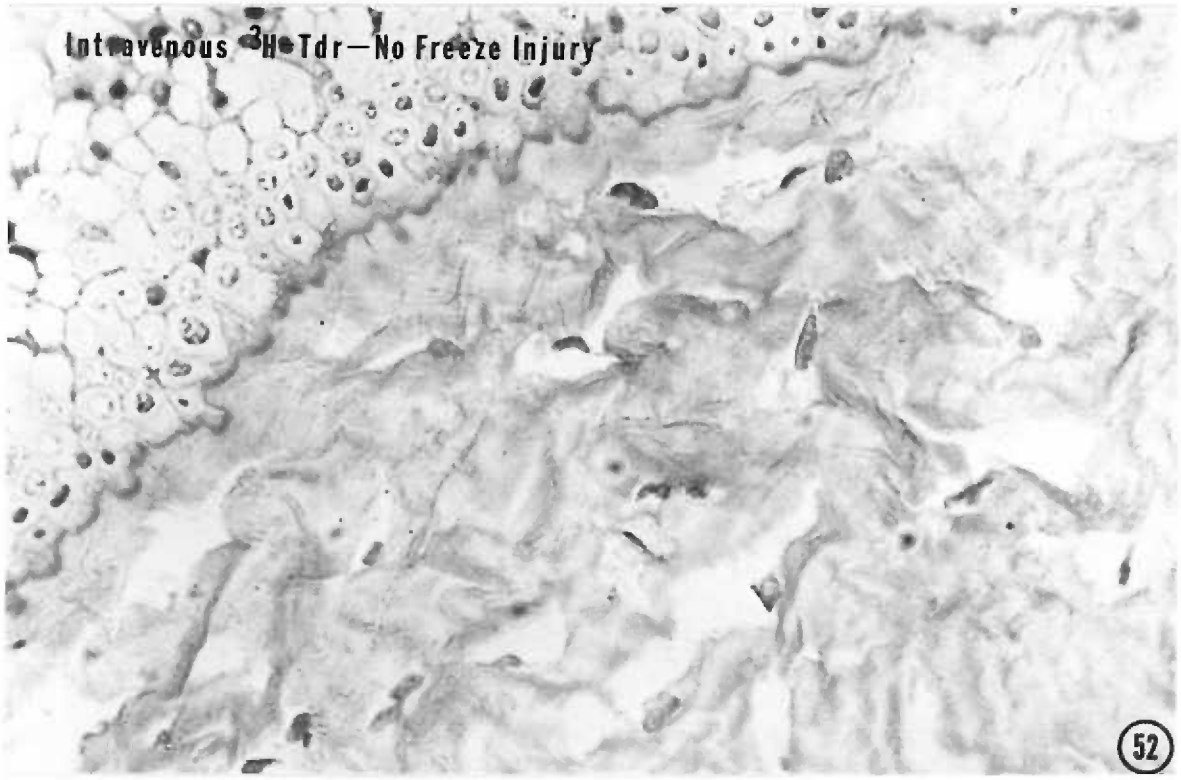
Figures 51 and 52 represent the first emulsion layer autoradiographic results obtained from those animals that had their mononuclear blood cells labeled with ^{3}H-Tdr. Half of these animals subsequently had their corneas frozen (group III-A). Following the repair of the freeze injured cornea, cell counts revealed that nearly 55 percent of the stromal cells contained ^{3}H (figure 51). As a rule the ^{3}H labeled cells were more numerous in the anterior one half of the corneal stroma. During the experiment an arbitrary number of 5 or more silver grains over a cell was defined as constituting a labeled cell. It is fortunate that the intercellular spacing of the stromal cells is relatively wide, because this aids in reducing the possibility of the β-particles of ^{14}C or ^{3}H from producing silver grains located over cells other than those from which they originated. In addition the background (silver grains appearing in the developed emulsion which are not due to radiation from the experimental source) was low in these experiments. Therefore a count of 5 or more silver grains over a cell is a reasonable standard to define a labeled cell.

There was no labeling of the stromal cells in those animals with labeled mononuclear blood cells that did not receive a corneal freeze

Figure 51. Autoradiogram showing the presence of labeled stromal cells in an animal that had its mononuclear blood cells labeled with ^{3}H-Tdr and later received the freeze injury. As a rule the ^{3}H-Tdr labeled cells were more numerous in the anterior one half of the corneal stroma. (X 1000)

Figure 52. There was no labeling of the stromal cells in those animals with ^{3}H-labeled mononuclear blood cells that did not receive a corneal-freeze injury. (X 400)


Intravenous ^{3}H-Tdr + Freeze Injury
51


Intravenous ^{3}H-Tdr—No Freeze Injury
52

injury (figure 52). This finding is not surprising since it has been well established that cell division is very infrequent in the fixed cells of the normal corneal stroma (140). It appears then that the majority of the ^{3}H arrives in the stroma as a constituent of the DNA of hematogenous cells--i.e., in all probability the blood borne-monocytes. Occasionally a ^{3}H labeled epithelial cell was seen in the non-freeze injured eye.

Could these ^{3}H labeled stromal cells be derived from labeled perivascular cells that were present in the limbus of the eye and later migrated to the wound site following the freeze injury? To answer this question, sections were prepared from the limbal tissue of animals that had undergone the ^{3}H-Tdr intravenous labeling procedure. The tissue samples were taken 8 hours following the last ^{3}H-Tdr pulse, which corresponded to that time period when the corneal freeze injury was performed on the experimental animals. Examination of the autoradiographs disclosed that labeled limbal cells were only rarely found.

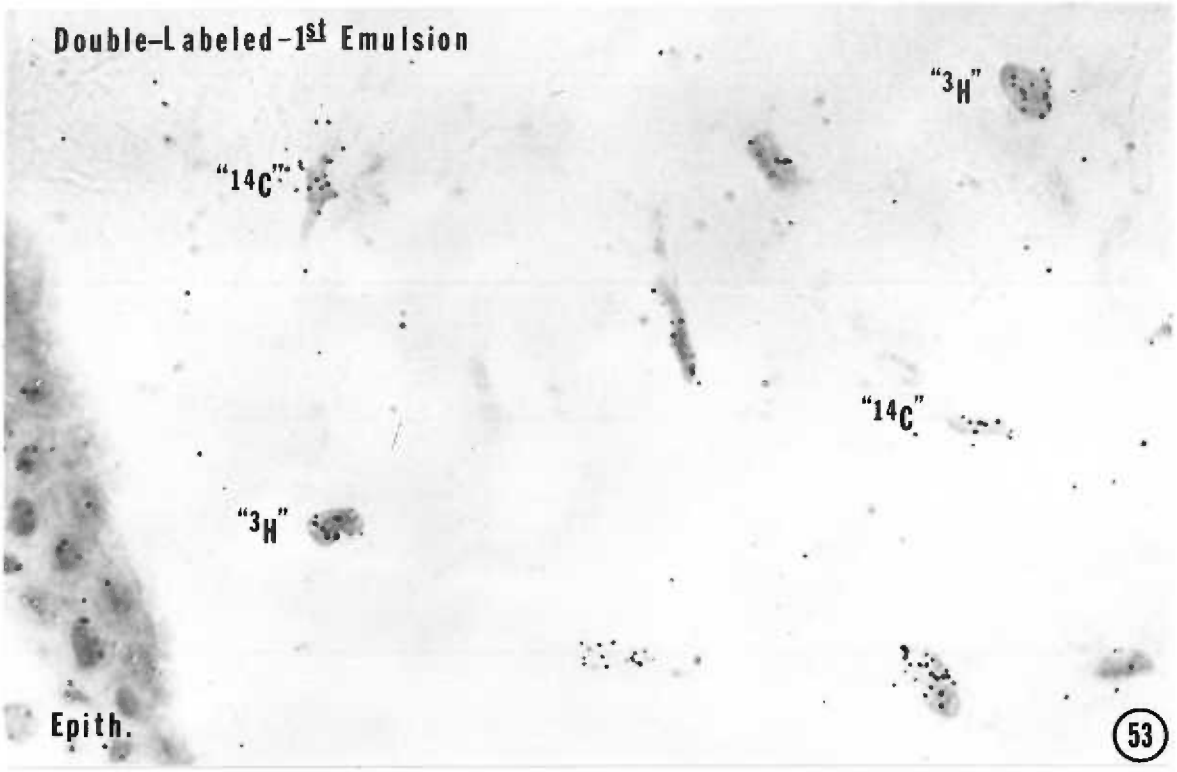
Double-Labeled Corneal Stromal Tissue--Single Emulsion

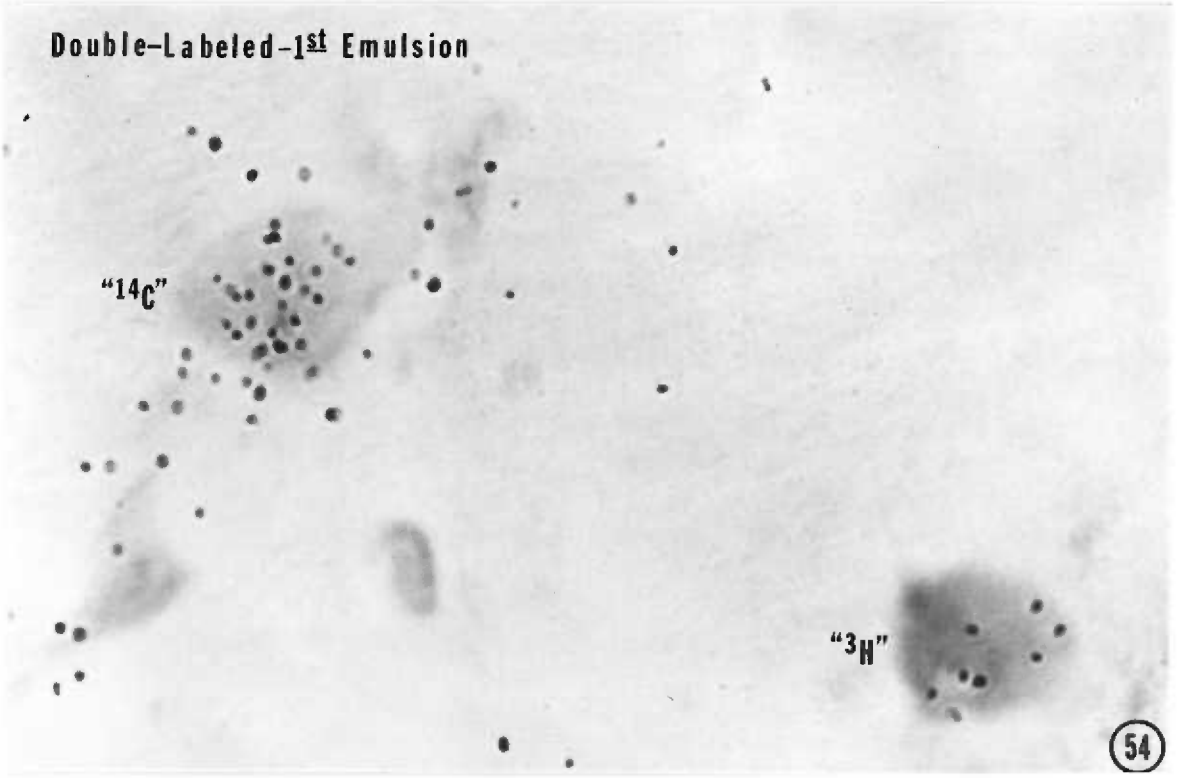
All double-labeled corneas were derived from animals having their stromal cells initially labeled with ^{14}C-Tdr via the anterior chamber and later their peripheral mononuclear blood cells labeled with ^{3}H-Tdr, followed by a standardized corneal freeze injury

(group I). Figures 53 and 54 are examples of data obtained following the processing of the first emulsion. As was described earlier it is quite possible with a single emulsion layer to roughly distinquish whether a cell is labeled with ^{14}C or ^{3}H. The two cells in figure 54 illustrate this point very well. The cell designated as containing ^{14}C displays the scattering of silver grains as a halo around the nucleus which is characteristic of ^{14}C β decay. In contrast, the cell designated as containing ^{3}H produced silver grains confined to the immediate area over the nucleus. To the casual observer the ^{3}H-labeled cell appears to have much less radioactivity than the ^{14}C-labeled cell (figure 54). This may not be the case. The unit which expresses the quantity of radioactivity is the curie. This unit is defined as a decay rate of 3.7×10^{10} disintegrations per second (141). During the process of radioactive decay the ^{3}H and ^{14}C atoms emit β-particles which may enter the emulsion of the autoradiographic preparation. The range of the β-particles in the emulsion is largely determined by their energy--i.e., the amount of kinetic energy with which the β-particle is emitted from the ^{3}H or ^{14}C nucleus. As the charged β-particle passes through an emulsion a latent image is formed by the transfer of kinetic energy from the trajectory of the particle to a silver bromide crystal. This transfer of energy results in the silver bromide crystal acquiring additional electrons which reduces some of the silver ions into elementary silver. In this state

Figure 53. Autoradiogram of a double-labeled cornea following the processing of the first emulsion. It is possible with the single emulsion layer to roughly distinguish whether a cell is labeled with ^{14}C or ^{3}H. The cells designated as containing ^{14}C display the scattering of silver grains as a halo around the nucleus which is characteristic of ^{14}C β decay. In contrast the cells designated as containing ^{3}H produced silver grains confined to the immediate area over the nucleus. (X 400)

Figure 54. Upon higher magnification the presence of ^{14}C or ^{3}H labeled cells becomes more discernible. Note that the silver grains over the "^{3}H" cell are in one plane while those silver grains over the "^{14}C" cell are located at various levels of the emulsion. (X 1000)


Double-Labeled-1st Emulsion
"3H"
"14C"
"14C"
"3H"
Epith.
53


Double-Labeled-1st Emulsion
"14C"
"3H"
54

the crystal is susceptible to reducing agents (developers) which convert more silver ions into metallic silver, thus yielding the final image. Therefore, for a given amount of radioactivity, (expressed in curies) the ^{14}C labeled cell will produce more silver grains in the autoradiographic emulsion than a ^{3}H-labeled cell because the ^{14}C β-particles have a higher mean energy value than those of ^{3}H.

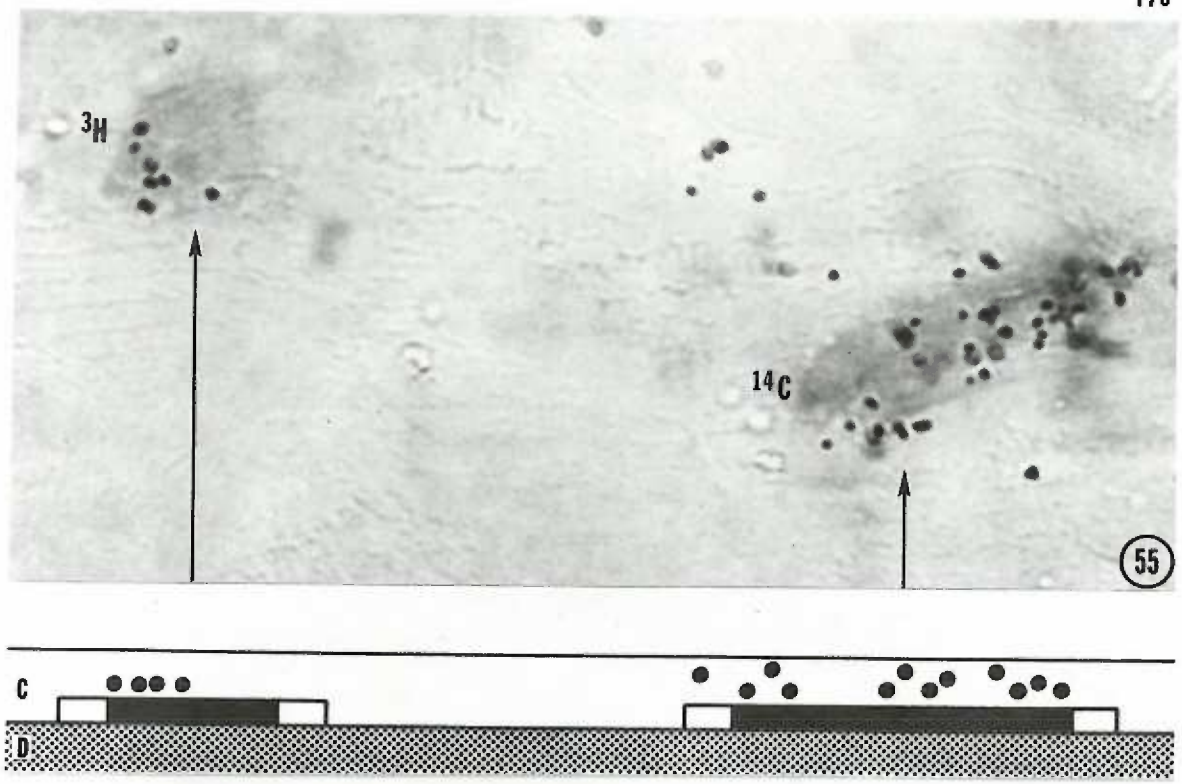
Double-Labeled Corneal Stromal Tissue--Double Emulsion

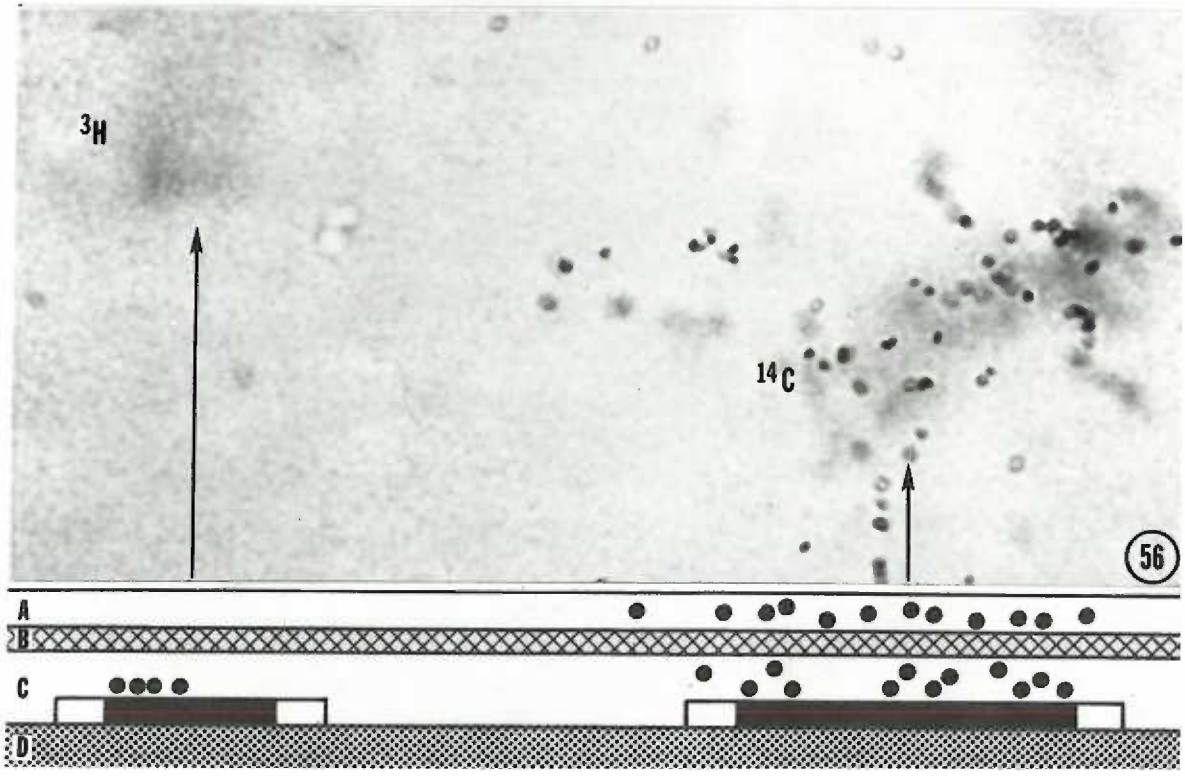
Figures 55 and 56 are graphic and photomicrographic montages that illustrate the method by which the double-label/double-emulsion autoradiographic technique is evaluated. Upon examination with an oil immersion objective, the first emulsion layer (figure 55) and the second emulsion layer (figure 56) can be separated by differential focusing. No difficulty was encountered in distinguishing whether silver grains were located in the first or the second emulsion because the collodion layer provided a sharp line of demarcation between the two emulsions. Both ^{3}H and ^{14}C-labeled cells were seen in the first emulsion, but as the field of focus was moved upward through the collodion layer and into the second emulsion only the ^{14}C-labeled cells showed evidence of β-particle activity..

To substantiate that only ^{14}C β-particles can reach the second emulsion, corneal tissue sections containing stromal cells labeled via the anterior chamber with ^{3}H-Tdr were prepared for double-layer

Figure 55. A line drawing and a photomicrograph are combined to illustrate the results seen in the processed first emulsion of the double-label/double-emulsion autoradiographic technique. Both ^{3}H and ^{14}C labeled cells are seen in the first emulsion. C = first emulsion; D = glass slide. (X 1000)

Figure 56. A line drawing and a photomicrograph are combined to illustrate the results seen in the processed first and second emulsions of the double-label/double-emulsion autoradiographic technique. As the microscopic field of focus is moved upward through the collodion layer and into the second emulsion, only the ^{14}C-labeled cells showed evidence of β-particle activity. A = second emulsion; B = collodion layer; C = first emulsion; D = glass slide. (X 1000)


^{3}H
^{14}C
55
C
D


^{3}H
^{14}C
56
A
B
C
D

autoradiographic evaluation. The results of this procedure are shown in figures 57 and 58. Although the cell is relatively heavily labeled for ^{3}H (figure 57) no evidence of β-particle penetration into the second emulsion was seen (figure 58). This finding illustrates that the lack of silver grain production in the second emulsion is not due to a deficiency in the amount of cellular isotope incorporation, but is rather a factor of the lower β energy of ^{3}H. Results similar to those seen in figures 57 and 58 were obtained in the double-labeled stroma when a relatively heavily ^{3}H-labeled cell was selected for examination (figures 59 and 60).

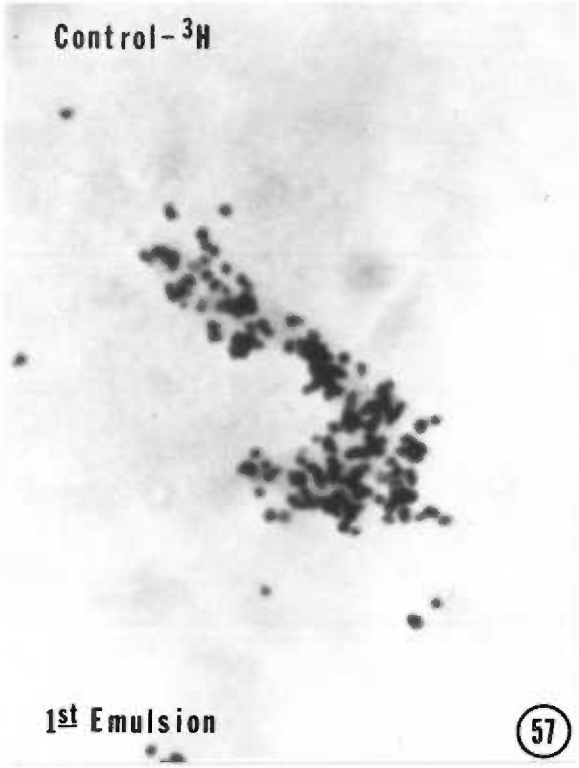
Can a cell containing a low level of ^{14}C activity be misinterpreted as being a ^{3}H-labeled cell? There exists a direct relationship between the degree of cellular ^{14}C-Tdr incorporation and the number of β-particles emitted by the cell per unit time. Therefore, the lower the ^{14}C-Tdr incorporation the lower the mathematical probability that the second emulsion layer could record the passage of β-particles. Upon examination of the double-labeled/double-emulsion autoradiographs it was noted that the autoradiographic technique was sufficiently sensitive to detect ^{14}C activity in relatively lightly labeled stromal cells. Figures 61 and 62 illustrate this finding. In figure 61 two lightly labeled cells can be seen when the field of focus was positioned immediately above the tissue section. As the field of focus was moved upward the silver grains produced by

Figure 57. To substantiate that only ^{14}C β-particles can reach the second emulsion, corneal tissue sections containing stromal cells labeled via the anterior chamber with ^{3}H-Tdr were prepared for double-layer autoradiographic evaluation. The first emulsion showed a relatively heavily labeled cell. (X 1000)

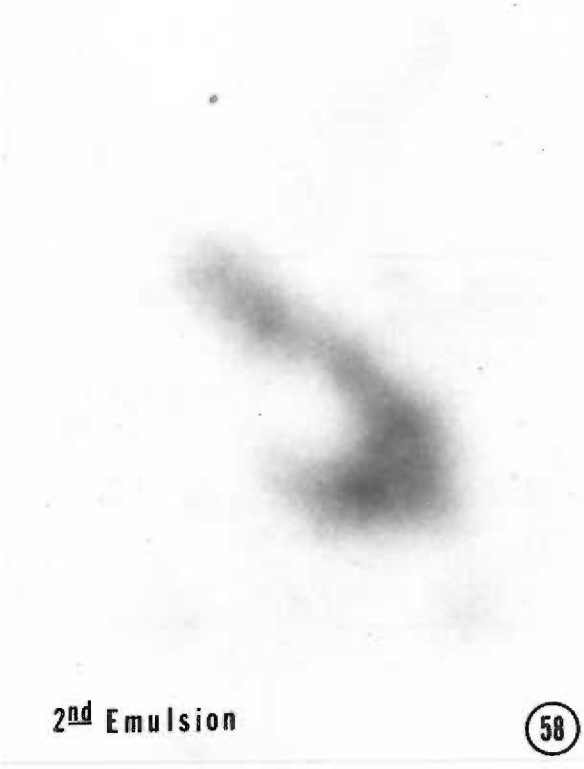
Figure 58. The second emulsion showed no evidence of ^{3}H β-particle penetration through the collodion barrier. (X 1000)

Figure 59. Results similar to those seen in figures 57 and 58 were obtained in the double-labeled stroma when a relatively heavily ^{3}H-labeled cell was selected for examination. Evidence of ^{3}H β-particle activity was seen in the first emulsion. (X 1000)

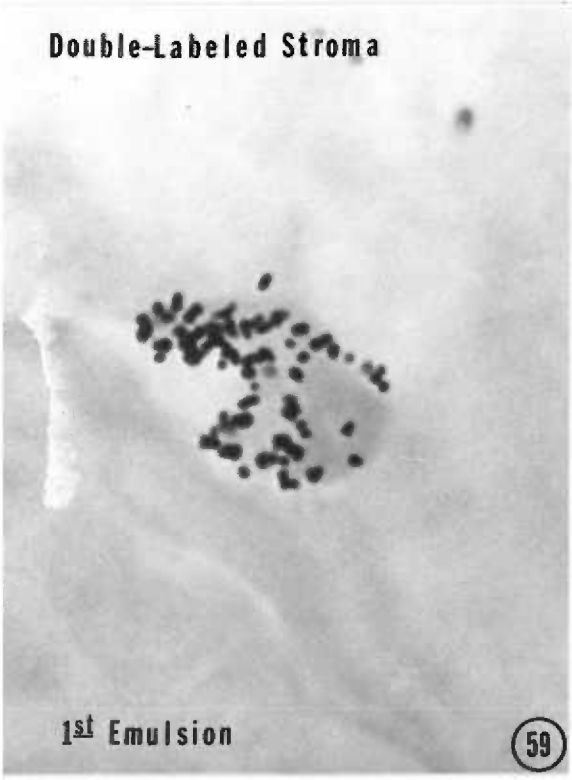
Figure 60. The second emulsion showed no evidence of ^{3}H β-particle penetration through the collodion barrier. (X 1000)



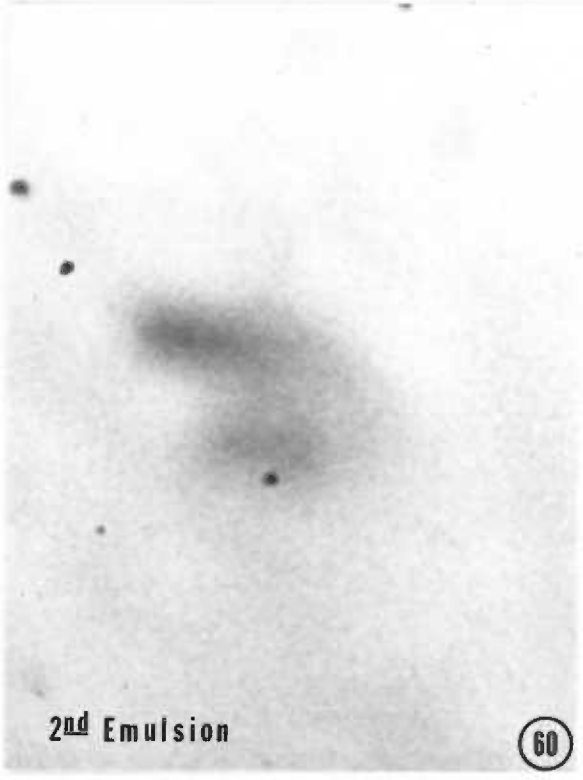
1st Emulsion (57)



2nd Emulsion (58)



1st Emulsion (59)



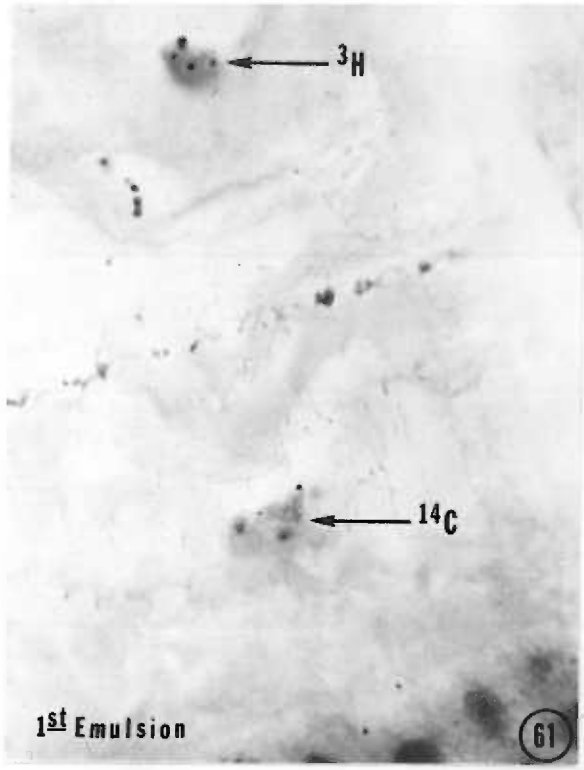
2nd Emulsion (60)

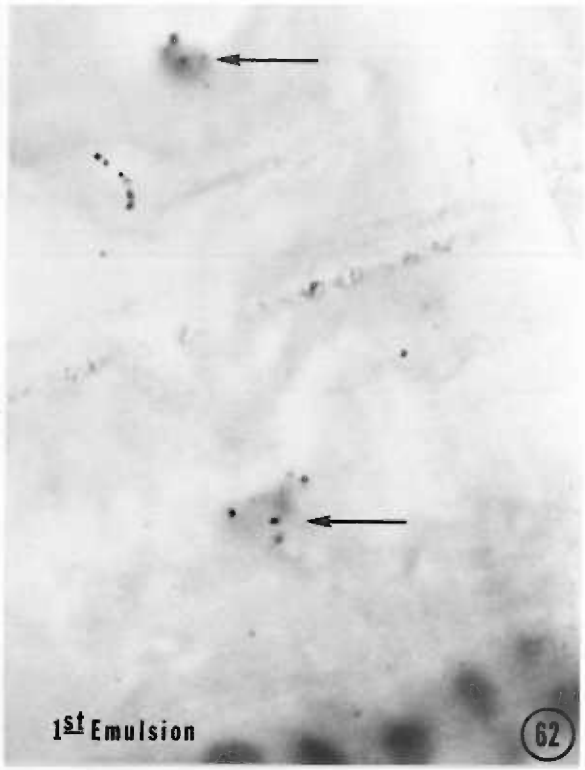
Figure 61. Two lightly labeled cells can be seen when the field of focus was positioned immediately above the tissue section. (X 1000)

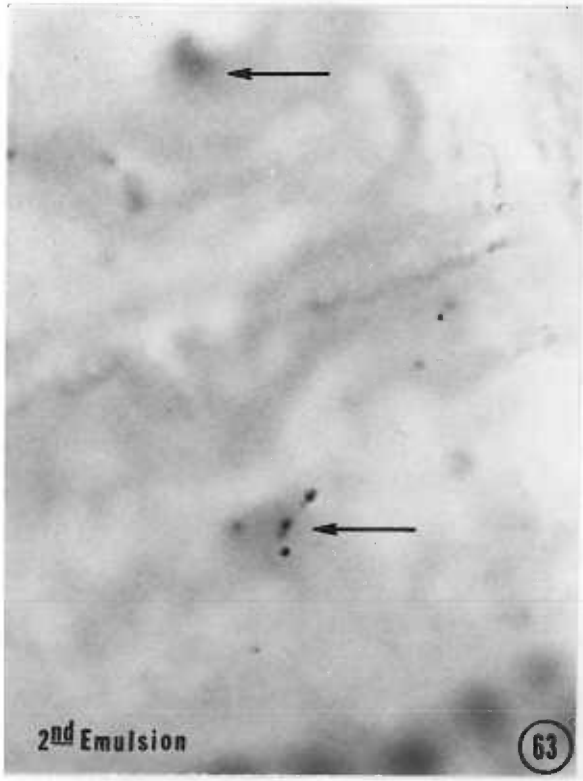
Figure 62. As the field of focus was moved upward the silver grains produced by the ^{3}H-labeled cell have begun to recede from view, while new silver grains have appeared over the ^{14}C-labeled cell. (X 1000)

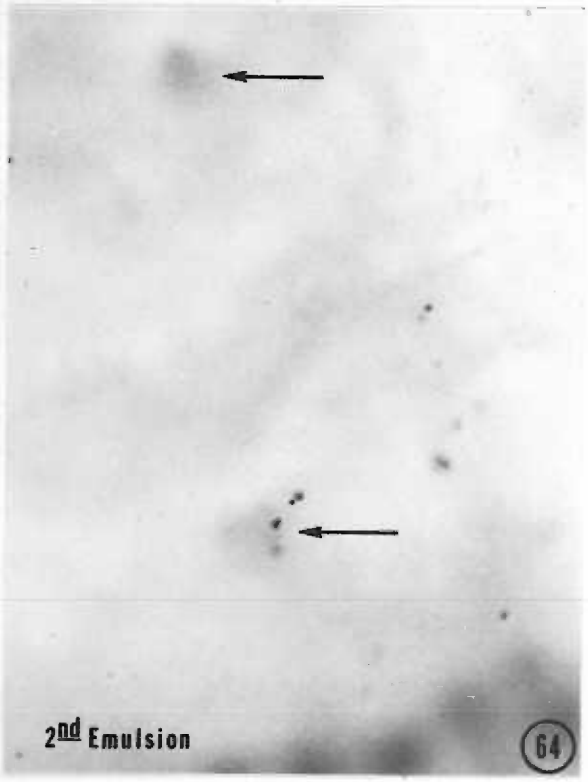
Figure 63. The ^{14}C activity was of sufficient energy to penetrate the collodion layer and record its presence in the second emulsion. (X 1000)

Figure 64. The silver grain tracks continued upward as the field of focus was moved higher in the second emulsion. This finding substantiated that the autoradiographic technique was sufficiently sensitive to detect ^{14}C activity in relatively lightly labeled stromal cells. (X 1000)


^{3}H
^{14}C
1st Emulsion
61


1st Emulsion
62


2nd Emulsion
63


2nd Emulsion
64

the ^{3}H-labeled cell have begun to recede from view, while new silver grains have appeared over the ^{14}C-labeled cell (figure 62). The ^{14}C activity was of sufficient energy to penetrate the collodion layer and record its presence in the second emulsion (figure 63 and 64).

Figures 65, 66, 67 and 68 have been included to illustrate further the results obtained by the double-label/double emulsion technique.

Cell Counts of Labeled Stromal Cells

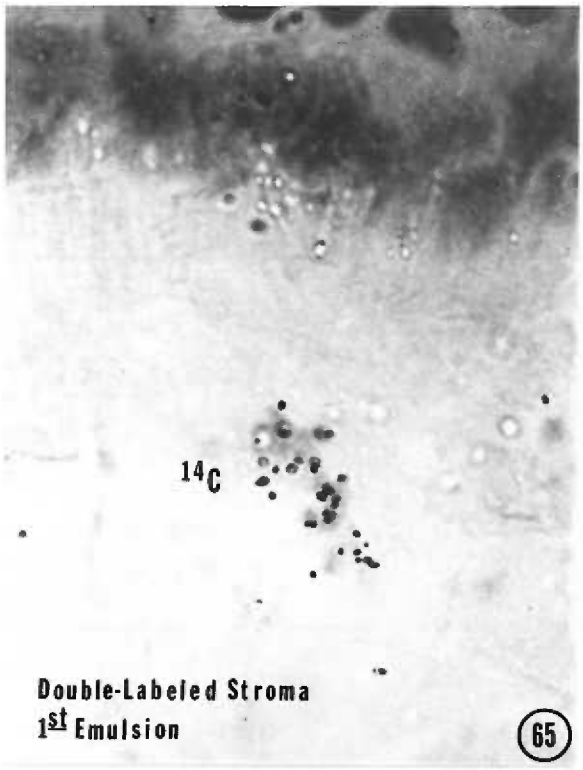
Table 3 is the numerical summation of cell counts done following the processing of the first and second emulsions. Data presented in table 3 and figure 69 were derived from the second experiment. The ^{14}C was too intense in the first experiment to allow accurate cell counting. The ratio of $^{14}C/^{3}H$ was such that the ^{14}C activity obscured those cells containing only ^{3}H. This condition was corrected in the second experiment by reducing the level of ^{14}C-Tdr and changing the first emulsion from NTB-3 to NTB-2. NTB-2 emulsion is less sensitive to ^{14}C activity, while its sensitivity to ^{3}H is equivalent to that of NTB-3 emulsion. Figure 69 is a graphic display of the information contained in table 3. In addition the range of the labeled cell counts in the different experimental groups is included in figure 69.

Figure 65. An example of ^{14}C activity in the double-labeled corneal stroma viewed at the level of the first emulsion. (X 1000)

Figure 66. The same ^{14}C-labeled cell, as seen in figure 65, viewed at the level of the second emulsion. (X 1000)

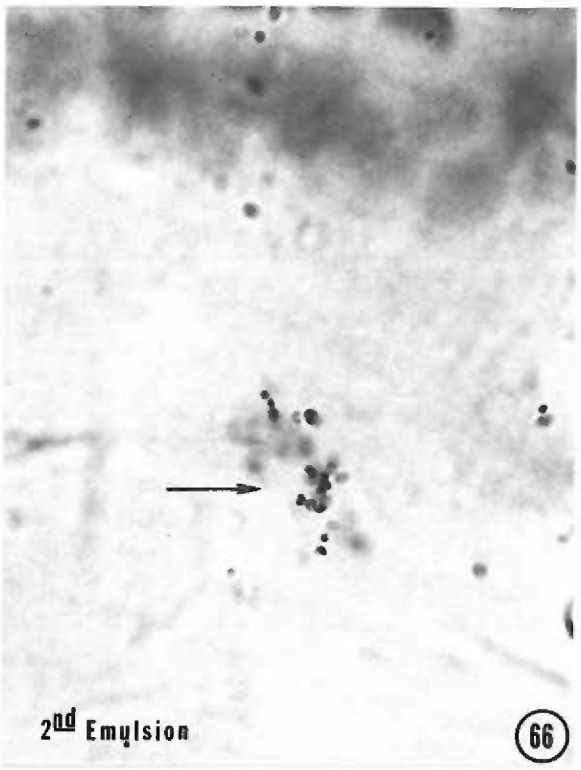
Figure 67. An additional example of ^{14}C and ^{3}H activity in the double-labeled corneal stroma viewed at the level of the first emulsion. (X 1000)

Figure 68. Only the ^{14}C activity is evident in the second emulsion. The ^{3}H activity was confined to the first emulsion. (X 1000)



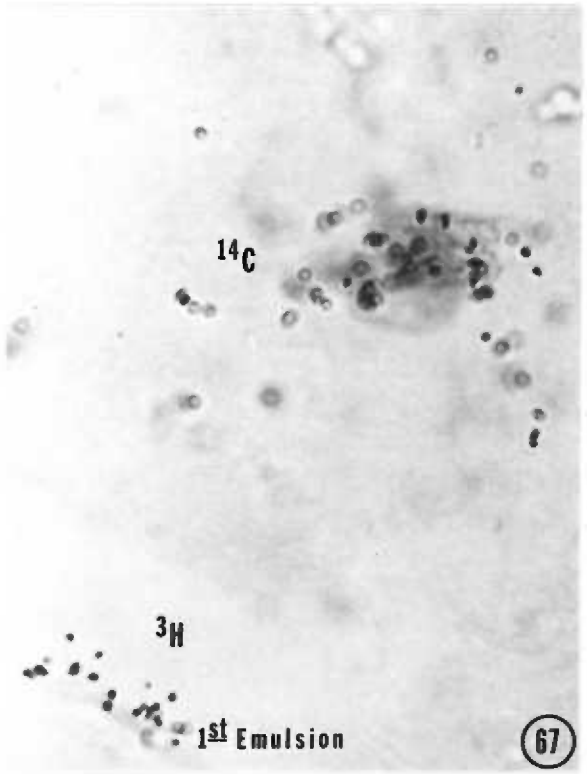
Double-Labeled Stroma
1st Emulsion

(65)



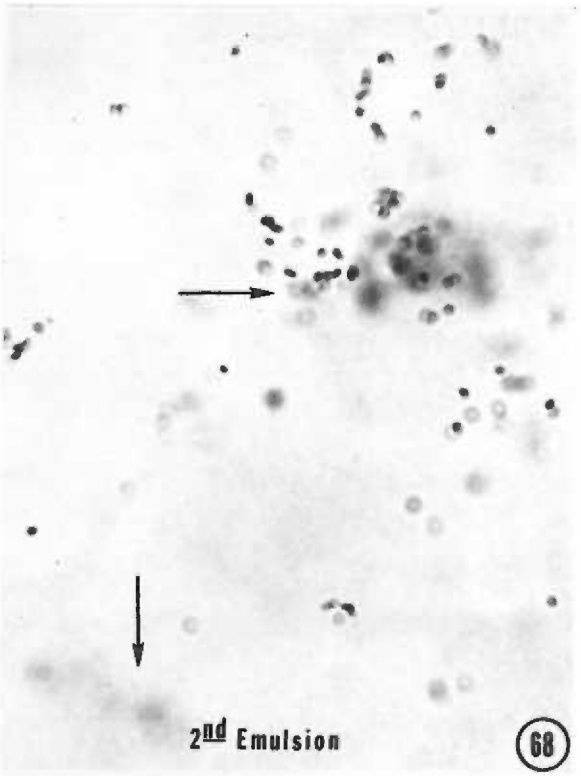
2nd Emulsion

(66)



1st Emulsion

(67)



2nd Emulsion

(68)

Table 3

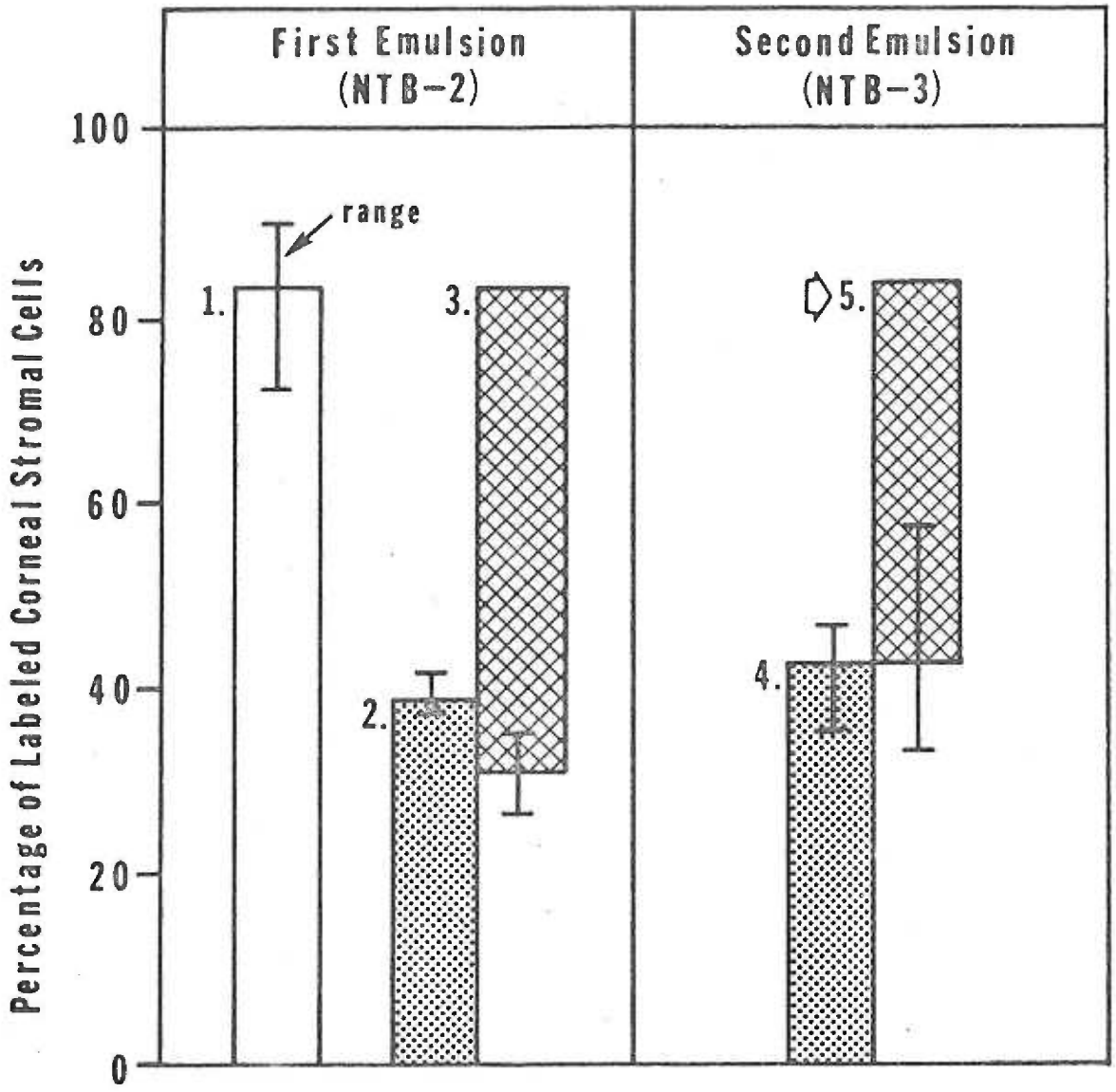
Counts of labeled corneal stromal cells

		Percentage of labeled cells		
		First Emulsion	Second Emulsion	
			^{3}H	^{14}C
Group–I	^{14}C-Tdr labeled stromal cells + ^{3}H-Tdr labeled mononuclear leucocytes + freeze injury	83±5.3*	41±8.9†	43±3.4
Group–II	^{14}C-Tdr labeled stromal cells, no freeze injury	63±3.6		65±1.8
	^{14}C-Tdr labeled stromal cells + freeze injury	40±1.9		42±3.8
Group–III	^{3}H-Tdr labeled mononuclear leucocytes, no freeze injury	neg.	neg.	
	^{3}H-Tdr labeled mononuclear leucocytes + freeze injury	52±3.3	neg.	
Group–IV	Control corneas – no ^{14}C-Tdr or ^{3}H-Tdr administered	neg.	neg.	neg.

* Standard deviation

† This value was determined from the composite results of the first and second emulsion – i.e., stromal cells showing evidence of beta–decay in the first emulsion but not in the second emulsion.

Figure 69. A graphic display of the information contained in table 3. In addition the range of the labeled cell counts in the different experimental groups is included.



1. ^{14}C-Tdr + ^{3}H-Tdr + freeze injury
2. ^{14}C-Tdr + freeze injury
3. ^{3}H-Tdr + freeze injury
4. ^{14}C-labeled cells in double-labeled corneas
5. ^{3}H-labeled cells in double-labeled corneas

⬡ This value was determined from the composite results of the first and second emulsion – i.e., stromal cells showing evidence of beta-decay in the first emulsion but not in the second emulsion.

DISCUSSION

The application of light microscopic autoradiography in studies of the cellular aspects of wound healing is not a new experimental procedure. However, the use of double-layer autoradiography as presented in this thesis report does involve a unique approach to the examination of the wound repair processes in the cornea. The technique has provided data that is in strong support of the contention of Weimar (15) that the blood monocyte does serve as an important precursor source of new connective tissue cells following corneal injury.

Cell counts from ^{14}C-Tdr/^{3}H-Tdr-labeled corneas following double-layer autoradiography showed that 41 percent of the stromal connective tissue cells contained ^{3}H-Tdr exclusively (table 3). It was determined that no ^{3}H-Tdr-labeled cells appeared in the corneas of a previously intravenously ^{3}H-Tdr labeled animal unless the corneas had received a wound (figures 51, 52, table 3). This finding was anticipated on the basis that in the adult animal DNA in the stromal cells of the cornea is of a nonrenewing type and normally (100) labeling of these cells by exposure to ^{3}H-Tdr is impossible. Since the labeled compound is rapidly removed from the circulatir blood (100) following an intravascular pulse and the cornea is a cular, the probability of ^{3}H-Tdr incorporation by the stroma

the cornea is extremely low. The diffusion of ^{3}H-Tdr into the aqueous humor from the anterior chamber vascular supply may constitute an additional route for the label to penetrate the corneal stroma. Apparently this avenue for acquiring ^{3}H-Tdr by the cornea is inconsequential, because the label after dilution in the blood is available only for a short period of time after intravenous injection. There is a subsequent rapid loss of label entering the anterior chamber as a result of the constant turnover of the aqueous humor (142). Therefore, the only reasonable explanation for the appearance of ^{3}H in the injured corneal stroma is that the label must have arrived at the wound site as a DNA constituent of emigrated hematogenous cells. The only cells observed entering the cornea following the freeze injury were the blood-borne monocyte and PMN leucocytes (figures 21, 22). Autoradiogrpahic results from the examination of the peripheral blood 36 hours following the intravenous labeling procedure did not show the presence of labeled PMN leucocytes (figures 44, 45). This finding is in agreement with the already cited observations of MacDonald (70) who reported that PMN leucocytes in rat cutaneous wounds were not labeled in autoradiographs for at least 4 days after an intraperitioneal injection of ^{3}H-Tdr, and then only very minimally. Van Furth (107) has also presented data that shows that the granulocytes do not appear in the peripheral blood as labeled cells for some days following a ^{3}H-Tdr pulse.

Following the freeze injury to the cornea, the PMN leucocytes were the most prominent cells seen during the first 12 - 24 hours (figures 15, 17, 18). Shortly after this time period the number of emigrating PMN leucocytes decreased while the number of monocytes steadily increased, reaching their maximum concentration by approximately the 36th hour. It would appear that the PMN leucocytes emigrating to the injured cornea during the first three days of wound repair contained negligible amounts of ^{3}H. Considering that a high percentage of the circulating blood monocytes were observed to contain label at the time of injury (figure 44), a valid assumption can be made that the monocyte does serve as the source of ^{3}H in the corneal stroma as determined by autoradiography. The unique stability of ^{3}H-thymidylic acid in cellular DNA normally precludes its transfer between viable cells. Therefore, the only pathway available to the stromal cell to acquire ^{3}H-labeled monocytic Tdr or its nucleotide is the death of the monocyte followed by the release of its catabolized DNA.

Experimental evidence strongly supports the contention that the potential life span of the monocyte after entering an area of inflammation may be a number of months in duration (110, 143, 144). This finding would suggest that following corneal injury the arriving blood-borne monocyte has a potential life span in the stroma which far exceeds the time required for complete corneal restoration.

Consideration of this long life span of the corneal monocyte would make it improbable that the cell would die, lyse and release its label for reutilization during the course of wound repair. If ^{3}H reutilization is negligible in the healing cornea, and if the emigrating monocyte is the source of ^{3}H, then those fibrocyte-like cells observed containing ^{3}H following wound repair (figure 51) must have evolved from labeled monocytes.

For many years the only physiological role universally attributed to the mononuclear phagocytes--i.e., macrophages and monocytes, was that of acting as scavengers capable of ingesting extravasated blood, bacteria, necrotic material and foreign bodies. Recent research has led to an increasing awareness that macrophages do, in fact, have a multitude of important functions above and beyond their ability to scavenge and dispose of particulate material. Functions presently ascribed to macrophages include among others: the production of interferon; detoxification of certain simple chemicals, exotoxins, and endotoxins; possible regulation of blood coagulation under certain special conditions; as precursor cells for Langhan's and foreign body giant cells; and immunological activities. Macrophages in various regions of the body may have specialized functions unique to their locations.

During the past decade the role of the macrophage in the antibody response has been studied extensively, both in vivo and in vitro.

Although the mechanism whereby the macrophage participates in the induction of immunological responses in higher vertebrates has not been solidly established, it is generally postulated that the cell has the capacity to engulf, process, and store antigens. In addition this cell apparently presents specific information to an immunologically competent cell in the form of trace amounts of antigen combined with RNA. This complex of antigen with RNA appears to enhance the ability of the antigen to elicit antibody production by serving as a "super-antigen" (145). The foregoing brief review of the biological properties of macrophages has illustrated the pluripotential nature inherent in these cells. This capacity for multidirectional development of the monocyte following its arrival at an area of inflammation implies that the cell can respond to local stimuli and undergo progressive specialization appropriate to the needs of the healing tissue.

As was discussed in the thesis introduction, there is general agreement that blood monocytes can mature into tissue and exudative macrophages (61, 103, 104, 105, 144, 146). Transformation of monocytes into typical macrophages as evidenced by their functional and biochemical characteristics has been observed in vitro as well as in vivo (147, 148, 149). On the basis of studies by Volkman and Gowans (103, 104), Kosunen, Walksman, Flax, and Tihen (150), and Spector, Walters and Willoughby (151) it was concluded that the macrophages which appear at inflammatory sites in response to

experimentally induced tissue injury are hematogenous in origin and are derived from rapidly proliferating bone marrow precursors.

In the present studies a single intravenous injection of ^{3}H-Tdr was administered at 18 and 10 hours prior to the corneal freeze injury to label the monocyte precursor bone marrow cells. It was fortunate that this labeling time sequence for optimum incorporation of ^{3}H-Tdr by the monocytes had been established by Volkman and Gowans (103, 104) and later confirmed by Gillman and Wright (61). Had the ^{3}H-Tdr pulse been given following the corneal freeze injury, autoradiographs may have revealed the presence of little ^{3}H following wound repair. Volkman and Gowans (104) using the skin window technique (a glass coverslip is applied to an abraded area of skin) reported that 57 percent of the macrophages on the coverslip which had been left in place for 24 hours were labeled by giving an injection of ^{3}H-Tdr 18 hours before the application of the coverslip. The authors noted that the percentage of labeling decreased progressively as the interval between labeling and application of the coverslip decreased. Labeling reached a minimum of less than 1 percent of the macrophages when the ^{3}H-Tdr was given 12 - 18 hours after the application of the coverslips.

Van Furth (107) has discussed the existence of a monocyte precursor or promonocyte in the bone marrow. He incubated bone marrow cells and noted that after a 6 hour period the mononuclear

phagocytes could be differentiated into two phagocytic cell types that adhered to a glass surface. Eighty to 85 percent of these cells did not differ from the peripheral blood monocytes in size, morphology, and function while the remaining cells (promonocytes) were larger with a diameter of 14 - 20 μ, a basophilic cytoplasm, and a large indented nucleus. The labeling index (percent of cells containing label) for promonocytes remained about 70 percent during the first 24 hours an in vivo pulse of ^{3}H- Tdr to mice. However, the mean grain count of these cells diminished during the same period. The labeling index of the monocytes continued to rise during the first 24 hours following the ^{3}H- Tdr pulse. These findings have led the author to the conclusion,

> ...the promonocytes are self-replicating cells giving rise to monocytes, which under normal steady-state conditions are nondividing cells (105).

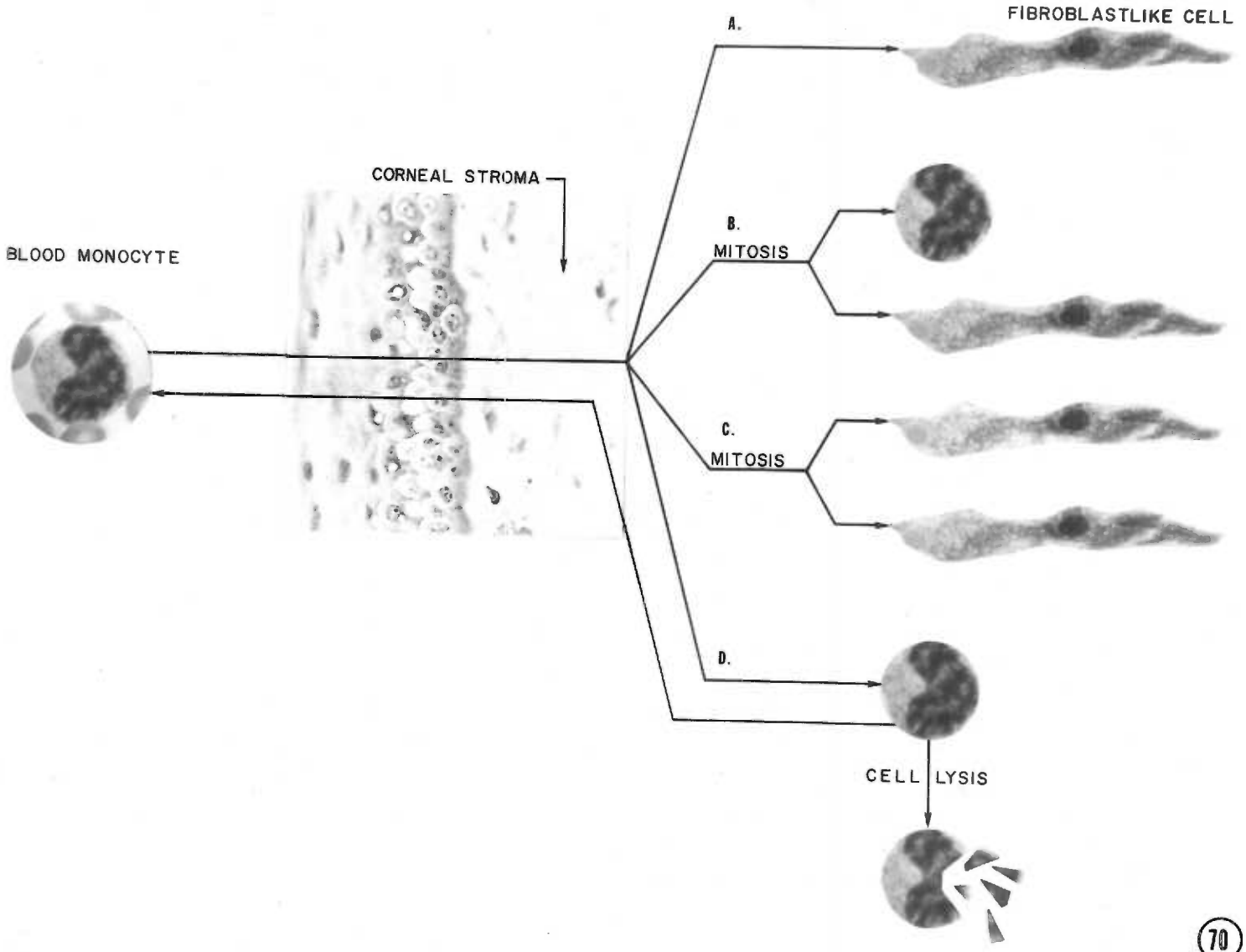
The freezing of the cornea elicits an immediate inflammatory response (figure 15). The emigration of leucocytes to the site of tissue damage is an integral component of this complex response. Although there is now a comprehensive picture of the morphological changes occurring during the emigration of leucocytes from the blood vessels, there is a considerable lack of definitive knowledge as to the intimate mechanisms involved in the stimulation of their migration (1952). In general, most studies have demonstrated that many substances which promote increased vascular permeability to protein have been found to have little or no leucocyte chemotactic properties

in vivo (153). Spector and Willoughby (153) have cited recent data showing that a number of endogenous tissue extracts are able to cause an intense, immediate, in vivo emigration of leucocytes. Included in this variety of tissue extracts are leucocytes themselves. Leucocyte extractions prepared in saline induce a considerable white cell emigration. The migration effect was enhanced when serum replaced saline in the extraction procedure (154). This finding may indicate that a tissue factor combines with or activates a substance in the serum that results in a specific mediator of leucocyte emigration. This activated serum substance may possibly be related to specific components of complement. Spector and Willoughby (153) speculated that following injury PMN leucocytes adhere to altered vascular endothelium and release an active principle responsible for large-scale emigration. The wound produced by freezing the central portion of the cornea was essentially aseptic and thus, the attraction of leucocytes to the injured tissue by bacteria and/or their products appeared to be minimal. Collagen-derived polypeptides produced as a result of the corneal freeze injury may have strong chemotactic properties. An in vivo method for measuring chemotaxis has been described recently by Chang and Houck (155) using Millipore filters. The authors demonstrated that soluble collagen was quite chemotactic. Products of collagenolysis produced by cutaneous collagenase were markedly chemotactic for PMN leucocytes. Bacterial collagenase-digested collagen was found not to be an effective chemotactic agent. It was suggested that since bacterial collagenase

reduces collagen to small tetrapeptides that these peptides may be too small to interact chemotactically with the PMN leucocytes (155). The existence of separate chemotactic mediators for the monocyte and PMN leucocyte has not been shown (153). Most likely both cells are responsive to the same chemotactic influences and leave the vessels over the same period of time, but the exuded PMN leucocytes subsequently die in situ conspicuously leaving the long-lived monocytes at the inflammatory site (153).

Once the monocytes have emigrated into the injured corneal tissue these cells may undergo maturation in a variety of ways according to the functional needs of the healing tissue. Figure 70 graphically illustrates these optional developmental pathways that the monocyte may follow during wound repair. As the monocyte matures within the tissue it enlarges, acquires a more elaborate lysosomal and mitochondrial apparatus, and becomes functionally more active. In this state the cell would be classified as a macrophage (156). During this stage of maturation it is generally believed that the monocyte becomes a phagocyte only and later leaves the corneal reaction site (figure 70-D). Weimar (15, 17) was the first to propose that the monocyte had a second major role to fulfill in the healing cornea. That role was to provide new connective tissue cells (figure 70-A). The findings of the present investigation confirms the contention of Weimar that the monocytes do seem to give rise to a proportion of

Figure 70. A graphic representation of the optional development pathways that the monocyte may follow during wound repair of the corneal stroma.


FIBROBLASTLIKE CELL
A.
CORNEAL STROMA
BLOOD MONOCYTE
B.
MITOSIS
C.
MITOSIS
D.
CELL LYSIS

the fibrocytes in the site of corneal repair. Additional evidence has emerged from the present studies that indicates that the transformed monocytes have become morphologically indistinguishable from classical stellate fibroblasts or spindle-shaped mature fibrocytes and were still present in the corneal stroma 3 weeks following the freeze injury. Other possible alternate ways in which the monocyte may achieve this fibroblast-like state are depicted in figure 70-B and C. The macrophage may undergo cell division producing either two fibroblast-like cells or, one mature macrophage and one fibroblast-like cell. Maximow's (55) well-known view on the pluripotentiality of blood mononuclear cells was accepted by few and discredited by many only a decade ago. It is now well established that, in vitro, some lymphocytes can be stimulated either by phytohaemagglutinin (PHA) or by suitable antigens, to transform into large and possibly pluripotential 'blast' cells which may later mitose (157). This same lymphocytic phenomenon has also been observed in vivo--i.e., some small lymphocytes react to transplantation antigens by enlarging and then dividing to produce more lymphocytes (158).

In the normal resting state only a small proportion of macrophages are in mitosis; however, Forbes and MacKaness (159) found that 50 percent or more of the peritoneal macrophages of immunized mice incorporated ^{3}H-Tdr in response to secondary antigenic stimulation. This finding suggests that blood-borne mononuclears may,

in appropriate circumstances, be stimulated to undergo mitosis and indicates that these cells are not end cells. No direct evidence was found in the present studies that demonstrated any extensive macrophage mitosis, although this possibility cannot be ruled out. Mitotic figures were occasionally observed in the corneal stroma during the first week following the freeze injury (figure 23). This would seem to eliminate the argument that the compactness of the stromal tissue morphologically distorts the dividing macrophage or stromal cell in such a manner as to prevent recognition from the non-dividing cell population. In the event that the large concentration of new cells at the wound edge (figures 31, 32) can be explained solely on the basis of multiplication of native stromal cells, then an abundance of different mitotic stages should be apparent in the histologic sections made soon after injury. As has been previously stated, this rapidly replicating cellular process was not seen in the present studies.

Colchicine was employed by Weimar (15) to estimate the contribution of cell division in providing the increased number of fibroblasts at the wound edge. The author noted that the rate of stromal cell division and/or transformation could only account for approximately 35 percent of the wound fibroblasts.

Allgöwer (24) emphasized the important fact that mitosis requires a minimum of 30 to 90 minutes for completion and is always followed by a resting stage, or interphase, of several hours duration.

This investigator was acutely aware of a mathematical paradox revealed by the results obtained from rabbit ear wounds. Starting and ending cellular quantities could not be correlated as a function of the rate of mitosis during the allotted time. Allgöwer concluded that this apparent discrepancy could be answered on the basis of the ability of the hematologic elements to form connective tissue.

To what numerical extent did the monocyte contribute to the total number of the stromal connective tissue cells present in the cornea following three weeks of wound repair? It must be emphasized that a comparison of the control corneas (figure 16) and the three week postoperative corneas (figure 36) disclosed at the light microscope level that there was no discernible histologic differences between the two tissues. Examination of the data presented in table 3 and figure 69 reveal that ^{3}H was found exclusively in 41 percent of the stromal cells in the double-labeled and wound repaired corneas. In these same corneas 43 percent of the stromal cells contained ^{14}C only. The double emulsion autoradiographic technique proved to be unsuccessful for differentiating those stromal cells that had incorporated both ^{3}H and ^{14}C in the double-labeled corneas. However, this information could readily be determined by an indirect autoradiographic approach using a single emulsion. Figure 69 graphically conveys how the percentage of double-labeled cells was ascertained. Cell counts revealed that 83 percent of the stromal cells in the

double-labeled corneas had incorporated radioactive Tdr (figure 69, bar No. 1). Corneas that were labeled with ^{14}C-Tdr only and then subjected to the freeze injury gave a percentage labeled cell count of 40 (figure 69, bar No. 2). In those animals that had their mononuclear leucocytes labeled with ^{3}H-Tdr and later received a corneal freeze injury, 52 percent of the cells were labeled (figure 69, bar No. 3). When the percentage values from the two singly labeled experimental groups (figure 69, bar No. 2 and 3) were added they totaled 92 percent. If no isotope reutilization had taken place then the projected level of isotope incorporation in corneal stromal cells of the double-labeled corneas should have been approximately 92 percent. This was not the case however, for only 83 percent of the stromal cells were labeled at the conclusion of the experiment with the double-labeled corneas. The 9 percent difference between the projected and actual cell count values would appear to be the average percentage of stromal cells containing both ^{14}C and ^{3}H. Approximately this same value can also be derived by subtracting the percentage of ^{3}H-labeled stromal cells in the double-labeled corneas (determined from the combination of the first and second emulsion) from the percentage of ^{3}H-labeled stromal cells found in the singly-labeled corneas.

Are there any plausible explanations for the existance of these double-labeled cells in the corneal stroma? There may be at least

two processes whereby the cell can acquire the second isotope. Mitosis of wound macrophages and/or native stromal cells could account for the presence of the double-labeled cells. Only a maximum of 5 percent of the stromal cell population need reutilize either ^{14}C or ^{3}H and later undergo cell division to result in a 10 percent double-labeled cell count. A second avenue for the cells to incorporate an additional isotope would exist if the increase in the number of nucleoli seen in wound fibroblasts (figures 27, 33) was accompanied by a concurrent increase in extra-chromosomal nucleolar-associated DNA. It is tempting to suggest that if a cell requires multiple copies of a particular cistron at some stage of wound repair to satisfy an elevated demand for the corresponding Ribonucleic acid (RNA) complements, that there exists within the nucleolus a mechanism for chromosomal cistron magnification. Admittedly this hypothesis is speculative in nature; however, there are examples of specific gene amplification in lower animals. Oöcytes of several amphibians, an echiuroid worm, and the surf clam increase their synthesis of ribosomes by replicating specifically the genes for 28S and 18S RNA early in oögenesis and later synthesize large amounts of ribosomal RNA from these additional genes. Following the production of these extra gene copies, the copies are isolated in multiple nucleoli (160, 161). These extrachromosomal nucleoli function only during oögenesis and are subsequently eliminated.

Pavan and Da Cunha (162) have reviewed the relevant biological studies that have dealt with the concept of gene amplification in animals. The authors concluded that

> ...if an excess of DNA is produced in chromosomes of the germline cell and afterward released to the nuclear sap, there are no valid reasons why the same phenomenon in a smaller scale could not occur in somatic cells.

The corneal stromal cells incorporate ^{14}C-and/or ^{3}H-labeled Tdr via the anterior chamber following the traumatic removal of the epithelium (figures 40, 47). Approximately 65 percent (table 3) of the stromal cells are labeled in this procedure with the labeled cells appearing to be randomly distributed throughout the stroma. A series of corneal histological sections taken at various time intervals following the removal of the epithelium showed little evidence of stromal cell mitosis. The stromal cells undergo marked morphological changes and become fibroblast-like in appearance. In these cells the number and size of nucleoli per nucleus increased (figure 39). By the end of the first postoperative week these cells have reverted to the more quiescent stromal fibrocytes. Why and how these activated stromal cells incorporate labeled Tdr poses an interesting question. They may acquire the label through a pathway similar to that of the freeze activated stromal cell, i.e., by an increase in nucleolar-associated DNA, or they may undergo the synthetic phase of the division cycle, but stop short of mitosis. An additional

mechanism for Tdr incorporation could be DNA repair. Although there is no strong experimental evidence to support DNA repair in the mammalian system subsequent to cell injury, it is known that trauma to the corneal epithelial and stromal cells results in an increased lysosomal fragility, followed by the release of degradative enzymes (159). These enzymes conceivably could damage, nonlethally, cellular DNA that may later undergo repair. Undeniably, this hypothesis is based on conjecture; however, the existance of mammalian DNA repair cannot be rigorously ruled out at this time.

In summarizing the results from the labeled cell counts (table 3, figure 69) the important finding was that a mean of 41 percent of the corneal stromal cells had incorporated ^{3}H-Tdr exclusively. This disclosure supports the concept that the monocyte has remained in the stroma and has assumed the morphological appearance of a stromal connective tissue cell by the completion of wound repair. Since all the corneal stromal cells were killed at the time of freeze injury, those cells containing only ^{14}C in the healed cornea represent labeled native stromal cells that have migrated into the devitalized cell-free zone. The finding of so few double-labeled stromal cells following wound repair in the double isotope corneas is suggestive that mitosis of existing stromal cells could not account for the rapid cellular repopulation of the damaged cornea.

Van Winkle (163) aptly expressed the opinion that "one of the

basic problems in the study of the fibroblast is to recognize it." Are the transformed monocytes fibroblasts or are they merely fibroblast-like in appearance at the light microscope level? The term fibroblast is used to denote a cell which is actively engaged in the formation of collagen and ground substance (8). No specific procedures were carried out to determine if the transformed monocyte had formed connective tissue matrix. The rationale for not directly ascertaining the production of connective tissue matrix by the transformed monocytes were: first, that the existance of the fibroblast can only be firmly established on the basis of fine structure characteristics as revealed by the electron microscope. The use of the electron microscope would have constituted a project of such magnitude as to warrant a separate study; second, the ability of the transformed monocytes to form extracellular connective tissue elements was not considered an absolute necessity to substantiate the hematogenous origin of new corneal stromal connective tissue cells. This is not to infer that the monocyte is incapable of synthesizing collagen and ground substance, but on the contrary there is reason to believe that the transformed monocyte does fulfill the criterion of a true fibroblast. It is generally concluded that every living cell nucleus in a metazoan organism contains the same complete genome as was present in the zygote nucleus. Numerous reports have provided evidence in support of this concept. An example of this work is the

fascinating studies that were conducted by Gurdon and Uehliger (164). In these studies a number of fertile adult toads were obtained from eggs whose only active nuclei were transplanted from differentiated intestinal cells. These striking results demonstrate that whatever the nature of the nuclear process leading to differentiation, it must be regarded as a reversible process. Thus, it is conceivable that the same external stimulus that provokes the corneal fibrocyte to undergo activation and form connective tissue components can also induce the monocyte to become a functional fibroblast. It appears that there is no necessity for the monocyte entering the wound site to contain specific information as to the type of collagen present in the cornea. Gross, Highberger and Schmitt (165) and Hodge, Highberger, Deffner and Schmitt (166) have demonstrated that soluble precursor can form collagen fibrils independent of cells. The present evidence supports the notion that the cell secretes collagen precursors (tropocollagen) into the extracellular region where they aggregate into new fibrils. These fibrils are assembled and arranged in a geometric pattern in the stroma mucopolysaccharide matrix, seemingly at the direction of the extracellular microenvironment (8). The ground substance of the corneal stroma differs from other connective tissues by being composed of keratin sulfate and chondroitin, in addition to chondroitin-4-sulfate (167). Peacock and Van Winkle (168), in reviewing the biological events in corneal healing, noted that there is evidence

which shows that the

> ...corneal fibroblasts, appear to be the only cells programmed for the production of this combination of mucopolysaccharides, and new cells formed by mitotic division of older cells do not commence to produce this combination of substances for many months after they mature.

In light of the results revealed by the present studies, it may not be a lack of native stromal cell maturity that prevents the production of the corneal specific mucopolysaccharides, but rather the formation by the monocytes of ground substance more commensurate with the biochemical makeup of the other connective tissues of the animal.

In planning the experimental protocal for these studies the prospect that the labeled Tdr might produce an isotope effect had to be considered. An isotope effect is observed when the chemical reaction rate or physical behavior of an isotopically labeled molecule is different from that of its unlabeled sister molecule. This effect is mainly due to the difference in mass which is introduced into the molecule by the isotopic label. Isotope effects, if not recognized, may lead to erroneous conclusions. No histological differences were observed during wound repair between the labeled and unlabeled corneas which had undergone identical surgical procedures. There was no significant variance seen in the total body weights between the labeled and unlabeled animal groups during the course of the experiments. These data indicate that the labeled Tdr did not have a deleterious effect on the corneal wound healing processes.

Are there other experimental methods in addition to autoradiography that could be utilized to determine the origin of new connective tissue cells in the corneal stroma during wound repair? Fluorescent protein tracing was unsuccessfully employed early in these studies in an attempt to identify the origin of the corneal stromal cells present at the wound site. Anti-rat monocyte serum was prepared by injecting rat peritoneal monocytes plus adjuvant into rabbit foot pads. The monocytes were obtained from rats by injecting mineral oil intraperitoneally and later aspirating the oil and cells. Cryostatic sections of both frozen and control corneas were treated with anti-rat monocyte serum. The serum was rinsed from the sections and replaced with a fluorescein conjugated anti-rabbit gamma globulin (produced in goats). The sections were then rinsed to remove excess antiserum, counterstained with rhodamine, and examined under an fluorescent microscope. The anticipated fluorescent labeling of the monocytes was obscured by non-specific binding of the antiserum to the dense collagen matrix of the corneal stroma. Although the fluorescent protein tracing technique did not prove applicable to the cornea, it might elucidate the role of the monocyte in wound repair in other tissues.

The use of parabiotic rats is another experimental approach that could yield meaningful data as to the origin of the corneal wound fibroblast. This technique has been employed recently be Ross

et al. (58) in studying healing skin wounds. Parabiotic rats were given total body irradiation except for shielding the femur of one rat whose marrow subsequently provided all the hematogenous cells for both animals. Simultaneous wounds were made in both rats, followed by the clamping of the anastomosing flap between the two animals and the injection of ^{3}H-Tdr into the "protected" rat on the day of wounding. Labeled blood cells in the circulation of both rats were found, but no labeled fibroblasts were detected in any wounds of either rat. Ross et al. concluded that fibroblasts were not derived from hematogenous cells. It is apparent that the authors disregarded the findings of Gillman and Wright (61) and Volkman and Gowans (103, 104) who unequivocally demonstrated that very few mononuclear cells were labeled if ^{3}H-Tdr was injected 24 hours postoperatively. The only alteration necessary to adapt the experimental protocol of Ross et al. to the study of corneal wound healing would be to label the bone marrow prior to a corneal freeze injury.

Three weeks following the freeze injury the cornea had undergone complete wound repair and was indistinguishable from the control cornea both in gross and microscopic appearance. This finding implies that not only was the normal stromal architectural pattern restored but that the destroyed stromal fibrocytes were replaced by cells of similar structure and function. Data has been presented which supports the concept that approximately 40 percent of the new

stromal fibrocytes were derived from monocytes. It is difficult to envision that the integrity of the cornea could be maintained solely by those cells derived from the native stromal cell population without the aid of the transformed monocytes.

These studies are in agreement with the statement made by Duke-Elder (28):

> Adult regeneration of the corneal wound thus appears to follow the same lines of the embryonic formation of this tissue, for the corneal corpuscles are formed from wandering mesodermal cells.

There is no reason to believe that the monocyte is restricted to the cornea in providing precursor cells for wound repair. The monocyte may aid any lesion requiring fibroblastic proliferation. However, it is more difficult to determine the origin of fibroblasts in more complex vascular tissues. Thus the cornea has provided an excellent wound model system.

Provided with the knowledge that monocytes do participate in the formation of new connective tissue cells, what are some of the practical applications of this finding?

Whenever the body is exposed to injury, whether secondary to physical, chemical, bacterial, viral or immunological agents, it responds by inflammation. This inflammatory reaction destroys and removes the irritant and repairs the damage. Following extensive injury with necrosis, or continued irritation, there may be excessive

proliferation of mononuclear cells and fibroblasts. Granulomatous diseases such as tuberculosis, sarcoidosis, silicosis, and histoplasmosis, which are characterized by increased number of monocytes and macrophages, also have prominent fibrosis.

A variety of disabling conditions in human beings are manifested by rapid synthesis of collagen which alters the physical properties of tissues and restricts normal function. Examples of such conditions are: cirrhosis of the liver following viral hepatitis, cardiac valvular deformity from rheumatic fever, and sclerosis of glomeruli in the kidney with systemic lupus erythematosis or following a streptococcal infection. It is generally accepted that the pathophysiology of these conditions involves antigen-antibody interaction, activation of complement, chemotaxis for PMN leucocytes, and tissue destruction from release of proteolytic enzymes from PMN leucocytes. Mononuclear cells are also prominent and their presence could be a source of new fibroblasts. Thus if the participation of the monocyte in wound repair is limited, excessive fibrosis and loss of function of the involved tissue might be reduced.

In conditions where there is retarded wound healing such as diabetes mellitus or with exogenous steriods, the proliferation of fibroblasts is deficient. Stimulation of monocytic participation in inflammation might promote accelerated healing. Wound healing in a healthy individual is quite adequate and does not require artificial

means of stimulation. However, every effort should be made by the clinician not to deter the participation of the monocyte in its role in the reparative process.

SUMMARY AND CONCLUSIONS

Previous morphological and enzymatic studies have strongly indicated the possibility that following injury to the rat corneal stroma, emigrating mononuclear blood cells do transform into fibroblasts and aid in wound repair. Additional evidence of the hematogenous origin of corneal stromal connective tissue cells has been derived from double isotope autoradiography. This autoradiographic technique utilizes two layers of sensitized emulsion separated by an inert layer of cellodion. Stromal cells containing only ^{3}H-Tdr, only ^{14}C-Tdr, or both ^{3}H-Tdr and ^{14}C-Tdr can be distinguished by this method.

Rats used in these studies were divided into four groups. The first group of rats had their corneal stromal cells labeled with ^{14}C-Tdr and their mononuclear blood cells labeled with ^{3}H-Tdr followed by a standardized freeze injury to their corneas. The second group of rats had only their corneal stromal cells labeled with ^{14}C-Tdr and half of these animals had their corneas frozen. The third group of rats had their mononuclear blood cells labeled with ^{3}H-Tdr and half of these animals had their corneas frozen. The fourth group of rats were maintained as control animals.

Corneal stromal cells were labeled by curette scraping of the epithelium followed by two ^{14}C-Tdr injections into the anterior chamber. The mononuclear blood cells were labeled by two intravenous

injections of ^{3}H-Tdr via the sublingual vein. Corneal freeze injuries were administered by a temperature controllable cryostatic probe. Upon completion of the labeling and freezing procedures the corneas were allowed to undergo complete wound repair.

Analysis of autoradiographic results revealed that 41 percent of stromal cells containing only ^{3}H derived from monocytes remained after completion of wound repair, while the percentage of ^{14}C labeled stromal cells decreased from 63 to 43 percent following the repair of the freeze injury. Some cells did contain both isotopes, but they were less than 10 percent. Therefore it appears that isotopic Tdr re-utilization is very slight. These studies indicate that the monocyte does serve as an important precursor source of new connective tissue cells in the healing corneal wound.

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