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Studies on Inflammation

III. Growing Capillaries: Their Structure and Permeability*

By

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With 23 Figures in the Text

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Introduction

During the past decade, electron microscopists have elucidated the fine structure of *normal* capillaries in a great number of tissues from a variety of animal species. Mature vessels have also been studied in several pathological states. However, no comparable progress has been made in regard to the structure and function of *regenerating* capillaries. In fact, modern histology has contributed little that was not known in the latter half of the nineteenth century. The most significant advances were made through the study of living tissues: careful, prolonged observations on amphibian larvae, and on regenerating vascular beds in mammals (made possible by the invention of the rabbit ear chamber in 1924) established the morphologic sequence of events at the level of the light microscope. This approach, however, suffers from considerable handicaps, because of the relatively low resolution afforded by the living preparations.

The following facts are generally agreed upon: Vascular regeneration always proceeds from preformed vessels. The endothelium gives rise to buds which grow in length and width to form sprouts. Their tips are thought to be solid cords of cells (possibly syncytia) that are secondarily canalized. Where growing sprouts meet, they fuse, giving rise to loops or cross-connections and, ultimately, to intricate networks. As this primitive system matures, certain preferred channels acquire coats characterizing them as arteries or veins; many of the capillaries regress.

With regard to the functional properties of newly formed vessels, the few observations available point to two abnormalities: an increased fragility and increased permeability. That new vessels are more *fragile* than their mature counterparts — or at least, more susceptible to bleed — was pointed out by CLARK and CLARK in 1935. Slight pressure on the window of the ear chamber forced blood through the walls of newly formed vessels, but did not affect older vascular segments of the same chamber. Growing vessels of the ear chamber were found to be more *permeable* to proteins as well as to Evans Blue, an azo dye, by ABELL (1939, 1946). COGAN (1949) also observed an enhanced escape of Chicago Blue, another azo dye, from recently formed vessels of the cornea. The limitations of dyes as indicators of vascular leakage, however, were recently stressed by MAJNO *et al.* (1961): the rapid extravascular diffusion of dyes makes the identification of leaking vessels extremely difficult.

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For many of the problems still unsolved, the *electron microscope* has become an obligatory tool for further exploration. The mechanism of sprout formation, the morphologic substrate of the abnormal permeability and fragility and the behavior of the basement membrane are especially considered in this paper. Pertinent data in the literature are fragmentary (ALBERTINI 1960, CLIFF 1961, MATSUHASHI 1961). In the present work, the site and nature of the leaking vessels, and the duration of the 'leaky' state, were studied topographically by means of a technique referred to as "vascular labelling" (MAJNO *et al.* 1961) which allows to identify an abnormally permeable vessel by light microscopy, at relatively low powers. Throughout this work, particular emphasis was placed on the *correlation between light and electron microscopy.*

This was, in part, a necessity, for it is practically impossible to identify a sprout in ultrathin sections, unless it has first been selected and carefully oriented at low powers. Hence the choice of experimental tissues: the cremaster muscle and the cornea of the rat. Both are membranous structures, in which vascular growth — consequent to local injury — is primarily directed in one plane. This offered two major advantages. First, the tissue could be cleared in glycerin and examined by light microscopy *in toto*, an essential step for topographic studies. Second, it was possible to embed the tissues for electron microscopy in thin, flat blocks of epoxy resin from which single vessels could be selected for sectioning, after the vascular bed had been recorded photographically.

Materials and Methods

Adult male albino rats of the Sprague-Dawley (Holtzman) strain, maintained on purina chow, were used. The animals were 2 to 3 months old, their weights varied from 200 to 350 g; they were not starved prior to sacrifice.

1. Muscle wound. A wound was produced in the midventral portion of the cremaster muscle sheet[1]. This site was selected since it does not contain major arterial and venous trunks; it was approached from the peritoneal surface to prevent excessive scarring and adhesion to the skin. The animal was anesthetized with ether and laid on its back. The lower abdomen was shaved, then depilated with a commercial preparation (Nair). This procedure was found necessary to prevent hair debris from contaminating the wound. Approximately midway between the *linea alba* and the inguen a small cutaneous incision was made on one side of the abdomen. The subcutaneous fat was cleaved by blunt dissection to expose the external oblique muscle. A surgical thread was pulled through the muscle in the caudal area of the incision and knotted; the two ends of this thread served as a hold on the muscle during the subsequent steps of the operation. A small buttonhole was then cut into the muscle adjacent to the ligature and widened with blunt scissors. A pair of fine curved hemostats was introduced through the opening, tips pointing up, and passed through the inguinal canal into the scrotal sac. The advancing points of the closed hemostat could best be followed from the outside if they were slightly pressed against the ventral surface during the insertion. This procedure also insured the by-passing of the epididymal fat bodies and the cephalad portion of the epididymis. The tips were advanced until they projected at the midventral area of the scrotum. The hemostat was then opened about one millimeter and, with gentle pressure from the outside, a small fold of cremaster was clamped (wider opening of the hemostat often lead to pinching of the epididymal fat bodies by the upper part of the instrument). The hemostat was then retracted while the testis was simultaneously

[1] The anatomy of the cremaster has been described previously by MAJNO and PALADE (1961) and MAJNO *et al.* (1961). Briefly, this muscle is an extension of the abdominal internal oblique which forms a pouch around each testis. Its vascular supply is derived from the external spermatic artery and a minor epididymal branch of the internal spermatic artery. The external spermatic enters at the proximal end, courses along the dorsal aspect and fans out into four or five branches that swing around to the ventral aspect, where they anastomose. The epididymal branch supplies the distal portion of the cremaster.

eased into the peritoneal cavity. In this way, the cremaster muscle could be partially everted through the incision. The tissue clamped in the forceps was cut off with scissors; thus a small, round, full-thickness buttonhole two to five millimeters in diameter was created in the cremaster, which promptly retracted while the testis was gently pushed back into the pouch. Muscle and skin were sutured separately, and the operation was repeated on the other side. No adhesions formed between the wound and the serosal surface of the testis. Despite the lack of aseptic precautions, no infection developed except, in a few instances, around the skin sutures.

2. Corneal lesion. The rat was anesthetized with ether and placed on its side under a dissecting microscope, so that one eye could be seen at enlargements of 6× to 10×. In order to immobilize the eyeball, the padded tip of a pair of forceps was pressed downward at the back corner of the eyelids so that mild exophthalmus resulted. A pointed crystal of silver nitrate was then briefly touched to the center of the cornea until a milky spot appeared.

3. Intravenous injections. Colloidal suspensions were injected, whenever indicated, into the saphenous vein. We used carbon (100 mg/cm³, Guenther Wagner, Hannover, Germany, No C 11/1431a) 0.1 cm³/100 g body weight; black mercuric sulfide (4%, Hille and Co., Chicago) 0.5 cm³/100 g body weight and gold (Abbott Laboratories, list No 4760), 0.3 cm³/100 g body weight.

4. Preparation of whole mounts in glycerin jelly. The *cremaster muscles* were prepared as described by MAJNO *et al.* (1961). Under deep ether anesthesia, the proximal insertion of the scrotal sac is clamped with two hemostats, the scrotum is removed *in toto* and immersed in buffered formalin. The animal is then killed by pneumothorax. The cremaster muscles are dissected out under formalin and pinned flat onto dental plate wax. After one or two days in fixative, connective tissue adhering to the dermal side of the muscle is dissected off with fine forceps. The tissue is then cleared in glycerin, trimmed, and after brief soaking in warm glycerin jelly, mounted on a microscopic slide in this medium.

To prepare the *corneas*, the animals were killed with ether. Under the dissecting microscope, the eyelids were removed, the eye was enucleated with the surrounding orbital tissues and suspended in 10% buffered formalin. After one or more days, the eye was cut equatorially; the lens was removed and discarded together with the posterior half. The remaining preparation was bisected with a razor blade. By grasping the cut edge of the ciliary body with watchmaker's forceps, the iris and retina could be peeled off, leaving the cornea with adjacent sclera and choroid layer. To flatten the cornea, radial incisions were made. The corneas were mounted on glass slides in glycerin, allowed to clear, and examined under the light microscope.

5. Examination of the tissues. The preparations, mounted in glycerin (corneas) or glycerin jelly (cremasters), were examined at 20—1800× with a Zeiss Ultraphot microscope. At higher magnifications, the image was blurred by diffraction due to the thickness of the preparation (cornea: 100—200 μ, cremaster: 250 μ). This effect was greatly reduced by introducing an opal glass plate between the microscopic stage and the slide. The lesions were also studied under a Zeiss dissecting microscope (6× to 40×), using epi-illumination.

6. Preparation of tissues for electron microscopy. *a) Cremaster.* Under deep ether anesthesia, the scrotal skin was removed and the exposed cremaster was irrigated with chilled fixative (PALADE 1952, CAULFIELD 1957). Fixative was also injected into the serosal cavity. The muscle was excised and pinned on a dental wax plate into a puddle of cold fixative using cactus needles (the use of metallic needles was avoided to prevent contamination of the preparation with oxidized metal). After approximately ten minutes, the area of the lesion, with some of the surrounding tissue, was transferred to a vessel with fresh fixative, post-fixed for 2 hours and dehydrated in graded alcohols (70%, 95%, 100%). During the first change of absolute alcohol the pieces of muscle were trimmed to leave a narrow rim of uninjured tissues around the lesion. Any loose connective tissue present on the dermal side was dissected off. The tissues were then prepared for Epon embedding according to LUFT (1961).

b) Cornea. The animal was deeply anesthetized with ether and placed under a dissecting microscope. One or both eyes were flooded with cold fixative for several minutes; the eyelids

were then removed and the eye flooded with additional fixative. The eye was enucleated, placed in fresh fixative for 2 hours, then dissected as described above for light microscopy while in the first absolute alcohol. Radial segments approximately 3 mm in width were prepared for Epon embedding.

7. Embedding, cutting and staining techniques. After infiltration with Epon, tissues of both groups were flat-embedded according to the method of BORYSKO (1956), slightly modified. The tissues were placed in a small bath of unpolymerized Epon, made by placing a polyethylene ring (cut from tubing) on a piece of cellulose acetate. The ring was covered with another sheet of cellulose acetate and weighted down by a microscopic slide. Polymerization was carried out in steps of 35° C, 45° C, 60° C, or by placing the tissues directly at 60° C. This method of flat-embedding gave a disc with such smooth regular surfaces that the embedded tissue could be successfully photographed by light microscopy. A photographic record was made of the embedded tissue, selected regions of the lesion were cut out, indicated on the photograph and cemented onto Epon blocks with either Duco cement or unpolymerized Epon. The blocks were oriented so that the sections were cut parallel to the plane of vascular growth. In cremaster preparations, sectioning was begun from the peritoneal aspect of the wound, in the cornea from the outer epithelial lining. The cutting facet was trimmed in the shape of an isosceles trapezoid pointing towards the center of the lesion. Sections were cut on the LKB Ultrotome, using glass as well as diamond knives. After each series of thin sections (gold interference color), several thicker sections were cut for light or phase microscopy. Thin sections were picked up on 200 mesh copper grids which were usually coated with a Parlodion film. The sections were stained with either of the following electron-dense stains: Lead hydroxide (WATSON 1958b), plumbite methods a and b (KARNOVSKY 1961), phosphotungstic acid and uranyl acetate (WATSON 1958a). The sections were examined with an RCA EMU 3D electron microscope at original magnifications from 850 to 11,000. Thick sections were transferred to microscopic slides, dried at 60° C on a hot plate and stained with Crystal Violet (MOORE *et al.*, 1960), Azure II-Methylene Blue (RICHARDSON *et al.*, 1960) or Toluidine Blue (TRUMP *et al.*, 1961).

Terminology. In describing vessels with abnormally increased permeability, several terms will be used which require clarification.

Vascular leakage (or *leaking vessel*) will be used in a broad sense, to include all the situations in which a blood vessel is abnormally permeable. The light microscope cannot be relied upon to demonstrate the underlying structural abnormalities. However, within the range of light microscopy, one can demonstrate the existence of leakage *indirectly*, and even infer as to the nature of the structural damage, by observing the behavior of visible colloidal material injected intravenously.

Carbon black and mercuric sulfide are particularly useful for this purpose, because under normal conditions they do not cross the endothelial barrier, and they are also retained by the basement membrane if the latter is exposed by an abnormal gap in the endothelium. In this case plasma escapes through the gap, while the suspended particles are literally filtered out (MAJNO and PALADE 1961).

Thus, if carbon particles appear *within the wall* of a blood vessel, it can be inferred that the endothelium has become discontinuous (while the basement membrane has remained intact). If carbon particles are found *in the extracellular spaces*, this indicates that both the endothelium and the basement membrane are interrupted. To describe the first situation, the term '*vascular labelling*' will be used, which implies that the leaking vessel is marked (MAJNO and PALADE 1961; MAJNO *et al.* 1961). For the latter case, we will use the term '*spilling*'. It is self-evident that intramural and extravascular deposits may form at the same site. It should also be emphasized that the spilling phenomenon can only be evaluated in animals killed shortly after the injection of carbon; one hour is a safe limit. In later stages, carbon deposits which were originally intramural tend to migrate into the extravascular spaces through the action of macrophages, thus simulating, to some extent, the pattern of 'spilling'.

Both labelling and spilling are strictly dependent upon the size of the colloidal particle which is being used. The great advantage of the carbon and mercuric sulfide suspensions, used in the present experiments, is that practically all the colloidal particles are retained by the intact basement membrane, as shown in histamine- and serotonin-induced lesions (MAJNO *et al.* 1961). Other preparations, such as gold for example, contain particles of a greater size range; the smallest are capable of traversing the basement membrane, thus labelling is often accompanied by a halo of spilling, and the distinction loses much of its significance.

In the absence of a colloidal marker, the occurrence of vascular leakage, at any particular time point, is practically impossible to detect in fixed tissues, except for the concentration of red blood cells. Extravasated erythrocytes and fibrin, precipitated in the vicinity of blood vessels, point to a high vascular permeability, but they give no reliable information about the time and site at which they may have escaped from the blood stream. In the case of fibrin, moreover, plasma may have seeped through the tissues for long distances before precipitation occurred.

Experimental Procedures and Results

I. Study by light microscopy

1. Muscle wounds. Seventy-one rats were operated bilaterally as described, then killed after 1, 2, 3, 4, 5, 6, and 7 days; 2, 3, and 4 weeks. Several animals were allowed to survive up to three months. One hour prior to sacrifice, most of these animals received the intravenous injection of carbon, mercuric sulfide or gold for the purpose of marking any leaking vessels. The one-hour interval sufficed to clear most of the injected substance from the blood stream, so that areas of leakage appeared in greater contrast. In addition, several animals were given 0.7 cm^3 per 100 g body weight of the carbon suspension and killed 2 minutes later. This quantity of carbon gave a dark gray coloring of the plasma, so that most vessels could be visualized. This more physiologic approach was preferred to post-mortem vascular injections which, in preliminary experiments, had given excessive extravasation in areas of new growth.

a) Overall process of wound healing. One day after the operation (Fig. 1)[1] the gap had filled with great numbers of large refractile cells and with hemorrhages of varying extent. Stumps of vessels abutted the cut edge. They did not appear to bleed, but the wound was surrounded by a zone of labelled vessels. Lack of circulation in several segments in the wound border was evident in animals with high amounts of circulating carbon. The bright red coloring of static vessels contrasted markedly with the dark gray plasma of patent vessels. The entire vasculature of the muscle was well injected, venous arborizations seemed especially prominent. At *48 hours* there was little change. Definite sprouts and club-shaped stubs were first detected on the *third day*. Many were distended with red blood cells and had, apparently, not been reached by the colloidal particles. Leakage in these preparations had occurred from most of the new tips as well as from vessels immediately adjacent to the wound edge. By the *7th day* (Fig. 2) the radial growth of vessels towards the center of the lesion was abundant; some smaller wounds were completely vascularized. The growing front was

[1] *Light micrographs* — Figs. 1 through 9 represent light micrographs of whole mount preparations of both cremaster and the cornea. Where no specific mention is made, the animals were given intravenous injections of colloidal carbon one hour prior to sacrifice. (*A* artery; *V* vein.) *Scale for all light micrographs: 100 μ.*

Electron micrographs: Key to abbreviations *B* basement membrane; *C* collagen; *c* carbon; *E* endothelial cell; *ER* endoplasmic reticulum; *F* fibrin; *f* fibril; *G* Golgi apparatus; *J* intercellular junction; *L* lumen; *m* mitochondrion; *N* nucleus; *P* platelet; *R* red blood cell; *r* ribosomes; *ve* vesicle; *xR* extravasated red blood cells. *Scale for all electron micrographs: 1 μ.*

always a dense brush of sprouts (Fig. 3); distinct loops and wide-meshed anastomoses were not seen in these preparations. Leakage, at that time, was localized predominantly near the advancing tips. Most wounds were completely vascularized by the *8th or 9th day* (Fig. 4). Once new vessels had made connection in

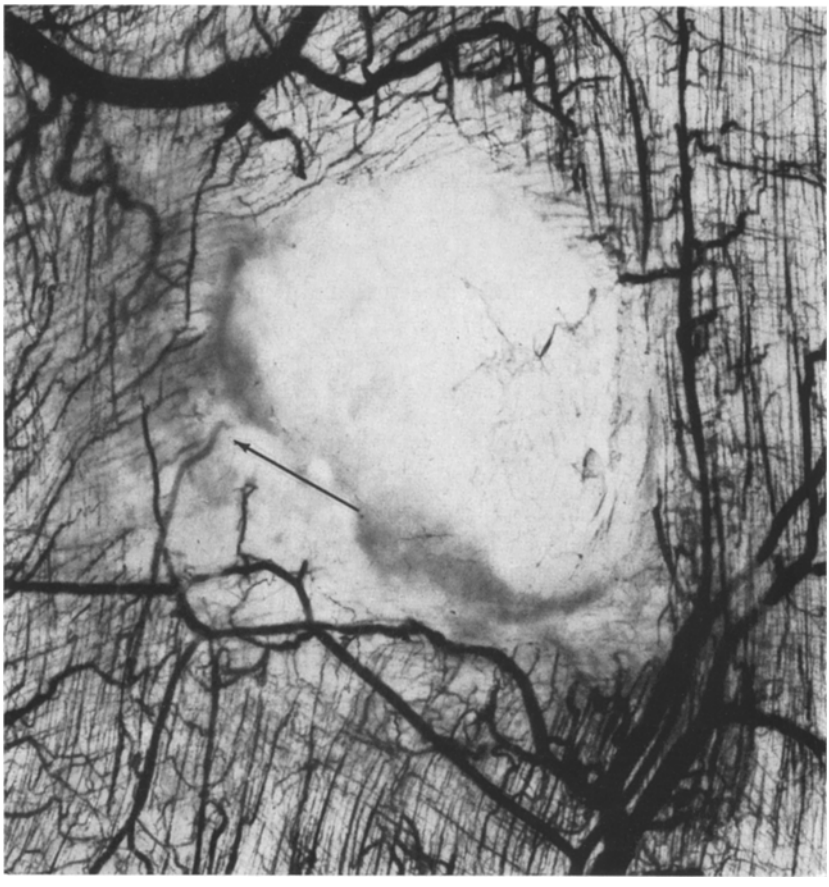

Fig. 1. Whole mount of a cremaster muscle one day after the operation. *2 minutes* prior to sacrifice, the animal was injected with a heavy dose of carbon, so that the plasma in patent vessels appears black in this photograph. The arrow points to several static vascular segments in which the blood had not become mixed with the colloidal particles. The short circulation time, and the presence of carbon within the blood vessels, do not allow identification of labelled vessels, which are present in the wound border on this day. (The blurring, especially evident in the upper left hand corner of the picture, is due to refractile cells adhering to the peritoneal surface of the muscle sheet.) Magnification, 20 ×

the center of the lesion, leakage was virtually absent. *Two weeks* after the operation, the former wound area had become an inconspicuous vascular tangle. The scars gradually diminished in size, became progressively more avascular and by two months were small whitish specks, occasionally tinged with clusters of macrophages containing hemosiderin.

Major vessels that had been severed at the time of operation were, apparently, not reconstituted: they showed distinct 'gaps' which persisted in scars eight or

more weeks old. This was especially true for arteries. Larger vessels circumventing the scar area were almost always veins.

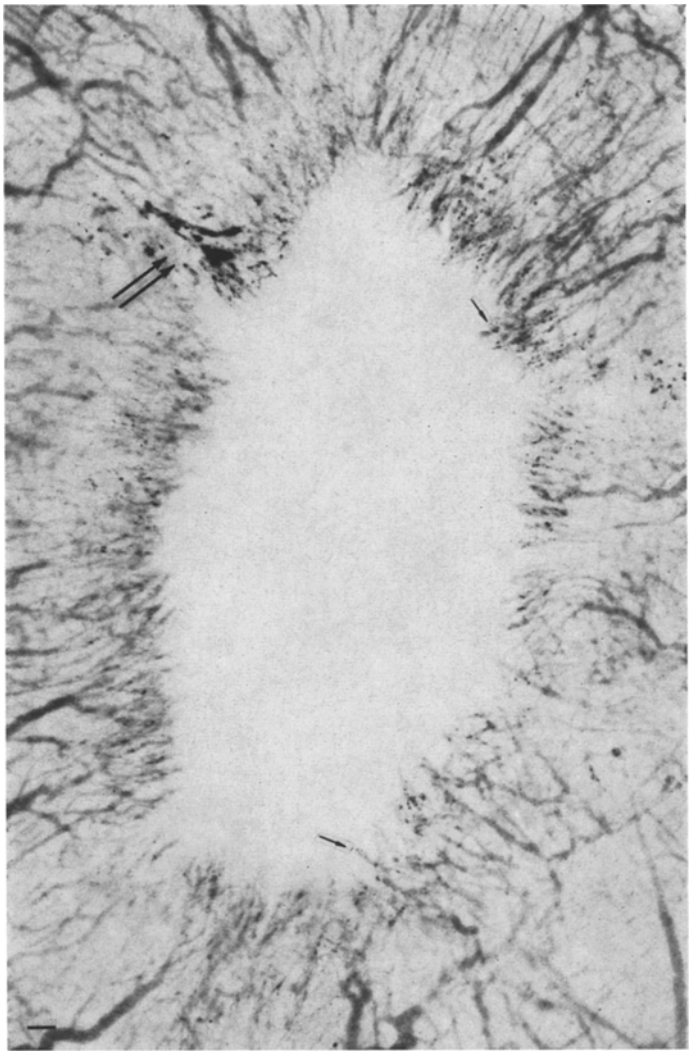

Fig. 2. Cremaster wound seven days after the operation. A dense ring of sprouts is growing towards the center of the wound. Leakage from new vessels has occurred predominantly near the growing tips of sprouts, and is evident as small black dots (arrows). A more heavily labelled vascular twig (double arrow) is also shown. Magnification, 40 ×

b) Aspect of the leaking vessels. Of the three colloidal particles utilized (carbon, mercuric sulfide, and gold), carbon gave the sharpest images of labelling, in that the leaks appeared relatively well localized.

The microscopic appearance of vessels labelled with carbon after histamine or serotonin injury has been illustrated by MAJNO *et al.* (1961). In these vessels, whether carbon deposits are in the form of granules, lumps or striae, the adven-

titial surface is almost always relatively smooth; granules in the extravascular space are encountered infrequently. In contrast, carbon leaking from regenerating vessels is rarely so well limited. Heaps of granules and larger clumps, at times

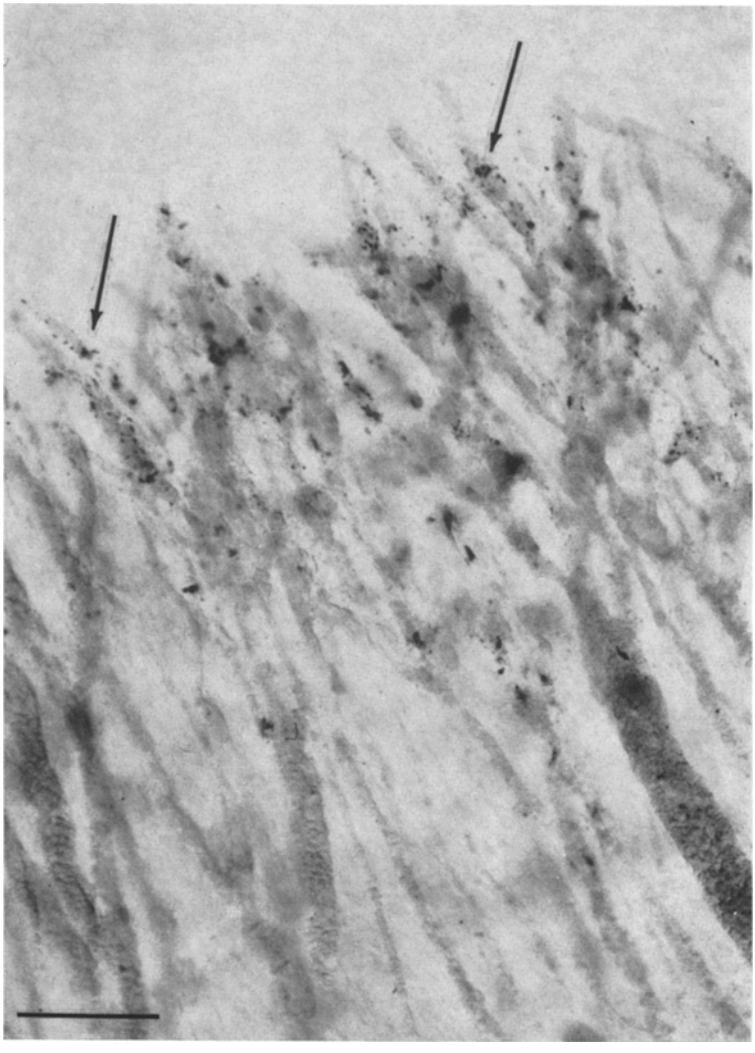

Fig. 3. Detail of sprouts growing into the seven-day wound shown in Fig. 2; note the numerous small leaks (arrows) near the growing tips. Magnification, 195 ×

scattered some distance from the vessel, are common appearances (Fig. 5). The impression gained was that the particles were not very effectively confined to the wall but had found access to perivascular spaces. This spilling effect was even more marked when mercuric sulfide or gold had been injected. Although lumps and aggregates of granules were present, they were often amidst an area of diffuse spreading.

c) Time and site of leakage. Labelling of preformed vessels around the margin of the wound was present before definitive sprouts could be identified. Once vascular sprouting had begun, no clear demarcation between old and new vessels

Fig. 4. Completely vascularized surgical wound of the cremaster, nine days after the operation. No signs of leakage (i.e. no carbon deposits) could be detected at this stage. Magnification, 35 ×

was possible. It was apparent, however, that in lesions three or four days old, new tips as well as their parent vessels must have leaked. Leakage ceased once new vessels had joined in the center of the wound. While growth progressed inward, leakage was constantly more pronounced at or near the growing front; vessels even a few days old had only sporadic deposits of particles. Solid blackening of vessels or entire segments of the vascular tree, as described by MAJNO *et al.* (1961) in histamine and serotonin lesions of this muscle, were not encountered. Most of the aggregates of colloidal particles appeared to be punctate leaks irregularly scattered along the vessels (Fig. 3).

2. Corneal lesions. Thirty-eight rats were treated as described, then killed after 2, 3, 4, 5, 6, and 10 days. Colloidal suspensions were injected as indicated for the previous group.

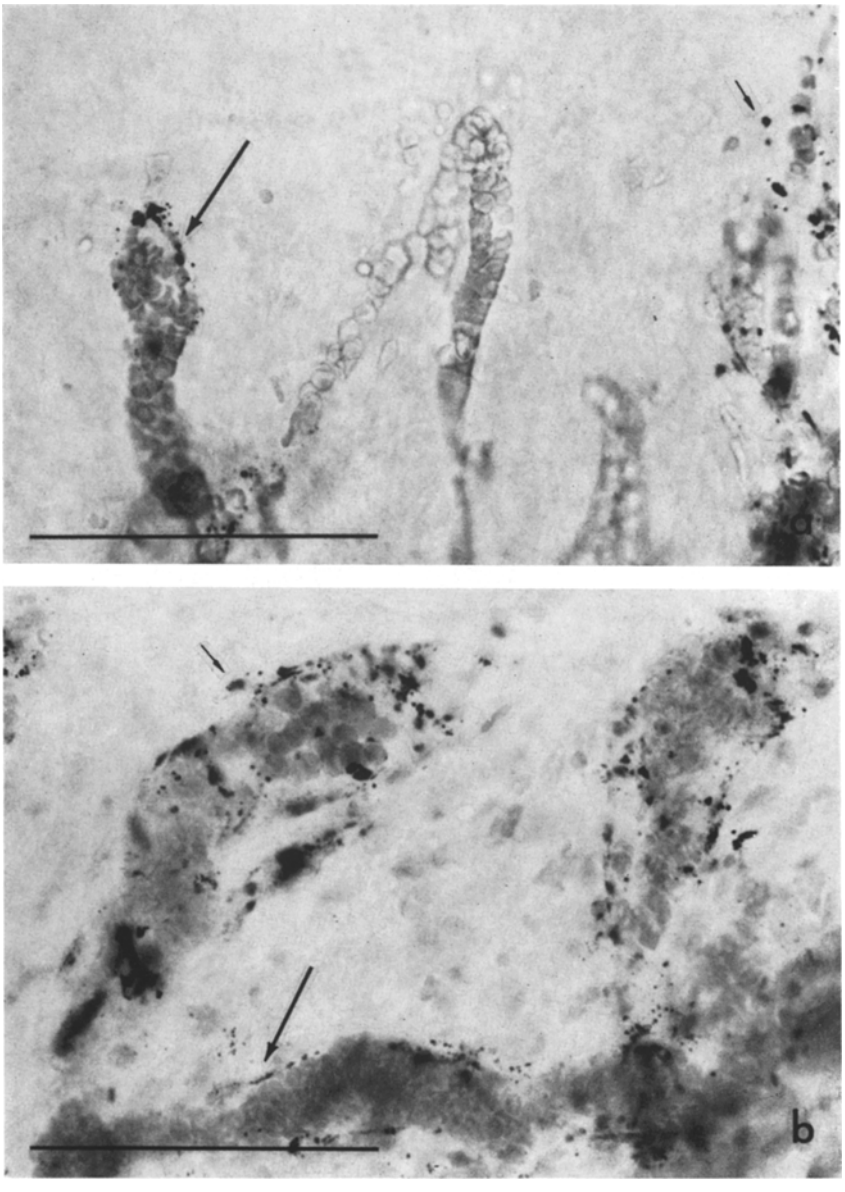

Fig. 5. Sprouts and club-shaped endings from the advancing vascular edge of the muscle wound, illustrating various aspects of the carbon deposits. Large arrows indicate clumps and granules near the vascular wall. Small arrows point to 'spilled' carbon some distance from the vessels. Figure b points out the dilatation which sometimes occurs in club-shaped endings. Magnification, 460 ×

a) Evolution of the lesion. Two days after the application of silver nitrate, the corneas of most animals had become slightly opaque and the major limbal vessels were dilated. Single sprouts as well as pointed loops were projecting

beyond the normal shallow arcs of the limbal vessels. Many tips of sprouts were packed with red blood cells, and small hemorrhages could occasionally be discerned ahead of the sprout. Leakage from new vessels was observed in all cases, most often near the tip of the sprouts and along the projecting loops. On the *third* and *fourth days*, the corneas were often markedly edematous and a conspicuous ring of new vessels had grown in towards the area of injury. Two types of overall growth patterns could be distinguished: *a meshwork of loops*

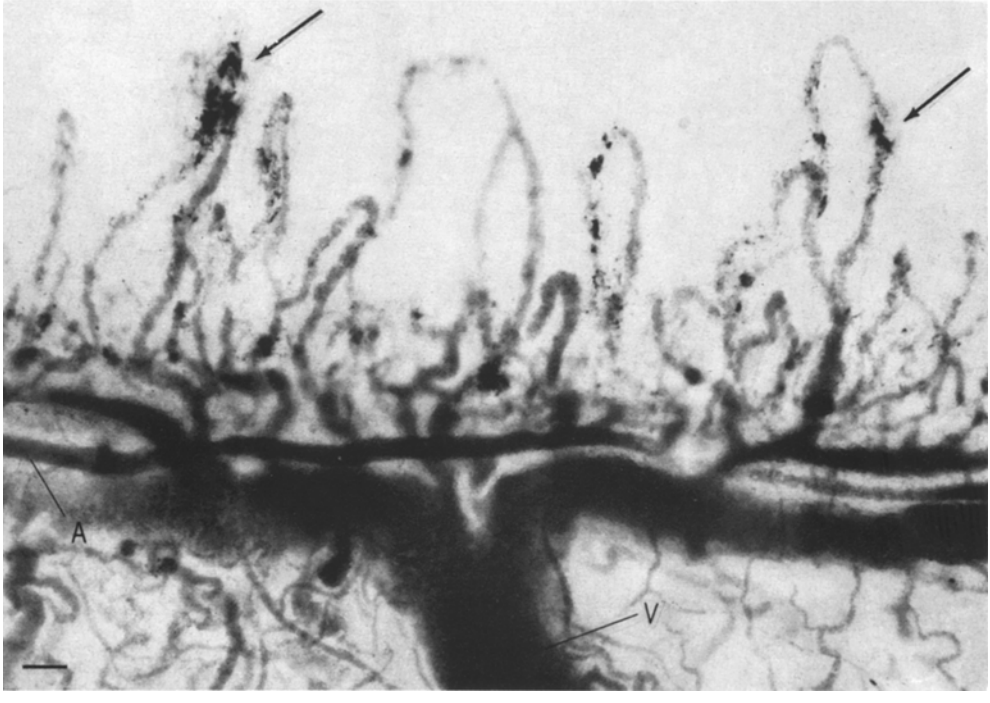

Fig. 6. Growth pattern of *loops* in a cornea two days after silver nitrate injury. 'Spilled' carbon (arrows) is scattered along the loops. Note dilated limbal vein. Magnification, 60 ×

(Fig. 6), and a very dense, and deep, *brush of sprouts* (Fig. 7). In the latter type, there was usually some involvement of and adhesion to the iris, indicating a more severe injury. Intermediate patterns with both loops and sprouts also occurred (Fig. 8). The growth of new vessels continued over the next two days and, by the *6th day*, had reached the area of injury. A dense plexus of vessels then formed around the silver nitrate spot, while a radiating system of freely communicating vessels permeated the cornea between the limbus and the site of injury. Vascular leakage was, by that time, most prominent in the plexus encircling the site of injury, although punctate leaks were seen throughout the cornea. The further fate of these vessels was not studied intensively, but, in agreement with numerous reports in the literature, regression occurred.

b) Aspect of the leaking vessels. The microscopic appearance of the leaks was essentially that described for vessels growing into the muscle wounds. Here too, the carbon deposits marked relatively discrete leaks (Fig. 9) while mercuric

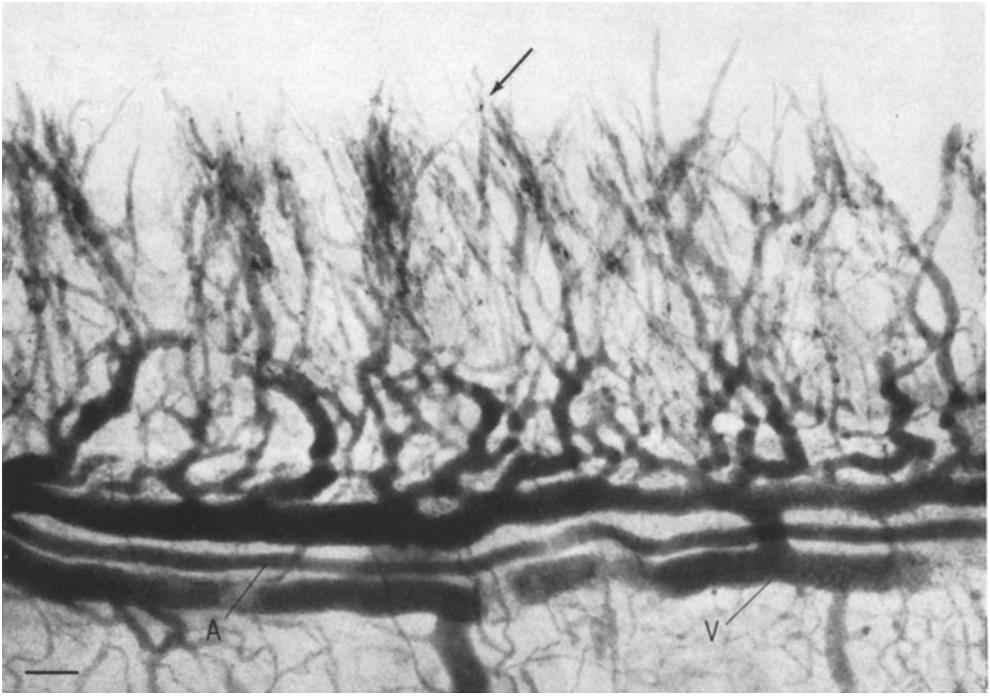

Fig. 7. Growth pattern of *sprouts* in a cornea three days after silver nitrate injury. One hour prior to sacrifice this animal received an intravenous injection of colloidal gold. A few discrete accumulations of gold (arrow) can be discerned. The anastomotic venous network at the base of the sprouts is especially conspicuous in this preparation. Magnification, 72 ×

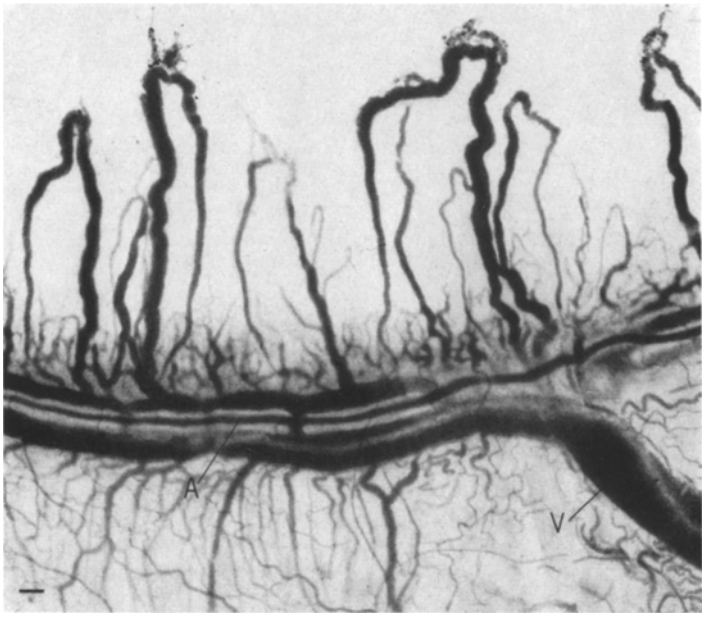

Fig. 8. Cornea, five days after silver nitrate injury. This preparation exemplifies an intermediate growth pattern, with both loops and sprouts. Magnification, 30 ×

sulfide and especially gold oozed into the neighboring tissues. Swarms of macrophages with ingested mercuric sulfide or gold sometimes obscured the growing tips. In corneas with very extensive growth (severe injury), such macrophages also formed a halo ahead of the sprouts and extended back to include the sclera. In these instances, a dense labelling of superficial venules at the limbus and in the sclera was not uncommon.

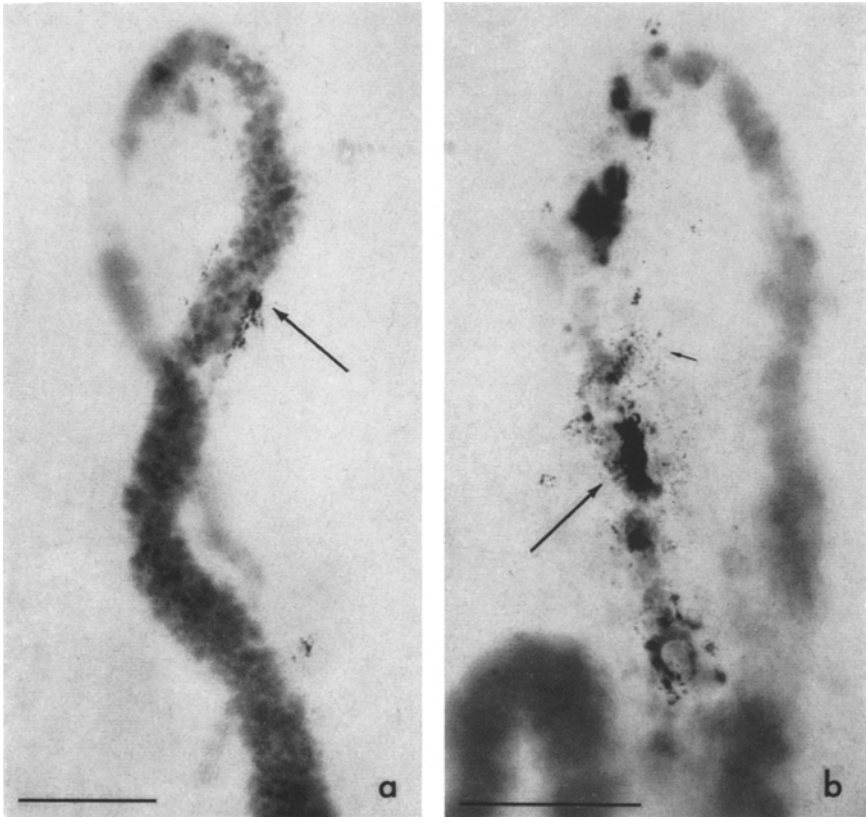

Fig. 9. a Relatively discrete leaks (arrow) in a corneal loop, 2 days after injury. Magnification, 185 ×. b Large leaks (large arrow) and spilled carbon (small arrow) along a vascular loop. Cornea, two days after injury. Magnification, 240 ×

c) Time and site of leakage. Leakage was present in all vascularized corneas during the period observed (10 days). It was always greater at or near the advancing front and, in corneas which had been more severely injured, included vessels of the limbus and the sclera.

II. Study by electron microscopy

The animals used for electron microscopy were killed 2 to 16 minutes after the intravenous injection of colloidal material. At this early time, many of the particles are still circulating; thus it was easier to identify vascular lumina. Eighteen selected fields from cremasters (3, 7 and 14 day stages) and nine selected fields from corneas (3 and 4 day stages) were used; special attention was given to the earlier stages.

1. Muscle wound. *Stage of 3 days.* A general description of the wound edge at this stage will help to portray the very complex structural milieu in which vascular growth occurred.

To facilitate orientation, the area of the wound will be subdivided into concentric zones. The samples for electron microscopy, as already stated, were shaped as isosceles trapezoids, pointing towards the center of the lesion. Thus, in a typical section, it is possible to distinguish three zones (Fig. 10). Zone A, containing part of the 'clot' and, if hemorrhage

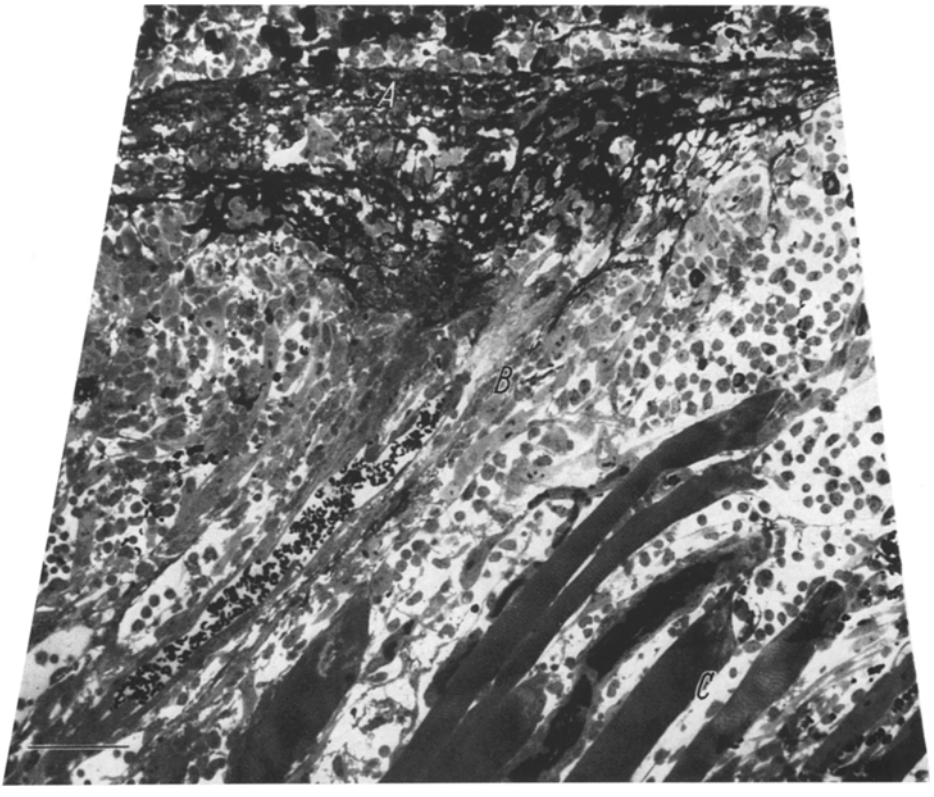

Fig. 10. Wound edge of the cremaster, three days after the operation. Light micrograph of an area sectioned for electron microscopy. *A* area of 'clot' and 'free cells'; *B* tissue breakdown area; *C* transition zone to surviving tissue (see text). Stain: crystal violet. Magnification, 150 ×

had not been extensive, 'free cells'; but no recognizable tissue. Zone B corresponds to the damaged margin of the muscle (zone of tissue breakdown). Finally, the transition to surviving tissue of the wound border is labelled C.

In *zone A*, the light micrograph suggests a relatively close packing of cells adjacent to the clot; however, when observed with the electron microscope, the same cells appear loosely scattered within a scant mesh of fibrin threads. In this area, leukocytes, macrophages, mast cells, eosinophils and, occasionally, plasma cells, were present. The outlines of these cells were extremely irregular, suggesting considerable membrane activity and movement. Wide pseudopodia and wavy processes of cytoplasm expanded into free spaces or seemed to wedge between wisps of fibrin. The surface of the clot was honeycombed by large macrophages in which bits of fibrin, cellular debris and erythrocytes in various

stages of digestion could, at times, be identified. Within the meshwork of the coagulum, several types of cells were entrapped by crisscrossing fibrin strands: erythrocytes, leukocytes and small oval cytoplasmic structures which resembled blood platelets. Extensions of the clot penetrated, often deeply, into the adjacent zone of tissue breakdown. In this area *(zone B)*, polymorphonuclear leukocytes and macrophages weaved through a scattering of muscle fragments, fibrin clumps and condensed cellular debris. Remnants of sarcolemma tubes were not uncommon. Large stellate or spindly fibroblasts with prominent granular endoplasmic reticulum, and many mitochondria, permeated this region. These cells were, at times, undergoing mitotic division. More peripherally, *zone C* contained surviving trunks of blood vessels and snags of muscle fibers projecting into the tissue breakdown area. The cross striation of many such muscle fibers appeared somewhat contracted near the degenerating stump. A hollow tube of sarcolemma, filled with mononuclear cells, projected beyond a layer of abnormal, finely vesiculated cytoplasm. A felt of disarrayed fibril fragments marked the transition of the normal fibrillar arrangement of the muscle fiber. Blood vessels, coursing with these fibers and piercing into the zone of tissue debris, were almost always dilated and frequently filled with stagnant blood. Those that had been reached by circulating particles showed deposits of this material, as well as erythrocytes, in clefts within the vascular wall. With regard to *new blood vessels*, despite the selection of suitable fields and the shaping of the block so that the direction of growth was indicated by the converging sides of the tissue section, the complexity of the tissue made it often difficult to ascertain whether the very tip of a growing vessel had been sectioned, or whether the cut was tangential some distance from it. Vessels which will be described, however, were unequivocally newly formed. Sprouts were most often found within or at the borders of the breakdown area, zone B. (Fig. 10). They appeared to grow into tissue spaces and clefts, but examples were seen where they lay embedded within compact clot areas. The lumina of many of these vessels were wide (Fig. 11) but their walls were thin and consisted of a single layer of endothelial cells with a faint basement membrane. In some cases, the latter was closely applied to a layer of fibrin shreds (Fig. 12). Within a short segment of the vessel, the thickness of the endothelial cell could vary from several micra, to cytoplasmic bridges so thin that the opposing aspects of the cell membrane almost made contact. Cellular junctions varied from a mere apposition of slender extensions to winding imbrications of adjacent cells. While adhesion plates could be demonstrated at cellular junctions, more often the endothelial cells fitted loosely. Endothelial nuclei were frequently distorted into ameboid shapes. Large numbers of mitochondria up to $4\,\mu$ in length and abundant granular endoplasmic reticulum, as well as numerous clusters of ribosomes were present in the cytoplasm (Fig. 12). Streams of fine fibrils were seen in many endothelial cells, sometimes so numerous that they formed entire tracts. 'Pinocytotic' vesicles, as described by PALADE (1953, 1961) were sparse; in fact, in certain areas they seemed to be completely absent. Profiles suggesting tubular cavities were noted at times. The most unusual finding in these vessels, however, were cytoplasmic extrusions arising from the outer aspect of the vascular wall and projecting into the tissue spaces (Fig. 12) (see below).

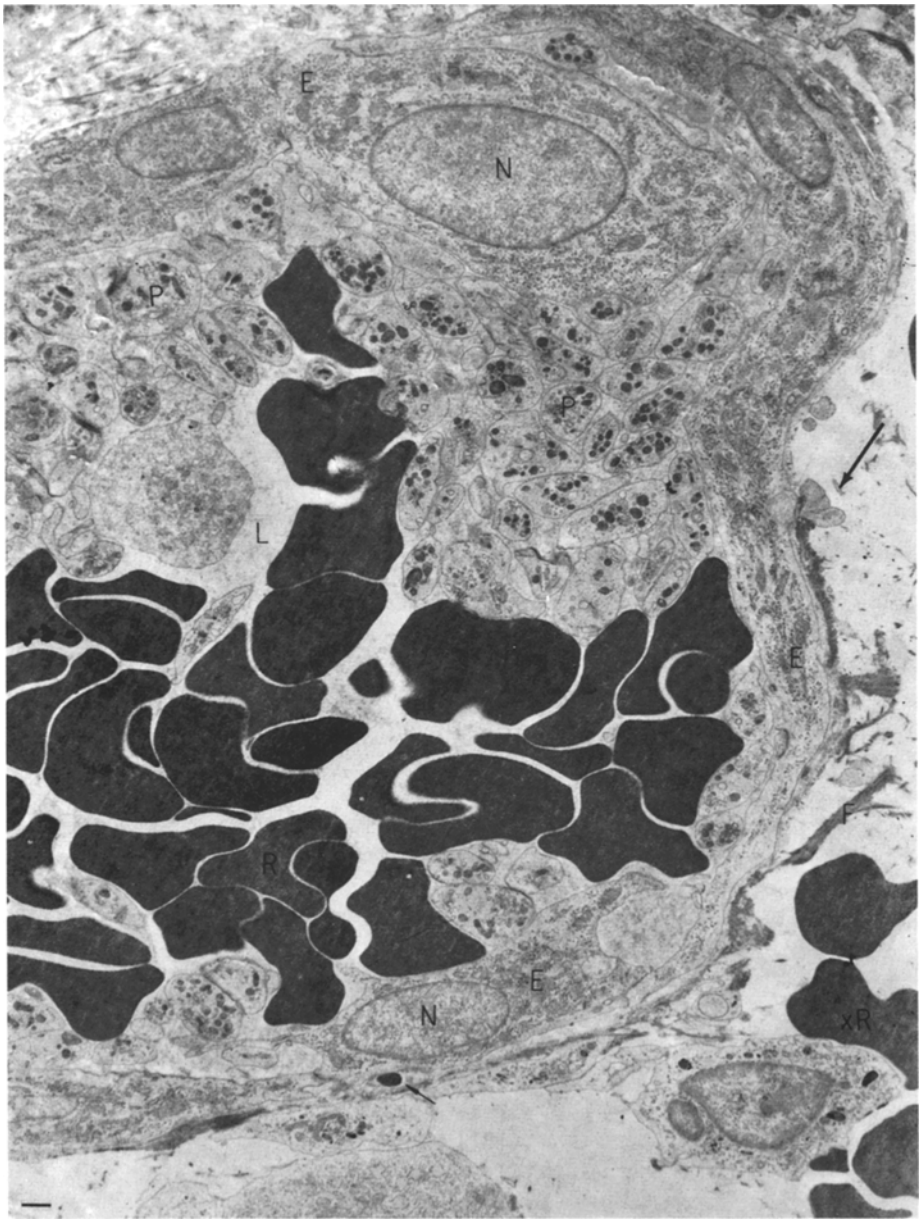

Fig. 11. Wide vascular sprout growing into a three-day-old muscle wound. The lumen of this vessel is filled with platelets and red blood cells. Extravasated erythrocytes are shown in the lower right hand corner. A fine layer of fibrin shreds coats the adventitial aspect of the vessel. Note the cytoplasmic extrusion (large arrow) of the endothelial cell. The small arrow marks a portion of a red blood cell within a cleft in the vascular wall. Stain: Karnovsky, method b. Magnification, 3800 ×

Stage of 7 days. At this time, the lesion contained a bewildering mosaic of cells. Outstanding were large rounded multinuclear myoblasts, packed with small granules and dense mitochondria. Periodic Z-lines with nearly fully formed fibrils were present in portions of many of these cells. Fibroblasts were abundant,

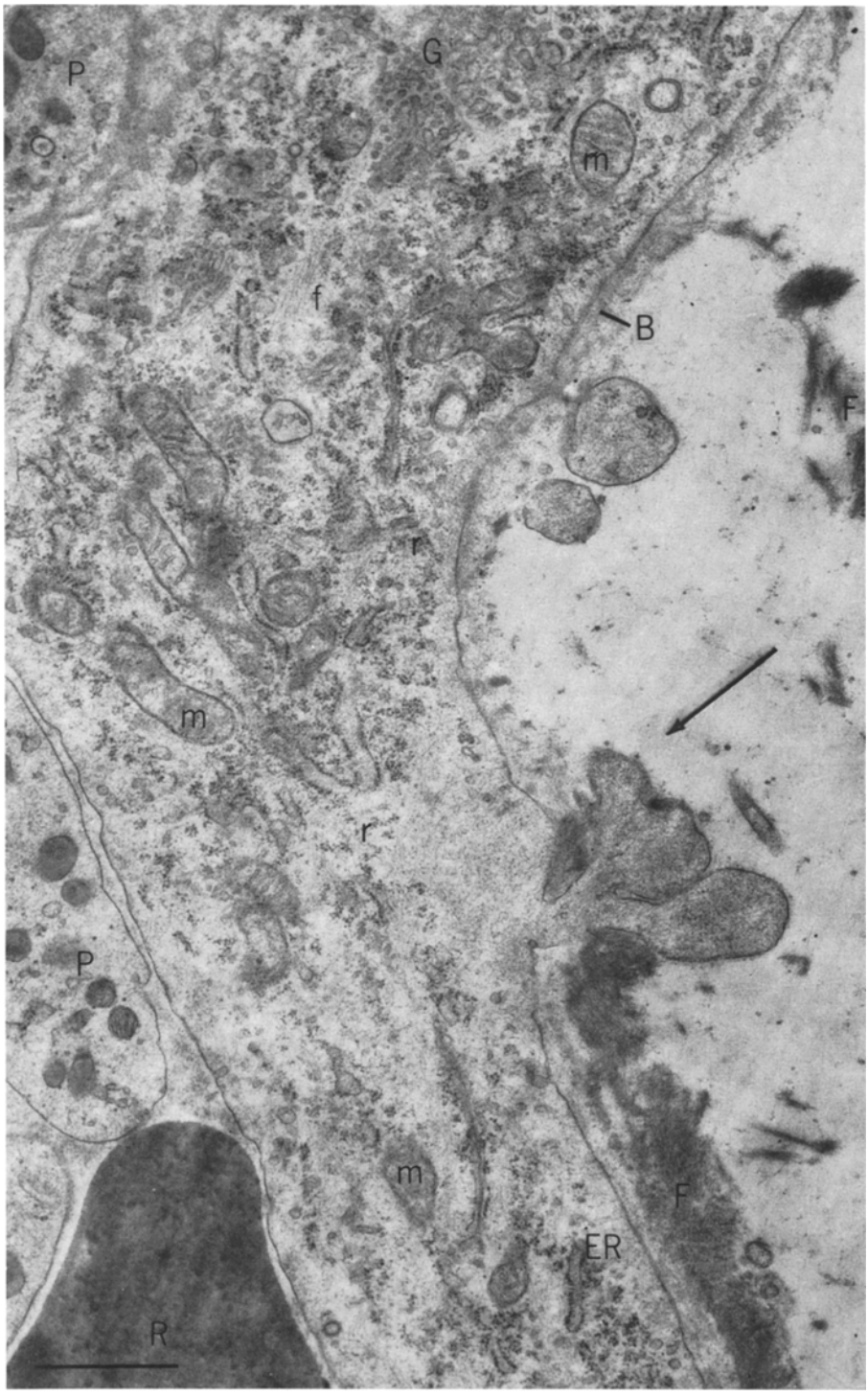

Fig. 12. Cytoplasmic processes (arrow) of the endothelial cytoplasm 'punching' through the basement membrane and a closely applied layer of fibrin shreds. Note absence of cytoplasmic inclusions in the portion extruding into the perivascular space. Serial section of the sprout shown in Fig. 11. Stain: Karnovsky, method b. Magnification, 20,000 ×

appearing as large pleomorphic cells filled with lamellae of endoplasmic reticulum. Intermingled throughout were macrophagic cells containing numerous dense vacuoles. Blood vessels in these areas were no longer thin-walled tubes. The endothelium was often composed of short plump cells. External to the basement membrane, connective tissue cells were, at times, closely applied. In certain areas, the adventitial aspect of the vessels bordered onto compact layers of collagen bundles. This was especially the case in a few pictures taken from a *14 day* lesion. However, the vessels in zones which still contained fibrin and cellular debris resembled those described for the earlier stages.

2. Cornea. *a) Normal cornea.* The corneal stroma consists of flat collagen ribbons which run parallel to the outer surface, interweave at many different angles and, near the periphery, fan out and interdigitate with scleral fibers (POLACK 1961). With the electron microscope, these ribbons are seen to consist of collagen fibrils, oriented parallel to each other and to the long axis of the ribbons. Clefts and channels between lamellae, at times containing corneal corpuscles (stromal cells), were described by JAKUS (1954). Near the limbus, the collagen fibrils are more variable in diameter and the interlamellar spaces contain stromal cells as well as macrophages and mast cells (JAKUS 1961). The blood vessels of the limbus have not been described.

In control animals the collagen bands run in groups which extensively intertwine and, in places, they separate to form relatively large clefts. These were at times empty, but often they contained stromal cells whose processes could be traced for long distances, following the contours of these cavities and penetrating between collagen lamellae. Limbal blood vessels lay between dense fiber tracts or near these clefts, sometimes extending into them. The vascular lumen averaged 10μ. Macrophages and polymorphonuclear leukocytes were frequently seen in the perivascular tissue. The endothelial lining was almost uniformly 1 to 2μ in thickness, except in areas occupied by the long flattened nuclei. The basement membrane was an inconspicuous layer, often fused with collagen bundles. Closely molded to the blood vessels were flattened cells, or slender bands of cytoplasm. These were usually separated from the vascular wall by a thin layer of collagen fibrils, but at times they were contained within the endothelial basement membrane. Platelet thrombi were present in certain segments of these vessels; one vessel was found with an intramural deposit of carbon particles.

b) Injured cornea. Corneal fields were selected from the advancing edge of the pannus. The corneal lamellae in areas of vascular growth were distinctly less compact and, in a number of specimens, the packing of collagen fibrils was cleaved by strands of fibrin (constituting fibrinoid as described by MOVAT *et al.* 1960). Since the latter were more electron dense than collagen, they tended to accentuate the fiber direction, particularly in relation to growing blood vessels (Figs. 13, 22). Large interlamellar clefts were encountered rarely, but in many fields, the perivascular tissue behind the actively advancing sprouts was permeated by polymorphonuclear leukocytes, macrophages and stromal cells. The latter contained prominent 'succulent' mitochondria and an extensive system of stacked granular endoplasmic reticulum. Macrophages with ingested erythrocytes and degenerating leukocytes were found along most blood vessels in proximal

regions. In the area of the advancing tip, leukocytes were not nearly as numerous but they were occasionally found even beyond the vascularizing zone. Bundles of nerve fibers, almost always unmyelinated, were seen in all areas investigated. Vascular sprouts appeared to follow primarily natural cleavage planes, although a certain amount of packing and crowding of collagen fibrils was at times evident close to the sprout (Fig. 13). Near the advancing tip of a vessel, streamers of

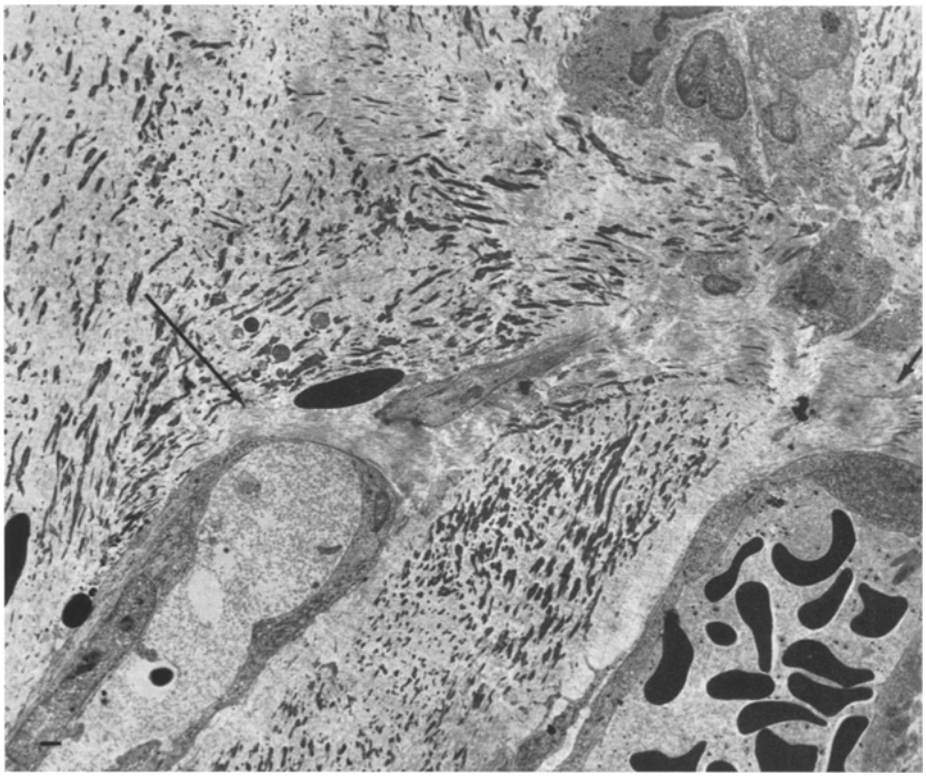

Fig. 13. Low-power electron micrograph from a cornea three days after silver nitrate injury. The dark streaks in the stroma are strands of fibrin packed between the collagen fibrils (thus constituting "fibrinoid": see MOVAT 1960). The orientation of collagen fibers around growing vessels is thus accentuated. Crowding of the stroma (arrows) is evident next to the newly-formed vessels. Portions of three extravasated erythrocytes can be seen near the center and the lower left corner of the picture. Stain: Karnovsky, method a. Magnification, 2100 ×

cytoplasm appeared to probe tissue crevices (Fig. 14). Newly formed vessels were usually rather wide, especially so in proximal regions, and anastomosed freely. Parallel vessels, separated by less than the width of a blood cell, were frequently encountered. The thickness of the endothelial lining was, as described for new vessels in the muscle wound, highly irregular. In contrast to the vessels in early muscle wounds, however, the outer surface of the endothelium was accompanied by long slender cells either closely applied or separated by a thin layer of collagen fibrils. In proximal areas, several cell layers could often be distinguished within the basement membrane. Bubbling cytoplasmic processes, reaching into the perivascular space, were encountered more frequently than in muscle preparations, and were far more extensive (see below).

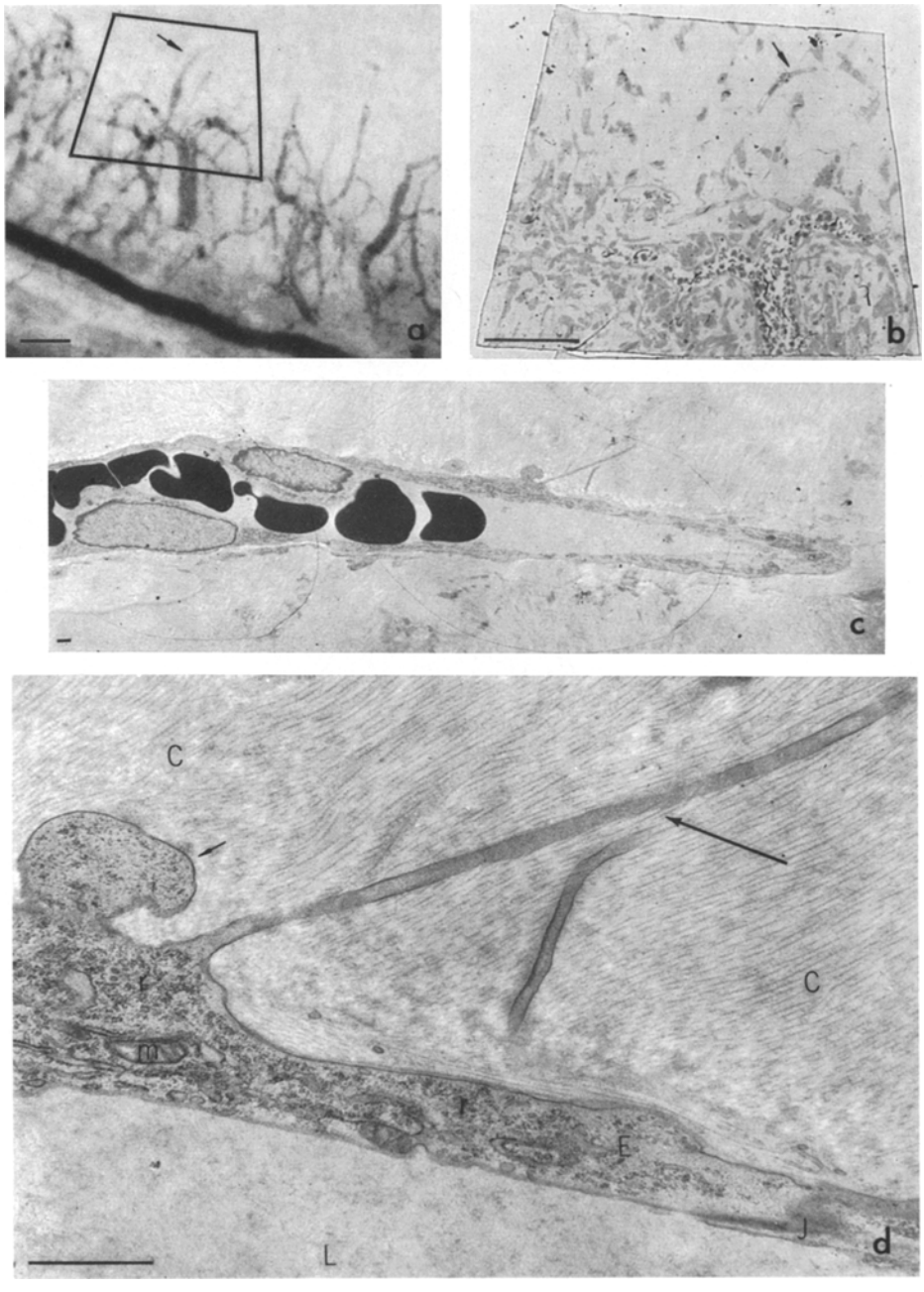

Fig. 14a—d. Tip of a sprout from a cornea three days after silver nitrate injury. a Light micrograph of the Epon block from which the field was selected. Magnification, 66 ×. b Light micrograph of a 'thick section'. The tip of the sprout, shown in figure a, is marked (arrow). Stain: Azure II-Methylene Blue. Magnification, 130 ×. c Low power electron micrograph of the tip of the sprout (thin section adjacent to b). Magnification, 1700 ×. d Streamers of endothelial cytoplasm (large arrow) penetrating into collagen lamellae. A knob-like endothelial extrusion is indicated by the small arrow. Stain: Karnovsky, method a. Magnification, 17,000 ×

3. Special features of blood vessels in muscle and cornea. Certain morphologic features of growing vessels which appear in muscle and cornea alike, deserve special consideration.

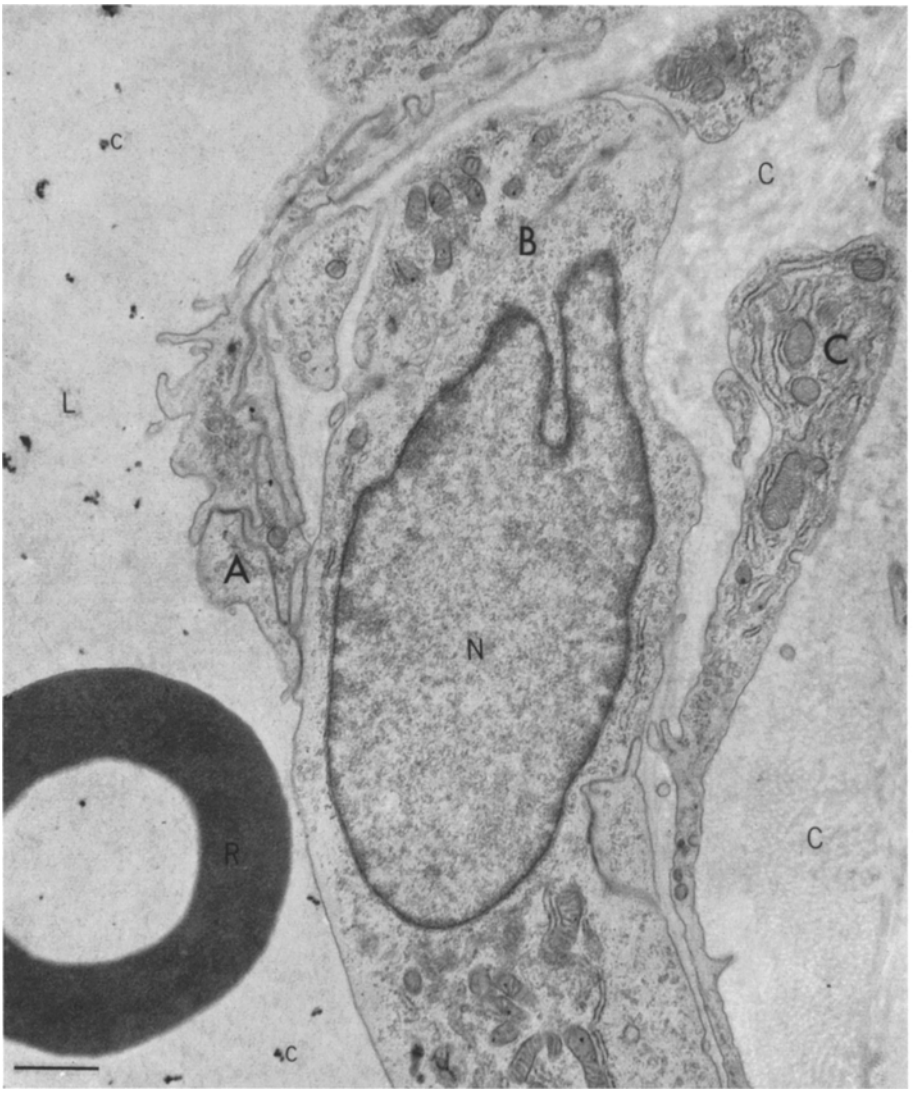

Fig. 15. Detail of the vascular wall illustrating a 'loose' overlap of adjacent endothelial cells, marked *A* and *B*. A third cell (*C*), partially embedded in the corneal stroma, is making contact with the inner endothelial lining in the lower portion of the picture. From a cornea, three days after silver nitrate injury. Stain: Karnovsky, method a. Magnification, 11,000 ×

a) Cellular junctions and leaks. The space between endothelial cells, in many vessels of both muscle and cornea, was 15 to 20 mμ. In the cornea, it was often possible to obtain sections in which growing vessels were cut longitudinally for long distances. This offered the possibility of comparing several cellular

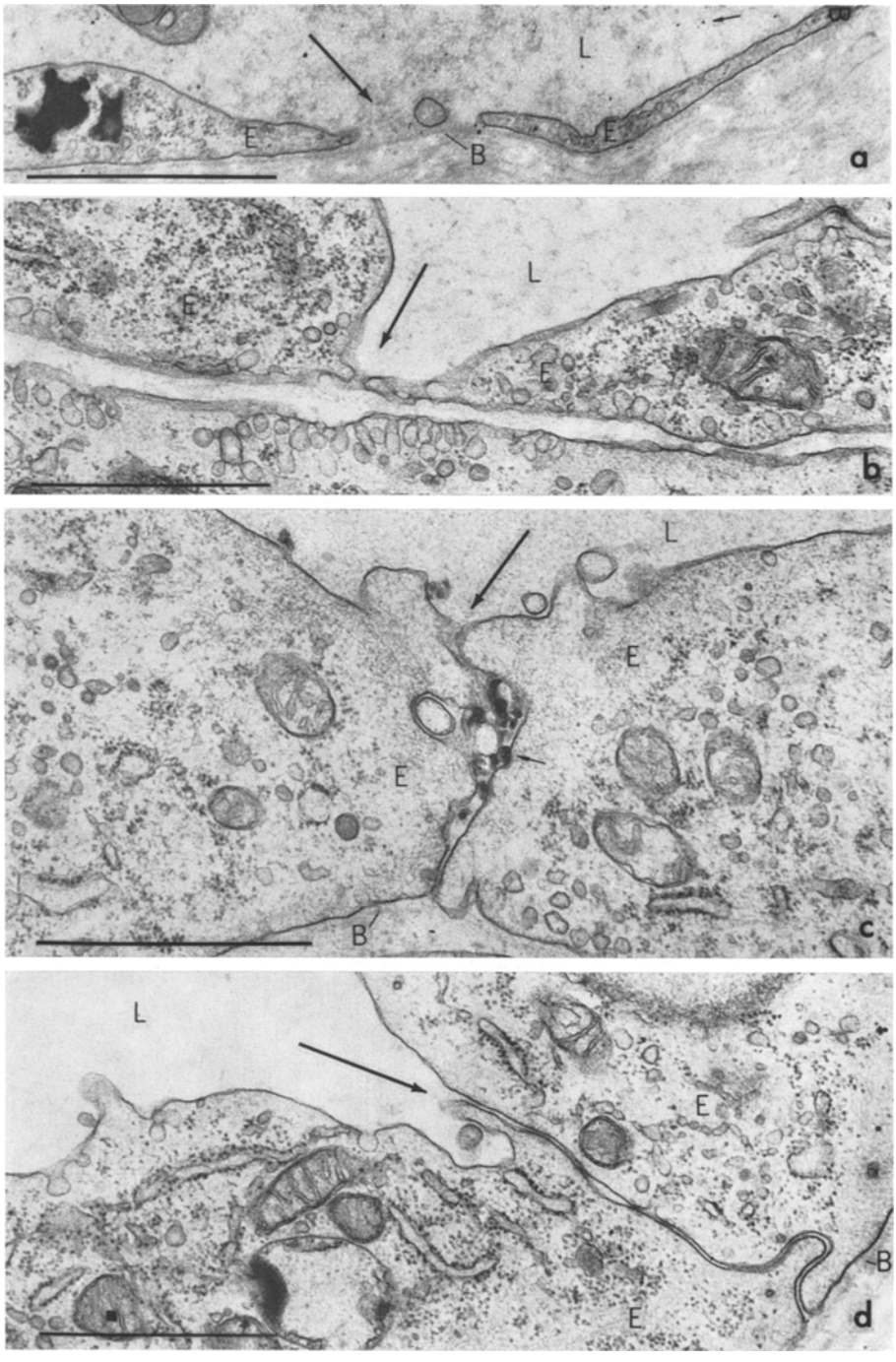

Fig. 16a—d. Cellular junctions (large arrows) from growing blood vessels in the injured cornea. a Endothelial cells separated by a wide gap. A few particles of colloidal gold (small arrow) may be seen in the lumen. A distinct basement membrane is present on the outer aspect of the endothelium. Magnification, 33,000 ×. b Cellular junction of the endothelium resembling a 'pore'. Clusters of vesicles, in a cell closely applied to the endothelial lining, seem to be especially prominent near this discontinuity. Magnification, 32,000 ×. c Endothelial cells which appear loosely apposed. A few carbon particles (small arrow) have penetrated. Magnification, 37,000 ×. d Simple cellular junction of the endothelium. Magnification, 32,000 ×. (All sections stained with Karnovsky, method a)

junctions within a given segment. In some areas, cells which at first appeared peri-endothelial, could eventually be traced to the inner vascular lining (Fig. 15). The junctions between endothelial cells ranged from extensive overlaps (20 μ

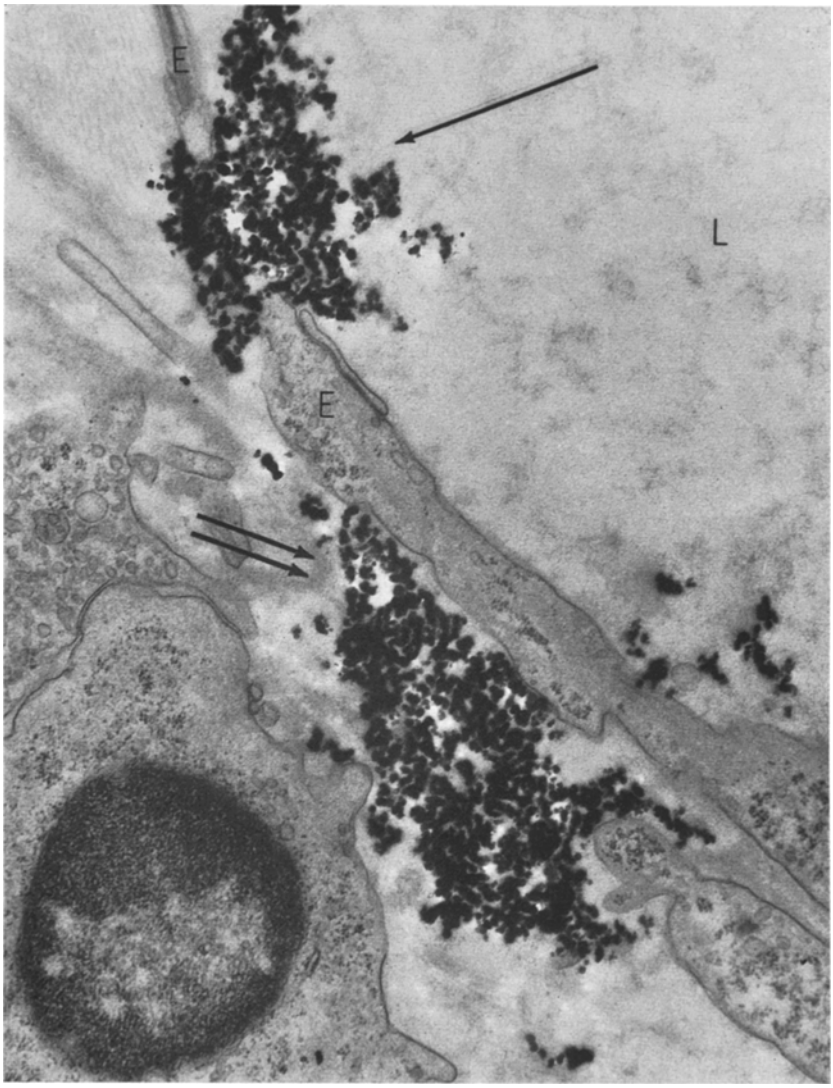

Fig. 17. Site of leakage in a corneal blood vessel. Carbon is escaping through an opening between endothelial cells (large arrow); it has also spilled into the perivascular tissue (double arrow). A structure corresponding to the basement membrane cannot be identified in this picture from a cornea three days after injury. Stain: Karnovsky, method a. Magnification, 21,000 ×

or more) to small areas of contact and even distinct gaps (Fig. 16a, b). Adjacent cells were at times extensively interdigitated, but often they simply made contact along a line perpendicular to the endothelial surface. Within an area of interdigitation, distinct gaps were not uncommon; a firmer attachment was usually present near the lumen. This impression of *loosely fitting cells* (Fig. 16c, d) was

strengthened by the fact that evidence of leakage (accumulation of colloidal particles) was always found near or in the cellular junctions. The particles appeared to percolate through spaces between loosely apposed cells (Fig. 16c) or to escape through wider separations of adjacent endothelial cells (Fig. 17). Frequently, red blood cells were seen passing through these openings (Fig. 18).

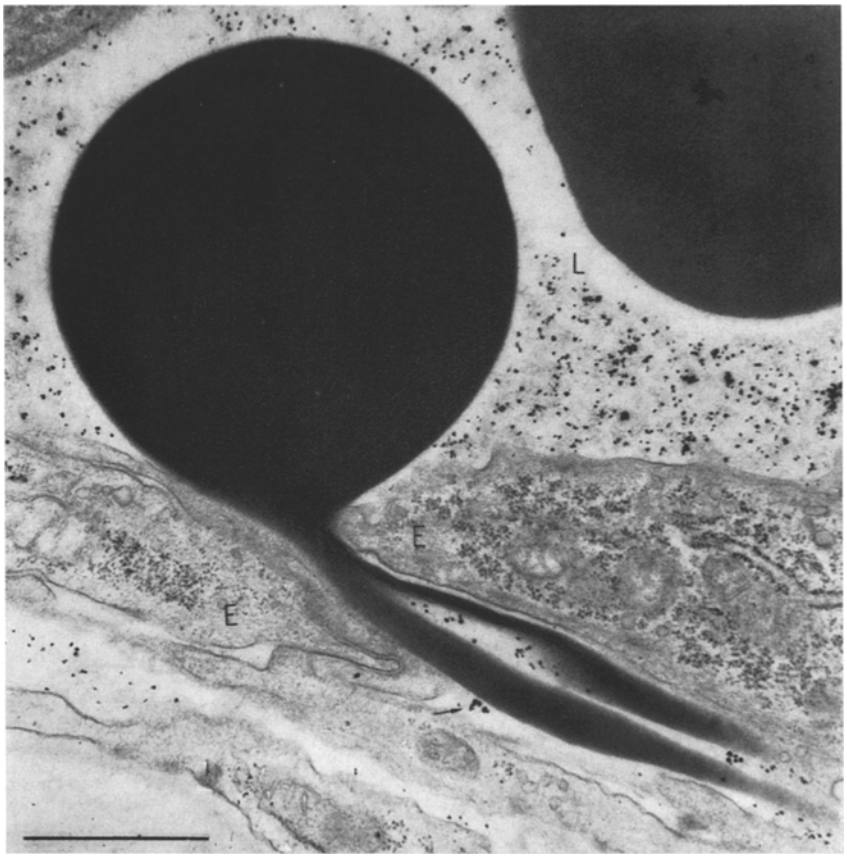

Fig. 18. A red blood cell and particles of mercuric sulfide (arrow) passing through a cellular junction of the endothelium. From a muscle wound three days after the operation. Stain: Karnovsky, method a. Magnification, 25,000 ×

Particles escaping from the blood stream were not always retained within the vascular wall, as was described by MAJNO and PALADE (1961) for mildly injured mature blood vessels, but appeared to spill into the perivascular tissue (Figs. 17 and 19).

b) Cytoplasmic extrusions and basement membrane. The basement membrane of growing vessels is exceedingly thin, and sometimes barely recognizable as a distinct entity. In the cornea, it was quite frequently difficult to see this layer at all. This finding suggested the reason for the spilling of particles into the extravascular space, and also related to a feature of the vascular sprouts which has not been described in normal, mature blood vessels. Certain peculiar cytoplasmic processes extruded from the outer surface of the endothelium, or from

the outer cell of the vascular wall. Sometimes these processes appeared to 'punch' through the basement membrane; their mushroom shape suggested that the cytoplasm squeezed through a narrow opening (Fig. 12). Often, however, a structure corresponding to the basement membrane could not be identified, and portions of the endothelial cytoplasm expanded irregularly into the sur-

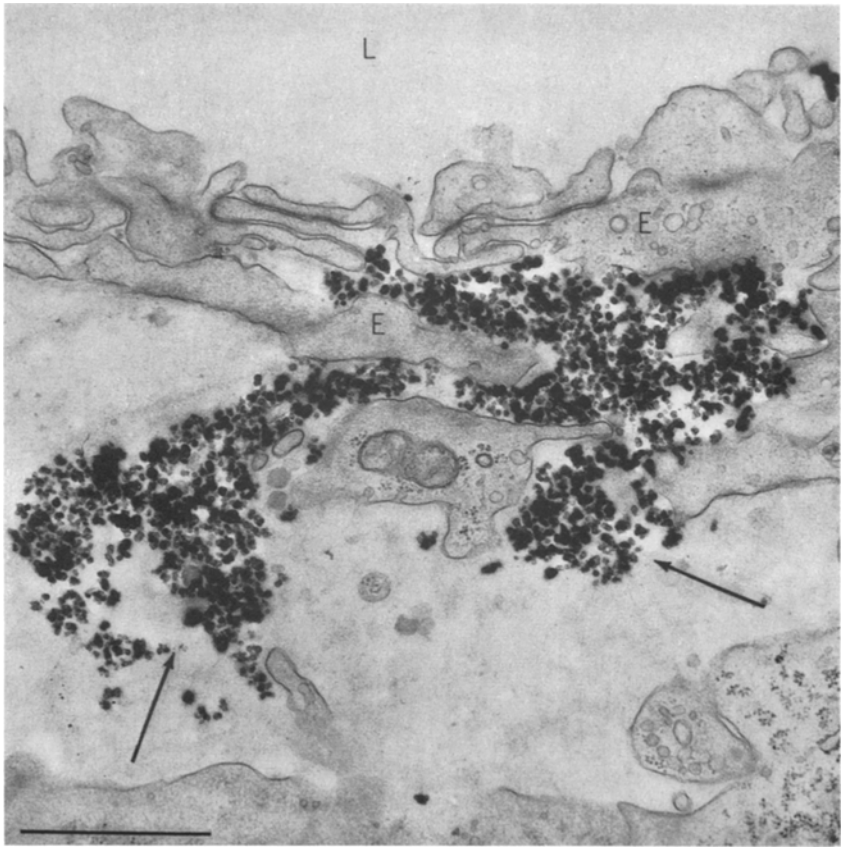

Fig. 19. Complex cellular junction that has permitted the escape of carbon from the blood stream The particles have spilled some distance into the perivascular space (arrows). A structure corresponding to the basement membrane cannot be identified in this field. From a cornea three days after injury. Magnification, 25,000 ×

rounding tissue (Fig. 20). In the cornea, these processes at times appeared as slender tentacles, penetrating between collagen bundles, or as stalked knobby protrusions filling an irregular tissue crevice (Fig. 14). The width of these processes is of the order of 0.1—0.2 μ. The length is difficult to estimate because only short segments may be contained within the plane of section, but in one instance (see Fig. 14) it could be followed for approximately 7 μ. Branching was seen occasionally and it may be inferred that small cytoplasmic bodies, often seen in the immediate neighborhood of the sprout, are in reality cross-sections of such processes. In some instances, in the cornea, this phenomenon reached great proportions, and appeared as a zone of extensive 'bubbling' of

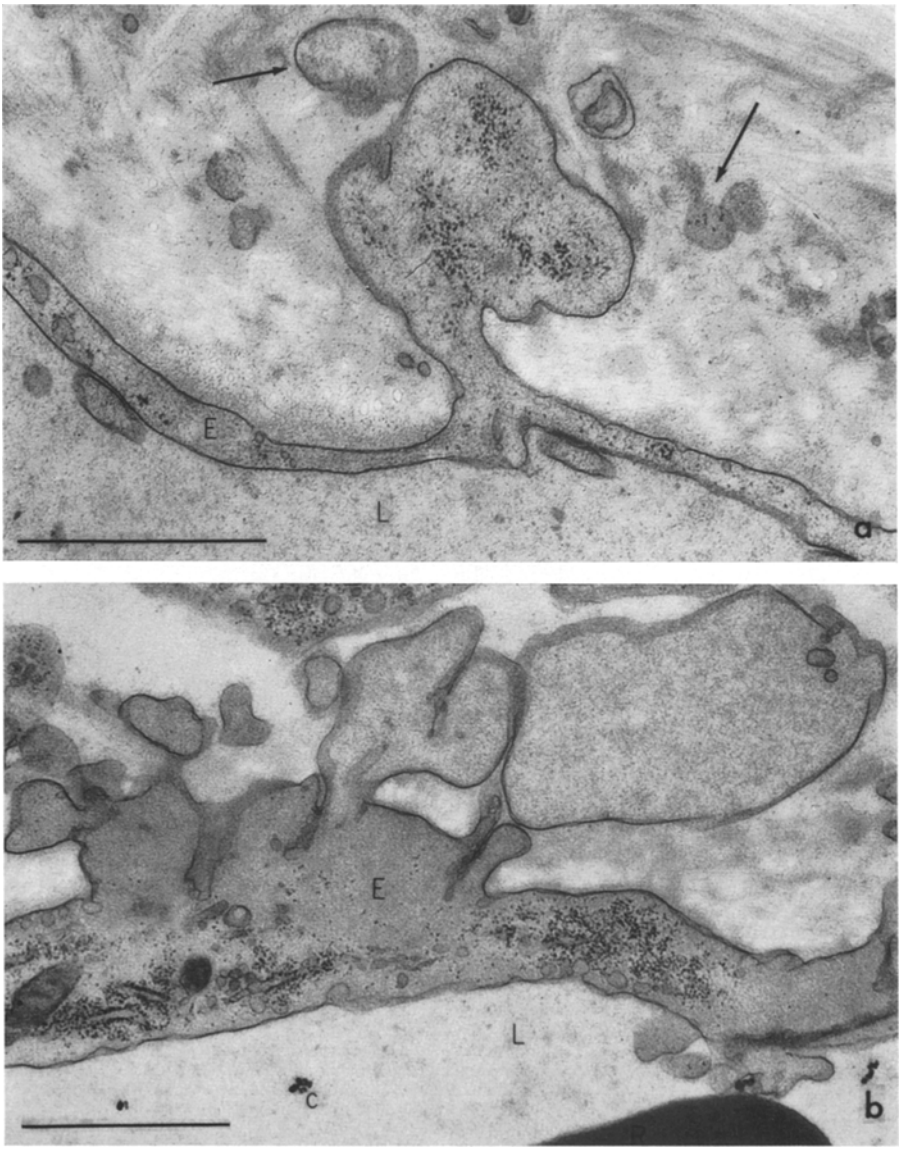

Fig. 20. a Cytoplasmic process projecting from the outer aspect of the endothelium. The membrane-bounded structures (arrows) in the vicinity of this cell process are thought to represent extensions, or branches of this endothelial tongue. From a muscle wound three days after the operation. Stain: Karnovsky, method a. Magnification, 34,000 ×. b Outer surface of the endothelium 'bubbling' into the perivascular space. Note 'watery' appearance of these cytoplasmic projections. A layer corresponding to the basement membrane cannot be identified. Cornea, three days after injury. Stain: Karnovsky, method a. Magnification, 28,000 ×

cytoplasm into the perivascular space (Fig. 20b). In these cases, it may be presumed that the basement membrane is entirely absent. At times, the second layer of the vascular wall appeared to extrude similarly into neighboring corneal stroma (Fig. 21).

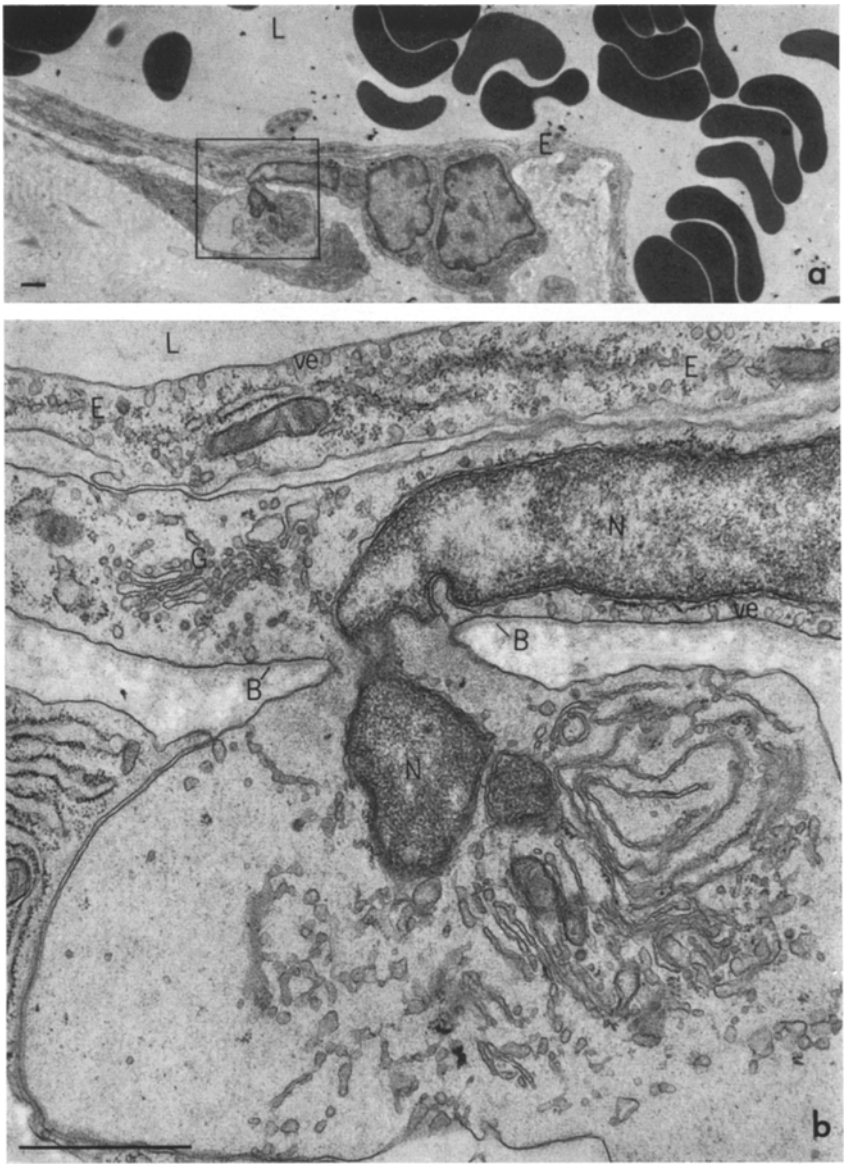

Fig. 21. a Cytoplasmic extrusion from the second layer of the endothelium; cornea, three days after silver nitrate injury. Magnification, 2800 ×. b (Detail of a). The extruding cell is tightly applied on the outside surface of the endothelial layer; the presence of vesicles suggests that this cell may also be endothelial in nature. Nucleus and cytoplasm appear to be squeezing through a narrow neck (the basement membrane is seen as a faint grey line, abutting against either side of this neck) then to expand into a broad, pseudopod-like structure. The latter contains abundant, clear cytoplasm, free of inclusions as is usually observed in the moving portion of migrating cells. (The smooth-surfaced tubular structures may represent endoplasmic reticulum, or part of the Golgi apparatus.) Stain: Karnovsky, method a. Magnification, 23,000 ×

c) Tips of the capillary sprouts in the cornea. The tip of most capillary sprouts observed in the cornea were invested with a continuous, though irregular, endothelial lining (Fig. 14). In several instances, however, the lining became

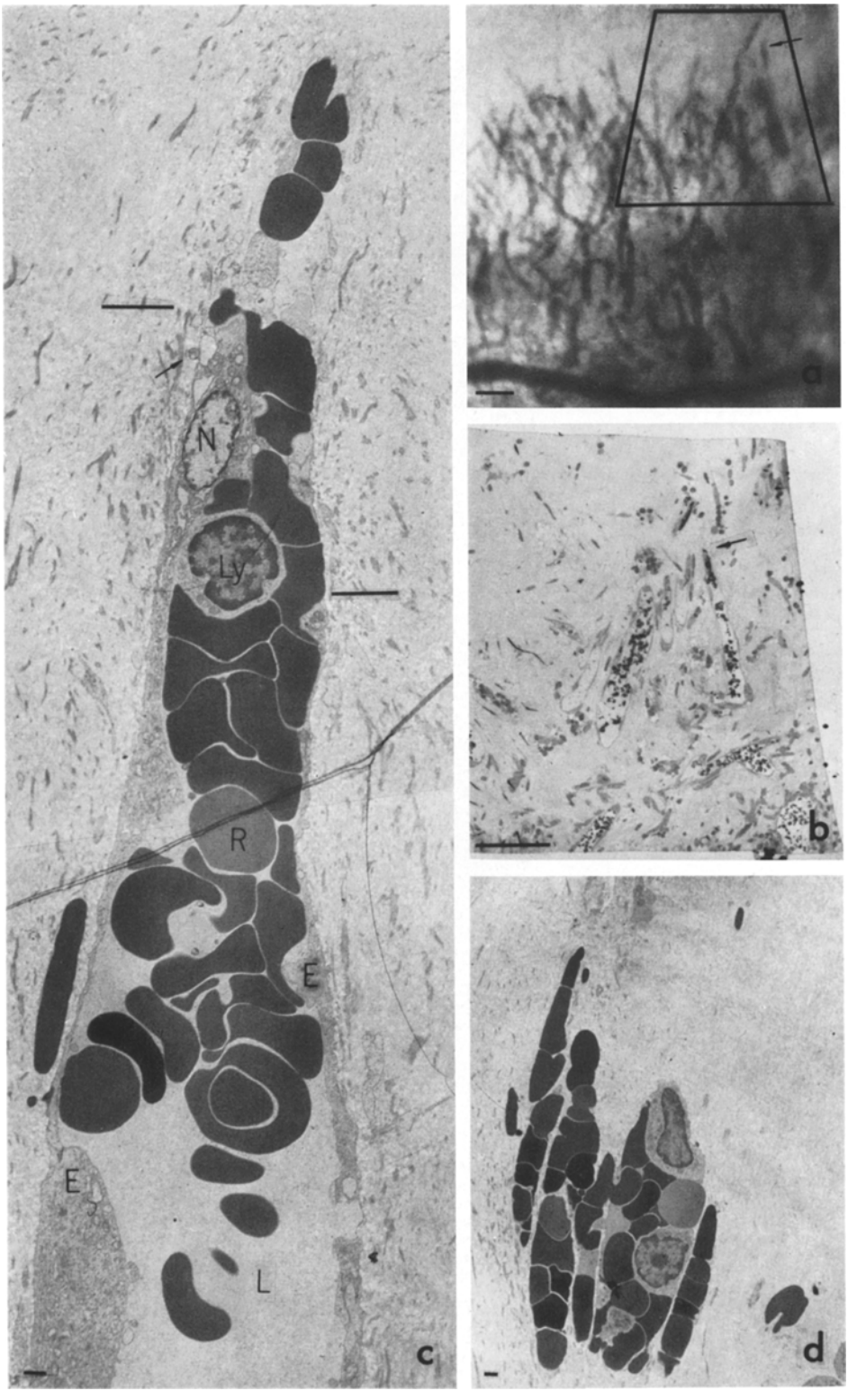

Fig. 22a—d

discontinuous towards the very tip of the sprout (Fig. 22), and the lumen, packed with red blood cells, appeared to be communicating with the extravascular spaces. The endothelial cytoplasm in these areas showed several abnormalities: rounded mitochondria with irregular cristae and greatly distended spaces of the endoplasmic reticulum. Small fragments of cytoplasm and even isolated cell organelles (mitochondria) could be identified in the adjacent corneal stroma, and among rows of red blood cells stacked between collagen lamellae.

Historical Survey

The literature dealing with regeneration of blood vessels is intimately related to that on wound healing. Although the latter subject can be traced back to the earliest recordings of medical history, its relevance to the growth of new vessels can only begin once Harvey's postulation of a heart-to-heart circulation, and Malpighi's as well as Leeuwenhoek's discovery of the capillaries, had established the concept of a closed circulatory system.

HENRI-LOUIS DUHAMEL DE MONCEAU (1741, 1742) is credited with the first experimental demonstration that severed vessels ultimately reconnect. He broke the leg of a chicken and, after callus formation, severed the entire soft tissue of the leg in successive operations. A mass injected some time later via the femoral artery was found to penetrate as far as the foot. BOERHAAVE, also in the first half of the 18th century, suggested that in a wound the vessels reconnect by elongation and fusion. VAN SWIETEN (1754, quoted by MARCHAND 1901) comments on the remarkable happening that vessels not only reconnect by an end-to-end process ("inosculation"), but must also reconnect in such a way that arteries join with arteries, and veins with veins, so that the new parts will resemble those that were destroyed. Whether all 'vessels' were thus restored was not certain, but it was acknowledged that the wound acquired at least two of the recognized types of vessels: the larger blood vessels and the 'exhalants'. A rather complicated circulatory system was envisioned at that time. Arteries were thought to branch and lead to veins "without an intervening substance" but partially they continued as *vasa exhalantia* into crypts and follicles, *vascula excretoria* and *secretoria*, as well as *vasa serosa* and *aquosa* (lymphatics). A corresponding diversity of veins was envisioned.

JOHN HUNTER (1792) distinguishes three kinds of arterial vessels: those conveying "debilitated blood" to the veins; others that "separate from the blood" or excretory; and a third kind that "contains the formers and supporters of the body". According to HUNTER, in a simple wound the ruptured vessels may close (which they do by muscular contraction), but they may also reunite: whether the cut ends exert a mutual attraction ("sympathy"), or whether the intermediate coagulated lymph forms a new connection, is not certain. "The very few instances where it [inosculation] can be observed, together with the want of accuracy in those who first introduced the term, would incline me to think that it arose from theory or opinion only". However, he felt that he could demonstrate this phenomenon in the inflamed vascularized cornea. In large wounds, several phenomena occur: adhesive inflammation (coagulated lymph is "thrown out" by exhalant vessels), suppuration, and finally granulation (formed by vessels which have been changed for that purpose). Old vessels are thought to extend into the wound, while new ones are formed *de novo*. Observing "a great number of spots of red blood" which he could not inject with mercury, HUNTER concluded: "I have therefore suspected that parts have the power of making vessels and red

Fig. 22a—d. All pictures were taken from a cornea three days after injury. a A portion of the whole mount from which the field (marked) was selected. The arrow points to the sprout shown at left. Magnification, 56 ×. b Light micrograph of a thick section cut adjacent to thin ones used for figure c. The sprout is marked as indicated for figure a. Stain: Azure II-Methylene Blue. Magnification, 110 ×. c Tip of the sprout, marked in the adjacent light micrographs. The endothelial lining becomes discontinuous at the levels indicated by the bars. Large dilated cisternae of the endoplasmic reticulum may be seen near the endothelial nucleus in the upper portion of the picture. The three erythrocytes at the top of the picture are lying within the corneal stroma. The small arrow points to a mitochondrion, located extracellularly. *Ly* lomphycyte. Stain: Karnovsky, method a. Magnification, 3200 ×. d Large numbers of red blood cells filling clefts of the corneal stroma near the tip of the sprout shown in picture c. (Section from a different depth in the block.) Stain: Karnovsky, method a. Magnification, 1700 ×

blood independent of circulation. This appears to be evidently the case with the chick in the egg." This analogy, according to JOSEPH MEYER (1852), insured that the concept of *de novo* formation would survive for half a century.

BICHAT (1818) believed that no blood vessels are present in the wound, but rather an abundance of 'exhaling' and 'absorbing' vessels, which are filled with blood since they are inflamed. BICHAT is said to have accepted a continuous capillary network between arteries and veins, but he also believed that not all capillaries are filled with blood. These 'white vessels' include the 'exhalant', the 'excretory' and also the 'absorbant' (lymphatics). The existence of these 'white vessels', however, is already seriously questioned in an addition to BICHAT'S anatomy by P. A. BÉCHARD in 1823.

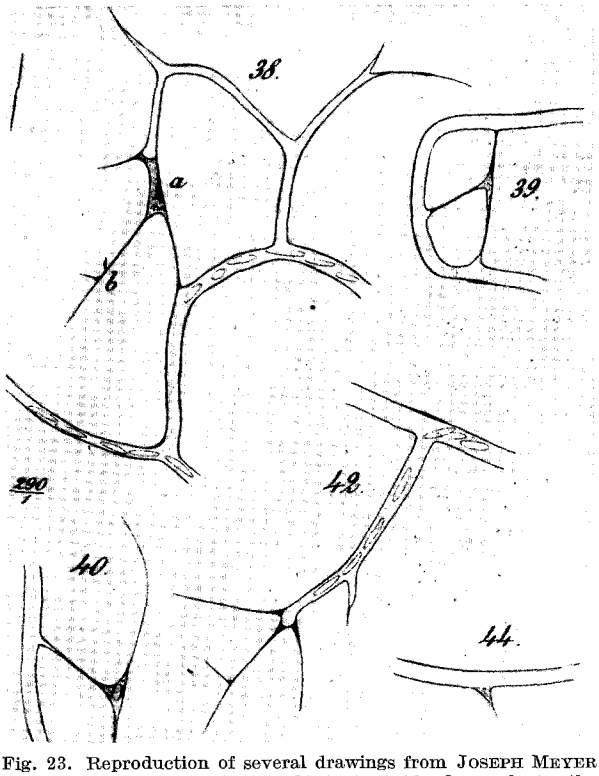

Fig. 23. Reproduction of several drawings from JOSEPH MEYER (1852) illustrating shoots ('Ausläufer') of blood vessels in the tadpole tail

A. N. GENDRIN, in 1826, provided ingenious experimental proof that old vessels can grow into a wound. In a series of surgical operations, he prepared skin flaps, which were promptly reapplied and allowed to heal, then recut after various periods in such a way that they would remain attached to the surrounding skin merely by the newly formed tissue filling the wound. With the aid of mercury injections, GENDRIN could show that ramification of arterial vessels extended through the wound and into the skin flap. From the survival of such skin flaps he also deducted that growth of vessels is variable; e.g. connection across a four-day wound sometimes sufficed for survival of the flap, while other times sectioning after fourteen days resulted in necrosis. Growth of vessels, he suggested, proceeds by a reopening of arterial ends, so that blood is forced into the clot, thus hollowing out a channel. By repetition of this process the vessel is extended into the wound.

The next significant contribution in this field, soon after the formulation of the cell theory, is an extensive treatise by JOSEPH MEYER in 1852 (Fig. 23). In a careful microscopic study of experimentally produced pseudomembranes, of skin wounds and of frog larvae observed *in vivo*, he describes the process of sprouting, capillary formation, differentiation into arteries and veins and finally regression of vessels in older wounds, essentially in the same form as it is accepted today. MEYER emphasized in particular: that growth of capillaries always proceeds from pre-existing vessels; the red "dots" and "lines", observed in early wounds and pseudomembranes (interpreted since HUNTER as analogous to blood islets, and taken as proof of *de novo* vasculogenesis), are extravasates; and that larger vessels in adhesions always develop from simple capillary tubes. It may be added that vasculogenesis without connection to the circulatory system was briefly revived by RANVIER in 1876. He based his theory on the observation of large stellate cells containing erythrocytes (cellules vasoformatrices). These cells, however, were later recognized as macrophages containing

phagocytized erythrocytes (YAMAGIWA 1893). In the year 1865, capillaries were recognized as cellular tubes (AUERBACH).

From the wealth of publications of the latter half of the 19th century, relating to newly formed vessels, only a few will be selected. Vieing with each other were schools which maintained that the formation of the vascular lumen was inter- or intracellular, and debated the question whether new vessels formed from endothelium only, or with the participation of connective tissue cells. KOELLIKER'S handbook of 1867 shows a diagram which pictures a sprout open to the tissue consisting of parallel extensions of endothelial cells. The participation of the connective tissue cells is thought possible, and envisioned as a 'rolling up' of these cells, presumably in the shape of a gutter, to fit longitudinally the cylinder of the extended sprout. Another theory of intercellular canalization was suggested by THIERSCH in 1878. THIERSCH proposed a loosening of cells in the vascular wall, leading to escape of blood into an intercellular system of canals which eventually become lined with cells. This theory was based on the observation that fine projections of a carmine-colored injection mass were constantly found radiating from vessels in granulation tissue.

ARNOLD (1871, 1872), saw the sprout as a syncytium whose core became liquid to form a nucleated protoplasmic tube which at first did not show cellular outlines with silver nitrate, but subsequently, by a process of furrowing, became divided into cells. ZIEGLER (1876), on the basis of his well-known experiments with implanted glass chambers, rejects the intercellular theory of THIERSCH since it does not explain how hemorrhage is prevented, and describes a process in which canalization proceeds *via* liquefaction within cells. MAXIMOW, in 1902, came to the conclusion that sprouts are formed neither by the inter- nor the intracellular theory, but that the endothelium migrates as a sheet; thus the sprouts are really outpouchings of the vascular lumen. Mention should also be made here of THOMA'S "histochemical laws", first proposed in 1893 (see THOMA 1911). The most debated of these laws has been one which maintains that the new growth of capillaries depends on the blood pressure within them. It was eventually refuted by the observations that vascular growth, in embryos, proceeded in the absence of the heart (CHAPMAN 1918), and that sprouting occurs with equal frequency from both arterial and venous segments. Finally, CLARK and CLARK and their collaborators, in a long series of publications, described the process of sprouting and capillary formation in the living tadpole (1918), and later in the rabbit ear chamber (SANDISON 1926, 1928, 1931; CLARK 1918; CLARK *et al.* 1931; CLARK and CLARK 1935, 1939). The advancing tip of the sprout is thought to be a syncytium (a concept primarily based on the observation of nuclear movement); connective tissue cells are not found to participate.

Since this time, little has been added to our knowledge of vascular growth.

Discussion

1. Patterns of vascular growth. It is generally agreed that the growth of new vessels takes place by the process of sprouting. There has been evidence, however, that sprouting, although more prevalent and obvious, may not be the only mode of expansion of the vascular bed. Several authors (KREIKER 1924, SWINDLE 1938) have even denied that *sprouting* occurs in the cornea, and stated that vascularization is effected by an *elongation of loops*, i.e. of the limbal arches. A carefully standardized study of wound repair in the mouse chamber by CHALKLEY *et al.* (1946) showed that there was a significant increase in the extent of the vascular bed prior to the appearance of sprouts. These authors suggest that one possible mechanism for this increase of vascular tissue may be the intercalation of young cells into the wall of pre-existing vessels, a process very similar to that proposed by MAXIMOW in 1902.

In the present experiments, the corneal preparations showed both types of growth, and despite a certain degree of admixture, it was obvious that sprouts prevailed in some cases, loops in others. There were consistent differences between

'loop' and 'sprout' preparations. Sprouts usually coincided with more severe injury, as evidenced by iritis, pronounced corneal edema, extensive venular leakage and cellular infiltration of the limbus. By contrast, when the silver nitrate burn was relatively small and shallow, and stromal swelling was slight, the vascular growth was limited to vascular loops projecting towards the site of injury. It is our impression that these different patterns may reflect different speeds of growth. With a mild stimulus, growth may be minimal, resulting primarily in a lengthening of pre-existing vessels; in fact mitotic activity of the endothelium, even with definite sprout formation, is said to be actually highest in vessels proximal to the actively advancing sprouts (VAN DEN BRENK 1955; FLOREY 1962). On the other hand, sprouts, according to the same concept, would reflect rapid vascular growth.

HEYDENREICH (1955) studying the vascularization of rabbit corneas after various types of injury, distinguishes two types of vascular patterns, distinct morphologically as well as functionally: 1. A shallow rim of growth ("randständige Kapillarneubildung") consisting primarily of loops, to which he assigns a resorptive function. 2. Extensive vascular arborizations ("pannoide Gefäßneubildung") effecting tissue repair, i.e. forming granulation tissue.

In cremaster wounds, vascular ingrowth seemed to occur by sprouts only; distinct loops were never seen (though a few might have escaped observation because of the thickness of the tissue). This predominance of sprouts may be a characteristic of muscle, as opposed to the cornea, but we are more inclined to correlate it with the relatively severe experimental injury. It is interesting to note here that in an experiment in which vascular growth was suppressed by ionizing radiation (VAN DEN BRENK 1955), mention is made of a pattern of tortuous loops, and that CHALKLEY *et al.* (1946) describe the same appearance of the vessels *prior* to sprout formation.

The experiments described here do not allow any conclusions as to the nature of the stimulus evoking vascular growth. Corneal vascularization is said to be initiated by any agent which loosens the stromal framework of the cornea, i.e. causes it to become edematous (COGAN 1949, HEYDENREICH 1955, SZEGHY 1960). It is also a common sequel of riboflavin deficiency (BESSEY and WOLBACH 1939, JONES *et al.* 1945). A possible role of material released from ruptured mast cells has also been suggested (SMITH 1961). Based on experiments in retinal vascularization, ASHTON (1961) has raised the possibility that the "endothelial cells themselves are in some way directly sensitive to oxygen — multiplying at low levels, resting at normal levels and dying at high concentrations". He summarizes the main conditions which seem to govern the growth of vessels in the eye as: 1. The presence of living tissue. 2. A low oxygen tissue-tension and 3. poor venous drainage. The accumulation of a metabolite may also be implicated. This concept of a chemical stimulus was originally proposed by LOEB in 1893.

Regeneration in wounds, as well as regeneration in general is still an imperfectly understood process and, as CLARK and CLARK (1939) pointed out "the same growth conditions which favored the formation of new blood vessels also stimulated the growth of other tissues in the same region". Vascular repair, concomitant with proliferation of connective tissue in general, has been found to be favored by tissue extracts (DANN *et al.* 1941, EDWARDS *et al.* 1960), by

maintaining the animals on balanced protein diets (CHALKLEY *et al.* 1946) and by increasing the histamine level of the tissues (BOYD and SMITH 1959). A retardation was noticed with cortisone treatment (ASHTON and COOK 1952, BANGHAM 1951) and with histamine depletion (BOYD and SMITH 1959). LANGHAM (1960) demonstrated that a mitotic poison, applied topically, reduced the vascularization of the cornea to about one fifth of that found in controls.

With regard to the path taken by the advancing vascular growth, our findings in both the corneas and cremasters suggest that the sprouts and loops pushed forward along a path of least resistance. This was especially evident in the cornea where vascularization followed cleavage planes between the collagen lamellae, in agreement with previous light microscopic observations (AUGSTEIN 1902, EHLERS 1927, COGAN 1949). In the cremaster, the vessels observed were growing primarily into areas of tissue breakdown which in the electron microscope was seen to be very loosely meshed. Occasionally vessels were found which extended along the remnants of sarcolemma tubes, as if the latter would provide some degree of surface guidance. CHALKLEY *et al.* (1946) have maintained that clotting of the exudate is a prerequisite to vascular growth, by furnishing a fibrin network for cell migration. The same conclusion was reached by FULTON *et al.* (1949) for growth *in vitro*. That migration of many cell types proceeds by "contact-guidance" has been shown by PAUL WEISS and associates (WEISS 1959, 1961). In fact, shreds as well as more compact layers of fibrin were often found adjacent to newly formed vessels, and might be interpreted in this way. In a few instances, however, new vessels of the cremaster seemed to be embedded in, or projecting into, relatively dense portions of the blood clot. A halo or liquefied zone, as observed by CLARK and CLARK (1939) in the ear chamber and interpreted as dissolved fibrin, was not observed.

2. Characteristics of the endothelial cells. In electron micrographs taken at relatively low powers (1000—3000×) the endothelial layer of regenerating vessels is not strikingly different from that of mature vessels of the same caliber. However, it is generally thinner, sometimes to the point of reaching a few hundred Ångström, and it is also unusual by the alternation of thick and thin portions. At higher magnifications, the difference from normal endothelium becomes more obvious, particularly toward the tip of the sprouts. The cytoplasmic inclusions indicate that the cells are at the same time more 'active' and less differentiated than normal: the granular endoplasmic reticulum is, as already noticed by CLIFF (cited by FLOREY 1962), richly developed; mitochondria are large and numerous and free ribosomes sometimes extremely abundant[1]. The latter have been suggested by PALADE (1958) as an index of relatively undifferentiated cells; in agreement with this interpretation, the 'pinocytotic' vesicles — a characteristic

[1] From an electron microscopic study of the vascularizing rabbit cornea, MATSUHASHI (1961) concluded that the endoplasmic reticulum and the mitochondria of young endothelium are *poorly* developed. The same author revives the contention that vascularization of the cornea does not necessarily proceed from pre-existing vasculature, and describes a process by which clefts within the corneal lamellae become lined with cells. Stromal cells as well as fibroblasts are said to be incorporated into this lining. However, the description of both the sampling methods and of the micrographs suggests the possibility that tissue spaces, rather than newly formed vessels, may have been included in the findings. A similar process was envisioned by PAU (1956).

of mature endothelium (PALADE 1953 and 1961, MOORE and RUSKA 1957, FAWCETT and SELBY 1958, FAWCETT 1959, BENNETT *et al.* 1959) — are scanty, irregular in size and shape, and sometimes absent over relatively large areas of the endothelial cells.

One cytological characteristic, which certainly distinguishes the endothelium of regenerating vessels, is the great plasticity or 'activity' of the plasma membrane. Endothelial processes extending into the blood stream have been described in normal vessels (KISCH 1957); however, in regenerating vessels we have observed cytoplasmic protrusions also on the *outer* surface of the endothelium, sometimes so extensive as to suggest a 'bubbling' activity. These extrusions, especially prominent in corneal specimens, were usually devoid of cytoplasmic organelles, and closely resembled the pseudopodia described for a variety of migratory cells (ALLEN 1961, ALLEN and COWDEN 1962). In fact, the electron micrographs sometimes suggested that not only the plasma membrane of the endothelium was active, but that the whole endothelial cell might be in motion. Although this conclusion may not seem warranted by observations of fixed tissue sections, this is by no means a new concept (MAXIMOW 1902, FLOREY 1962, VAN DEN BRENK 1955) and is well supported by observations *in vivo*. SANDISON (1931) remarks to the fact that the "nucleus is never stationary", and in a series of publications by CLARK and CLARK and their collaborators (CLARK 1918, CLARK *et al.* 1931, CLARK and CLARK 1935, 1939) a constant remodelling of the new vascular bed is described. The motion of the nuclei, together with the inability to obtain intercellular lines with silver nitrate, prompted these authors to consider the growing tip of a sprout a syncytium (see also VAN DEN BRENK 1955). The nuclei were described as migrating from the parent vessels to the sprout and "up and down the wall", as sliding by one another, as sending out and withdrawing processes, and even as traversing the lumen (1939). An ameboid movement of the "endoplasm" was already suggested by CLARK and CLARK (1935). The migratory capacity of the endothelium has also been shown by POOLE *et al.* (1958) in experiments on regeneration of the larger vessels (endothelial cells were found spreading out over the edge of the denuded area by 24 hours) [see also FLOREY *et al.* (1961)] and is especially emphasized in a recent publication by MACKENZIE and LOEWENTHAL (1960). These authors estimated that the endothelium migrated at a rate of 0.10 to 0.15 mm per day within vascular prostheses, and stated that this migration preceeded the growth of fibroblasts. A migration of endothelium in tissue culture, and of entire vessels as well, was shown by LEWIS in 1931, by FULTON *et al.* (1949) and by WHITE (1954).

Among the electron microscopic findings which appeared to agree with these observations were frequent images of nuclei distorted into peculiar, ameboid shapes. In one instance the nucleus, tightly applied against the cell membrane, was bulging towards the lumen in such a manner as to appear almost 'free'; with the resolution of the light microscope, an image of this kind could quite conceivably suggest a migration of the nucleus alone across the vascular lumen. The irregular shape of endothelial cells, also noted in electron microscopic investigations by CLIFF (cited by FLOREY 1962) may be taken as another indication that these cells are not in a passive resting state. It is our impression that the

nuclear displacements observed *in vivo* should be interpreted as an indication that whole cells move, rather than nuclei alone.

Electron microscopic images of mitoses in endothelial cells, in more proximal regions, were encountered in several instances. Two *resting* nuclei within one cell were never observed, hence we have no evidence in support of the syncytial nature of the sprouts (see also W. J. CLIFF, cited by SANDERS 1961).

3. Permeability of the vascular wall. The abnormal permeability of newly formed vessels can be demonstrated at the level of the light microscope, with the use of dyes (ABELL 1946, DIHLMANN *et al.* 1961) or of colloidal particles, as shown in Figs. 2, 3 and 5 to 9.

A high permeability of new vessels may be inferred from TEICHMANN'S experiments (1942) which demonstrated a rapid escape of fluorescein from capillaries in the border of the tadpole tailfin; it may also have been the cause of thorotrast 'shadows' described by BELLMAN and STROEMBECK (1960)[1]. ABELL (1939, 1940), with the use of moat chambers of his design, demonstrated that the escape of proteins was higher in ear chambers with actively growing vessels than in those fully vascularized. CLARK and CLARK (1937) describing the growth of lymphatic capillaries into ear chambers state these vessels are often "separated merely by a clear space" from adjacent thin-walled blood vessels. They mention widening of lymphatics concomitant with increased local blood flow (induced by heating of the ear) and observed an almost immediate coloring of the lymph after intravenous injection of methylene blue. Zones of free fluid with oscillating cells around newly formed vessels are described as a common appearance in the ear chamber (CLARK and CLARK 1935, FLOREY 1962) and are said to be preceeded by an increased blood flow. Finally, COGAN (1949) reported an enhanced escape of dye from sprouts growing into the rabbit cornea[2].

The morphologic substrate of this abnormal permeability is demonstrated by the electron micrographs: abnormalities exist at the level of both filtration barriers, the endothelium and the basement membrane. Whether the endothelial cells themselves may be "more permeable" — actively or passively — cannot be argued from the present evidence; the vesicles were fewer than normal, and images of phagocytosis of colloidal particles by the endothelium were practically absent, hence the evidence for an active transport is (if anything) less prominent than in the normal. On the other hand, it was frequently observed that the junctions between endothelial cells were 'loose' (see also MATSUHASHI 1961), a condition which is perhaps not surprising in view of the evidence discussed above, which indicates that the endothelial cells of these growing vessels are in constant motion.

The cellular junctions of mature endothelium in blood vessels from a number of tissues (including muscle and connective tissue) have been shown to be rein-

[1] These authors produced cold-injury in the rabbit ear. The longer periods of freezing caused lesions in which "revascularization" proceeded inward from the marginal zone. In high-resolution radiographs, shadows of thorotrast were found starting with the third day and "disappeared" after one to two weeks. The authors state that "it was often not possible to determine whether the increased vascular shadow was due to an increased size of existing vessels, diffusion of contrast material, or appearance of new vessels". Their illustrations also point out several extravasations.

[2] In the present experiments, a few minute leaks were seen in the limbal arches of several control animals. Rats from the Sprague-Dawley strain are often infected with a virus before they reach the laboratory (NEWBERNE *et al.* 1961). The disease becomes acute in older animals and it may, at that time, include keratitis. Isolated leaks present in whole mounts and found in one field in the electron microscope, were interpreted as mild grades of inflammation brought about by this infection.

forced by specialized attachment structures (BENNETT *et al.* 1959, ROLLHAEUSER 1959). In certain species these may have the form of desmosomes, as described by FAWCETT (1961) for the rete mirabile of the fish swim bladder, or represent attachments "like miniature terminal bars" near the lumen (ibid.). In a recent study, MUIR and PETERS (1962) describe the fine structure of terminal bars in mammalian endothelium as a quintuple-layered structure near the lumen, presumably analogous to the 'tight intercellular junctions' shown by FARQUHAR and PALADE (1961) and, as the latter authors point out, structurally similar to the "external compound membrane" of ROBERTSON (1959). The magnifications employed in the present experiments did not have the resolution to state definitely whether this tighter bond may be present also in recently formed vessels, but a survey of our material did suggest that the adhesions between neighboring endothelial cells were often firmer towards the vascular lumen. In certain respects, however, they often resembled the areas of higher density of adjacent cell membranes shown in premigratory aggregates of slime mould cells (MERCER and SHAFFER 1960), separated by a "narrow gap" of approximately 15—20 mμ.

In contrast to mature endothelium, there was also a great variety of ways in which endothelial cells formed junctions, suggesting a provisional nature of the endothelial lining. The extensive shingling, and long overlaps (up to 20 μ) of adjacent endothelial cells, may be interpreted as another index of endothelial shifting. In addition to 'loose junctions', true *gaps* between endothelial cells were also observed. Faults of this nature have not been described in mature blood vessels of either muscle or connective tissue (there is, however, indirect evidence that small gaps may exist: GROTTE 1956, MAJNO and PALADE 1961). A finding similar to that here described was reported by ALBERTINI (1960), who studied the fine structure of vessels in granulation tissue of various sources, and reported "intercellular clefts". That these endothelial defects actually allow the escape of plasma is clearly demonstrated by the accumulation of colloidal particles (Figs. 16c, 17—19).

This raises the question of the presence, and function, of the *basement membrane*. In the muscle, a thin amorphous layer corresponding to the basement membrane was usually found, but the existence of endothelial processes 'punching' through it suggested that it did not constitute a substantial barrier. As mentioned earlier, this structure was often difficult to recognize at all in injured corneas. Figs. 16a, c, d demonstrate the thin layer present around some of the vessels. Figs. 19 and 20 exemplify areas where it seemed to be wholly absent (discontinuities of the basement membrane of vessels in granulation tissue, at the light microscopical level, have been reported by HUECK as early as 1935). In general, it appeared to be relatively incomplete in recently formed vessels, and better defined around vessels somewhat older, as also noted by MATSUHASHI (1961). No information could be gathered on the formation of this membrane, other than this fact, that it gradually thickens as the vessel matures.

This morphologic observation is supported by functional evidence, which indicates that the basement membrane of younger vessels is not a very effective filter. When the basement membrane of mature vessels is bared to the plasma, through an endothelial gap, created e.g. by histamine, it behaves as a filter

of surprising strength and efficiency. It is able to withhold a layer of colloidal particles two to three micra in thickness, and the resulting light microscopic image is that of a discrete, well-defined intramural deposit (MAJNO *et al.* 1961). In growing vessels, this appearance of well-defined 'vascular labelling' is the exception. The colloidal particles usually tend to spill into the surrounding tissues. This is observed by light as well as by electron microscopy.

4. Fragility of the newly-formed vessels. Newly-formed vessels bleed very easily; hence it is generally assumed that they are more fragile. Although this property has never been, to our knowledge, specifically tested experimentally, there is evidence which supports this conclusion.

The tendency of granulation tissue to bleed is common surgical experience. However, the clearest indications are provided by the ear chamber: SANDISON (1926, 1928) and CLARK and CLARK (1935, 1939) have shown that erythrodiapedesis occurred when blood flow was increased; and when slight pressure was exerted on the cover glass of the chamber, red blood cells were forced out of the new vessels, but not out of older ones more proximally. A zone of hemorrhage, described as the usual accompaniment of growth in the ear chamber, was absent in animals treated with cortisone (ASHTON and COOK 1952). The authors describe a "somewhat empty" network due to the constriction of larger vessels, and conclude that the absence of the hemorrhagic zone was due to a decreased flow of blood.

In the cornea, pinhead-sized red spots (extravasates) were already described by ARNOLD (1872), and COGAN (1949) describes the bursting of "protuberances, or saccular aneurysms" of veins and capillaries on the side facing the injury (hence at the site of growth), as a regular occurrence in the vascularization of the rabbit cornea. In one instance, he observed the actual eruption of such an outpouching. Small columns of blood were forced into the adjacent stroma and came to lie, apparently along lamellae of the corneal stroma, as radiating spicules some distance from the vessel. A dye, injected intravenously at a later time, did not reach this area of extravasated erythrocytes. The author interprets these events as a manifestation of the loosening of the corneal lamellae, i.e. "a decrease in the external pressure through lessened support on the wall of the blood vessel, rather than an increase in the intravascular pressure".

These findings are cited in some detail here, because they may offer one possible explanation for several electron microscopic observations in the cornea, such as vascular sprouts literally open at the very tip (Fig. 22) with many erythrocytes filling lamellar clefts in the vicinity (see Fig. 22d). Abnormalities in the endothelium of these open tips (dilated cisternae, rounded mitochondria) and the occasional extracellular scattering of cell organelles, suggest that a burst similar to that observed by COGAN, may have taken place.

The electron-microscopic image of an 'open tip' does not necessarily mean that a previously 'closed' sprout has burst. It could also mean that some vascular sprouts advance as open-ended tubes. Also, the pathologic endothelial changes could depend on local conditions independent, of a burst e.g. anoxia. However, the general characteristics of the endothelium in the newly-formed vessels certainly suggest that they are very fragile, i.e. very susceptible to burst as a result of sudden changes in intra- or extra-vascular pressure. The same structural features which account for abnormal permeability — loose junctions and gaps in the endothelial lining, thinness or absence of the basement membrane — should also determine an abnormal fragility.

5. Blood flow in the newly formed vessels. The circulation of blood in regenerating capillaries can be expected to be abnormal for a number of reasons:

most important of these, the wall is excessively permeable; and furthermore, the sprouts represent dead-end canals.

The flow through newly formed vessels, observed *in vivo*, is usually described as very irregular. An ebbing and flowing into blind ends at the advancing edge is mentioned by ARNOLD (1871), and further described in most of the publications originating from CLARK and CLARK and their collaborators. Although the pressure in newly formed vessels has never been measured, several considerations suggest that this pressure may vary within a wide range. The influx of arteriolar blood into an extensive anastomotic bed of relatively wide vessels with a conspicuous venous drainage (see for example Fig. 7) would suggest relatively low pressures. On the other hand, the observation of pulsations in sprouts at one point and a collapse of blind ends and vessels at another, make a generalization hazardous. Capillary pressure in the *normal* vascular bed is "an extremely variable quantity, changing in the same capillary from moment to moment and differing widely in adjacent capillaries connected to the same arteriole" (LANDIS 1934); it may well be an even more fleeting quantity in areas of regeneration.

If we consider the case of a sprout, which is closed at one end, it is obvious that no real *blood flow* can take place. Because the wall is leaky, only a flow of plasma could conceivably occur. The plasma may penetrate into the mouth of the sprout and then seep out through the loose intercellular junctions (a possible nutritional as well as morphogenetic role of this fluid has been suggested by HADFIELD 1951). The circulatory condition in these sprouts is quite comparable, in principle, to the transient condition of a capillary, submitted to localized compression by means of a blunt rod to stop the blood flow, thus creating a blind end (LANDIS 1927): on the arterial side, fluid is filtered off and red blood cells become concentrated. Although complete stasis did not result in normal capillaries thus treated, the high permeability of newly formed vessels would favor a more substantial concentration of blood. Stasis was indeed present in many sprouts (Figs. 5, 11, 22). A further indirect sign of poor flow in young loops and sprouts is the irregular labelling, as shown in whole mounts after the injection of colloidal particles. Abundant deposits may form in one vessel, and none in the neighboring one though it is presumably of similar nature (Fig. 3).

In agreement with the light microscopic observations by STEHBENS and FLOREY (1960), we found no evidence for the supposed 'stickiness' of newly formed vessels (ZWEIFACH 1954). As these authors pointed out, if a "soft cement" coated the luminal surface, colloidal particles could be expected to stick to it. It may be mentioned in passing that intravascular fibrin formation was observed in a few instances, within the lumen of newly formed vessels.

Another circulatory phenomenon may be expected in sprouts. Both the angle of branching from another vessel, and the alternating periods of slow and rapid flow, should favor *plasma skimming* as observed by LANDIS (1926). That this does occur is suggested by the frequent finding of platelets in large numbers within the lumen (Fig. 11). It is also quite likely that ARNOLD, when describing a "crumbling of protoplasmic particles" within the syncytium of the sprout (an observation which prompted him to suggest the forming of a protoplasmic tube from a syncytium) was really observing an effect of plasma skimming.

6. Concluding remarks. If we combine the present findings with those of the literature, it appears that the growth of new vessels depends on two morphologically detectable processes. Proliferation of endothelial cells, in more proximal areas, and cell migration which is most pronounced in the vascular border. At the present time, evidence tends to support the concept of MAXIMOW that the endothelium advances as a sheet of cells.

The process of sprouting begins, in all likelihood, at the cellular level: cytoplasmic extrusions arise from the endothelial cells (possibly also from periendothelial cells) and reach into the perivascular space. In doing so they may either pierce the basement membrane, or an initial softening of this layer may allow the escape of the cell from the confines of the vascular wall. As endothelial cytoplasm flows out in this fashion, the cell gradually displaces itself, i.e. it performs amoeboid movement. Adjacent endothelial cells are either pulled along, or follow the lead actively, so that the endothelium migrates in the direction of the stimulus as a loose and plastic sheet of cells. That endothelial cells retain contiguity may be inferred from observations of growing vessels *in vivo*; it is especially evident in tissue culture (see e.g. LEWIS 1931). The way in which this sheet is pouching out will determine the shape of newly-formed vascular segments. Thus these early sprouts may be pointed or club-shaped, or even branched, depending on the irregularity of the substrate along which the endothelial cells advance. They may expand into a tissue cavity, forming a clubbed sprout, or they may wedge into clefts and crevices, thus giving the appearance of 'solid sprouts'.

During their migration, adjacent cells may become temporarily separated, allowing plasma and formed elements to escape from the blood stream. In very young tips where the basement membrane is incomplete or absent, the vascular contents spills directly into the extravascular space. In more differentiated segments which are already invested with a basement membrane, particles and cells are filtered out at this barrier; a situation comparable to that found in mildly injured adult vessels. The finding that leakage was confined to a narrow zone at the advancing edge suggests that new vascular elements approach normal permeability characteristics in a very short time.

Summary

The present study deals with the fine structure of growing capillaries and their permeability to colloidal particles. The blood vessels growing into a wound in striated muscle, and those vascularizing the injured cornea, were investigated in the rat. Intravenously injected colloidal particles (carbon, mercuric sulfide and gold) were used as markers of vascular permeability.

Observations of whole mount preparations of muscle wounds, with the *light microscope*, revealed a growth pattern of sprouts. In the cornea, loops occurred as well. It is suggested that sprouts may be the morphologic expression of rapid growth, as contrasted with a slower progression accomplished by elongation of loops. Newly-formed vessels were found to be abnormally permeable; the site of abnormal permeability is located at or near the advancing vascular tip. Microscopic images of vessels that had leaked suggested that the colloidal particles were not always retained within the vascular wall, but had found

access to perivascular spaces. With the *electron microscope*, the endothelial cells of growing blood vessels appeared to be very 'active', as suggested by the numerous mitochondria and free ribosomes, and a well developed endoplasmic reticulum. Cellular junctions were more tenuous than those described for mature endothelium in muscle and connective tissue. They varied from long overlaps of adjacent cells to areas in which the cells appeared loosely apposed. Distinct gaps were also found. Colloidal particles, injected into the blood stream, escaped through these loose junctions or gaps. Often they spilled into the perivascular tissue, especially in areas where a basement membrane could not be identified. The *fragility* of newly-formed capillaries may be explained by the same morphologic features which underlie the abnormal permeability. The finding of 'open' vascular tips, surrounded by extravasated red blood cells, was also interpreted as a possible sign of fragility.

Another finding related to the absence or thinness of the basement membrane was the presence of cytoplasmic processes arising from the *outer* aspect of the endothelial cell, and projecting into the surrounding tissue. These cytoplasmic tongues had the morphologic aspect of pseudopodia, and were interpreted as indicating ameboid movement of endothelial cells, a concept which is supported by observations *in vivo* in the literature.

The supposed syncytial nature of the vascular tips could not be confirmed.

Untersuchungen über Entzündung.
III. Struktur und Permeabilität der wuchernden Capillaren

Zusammenfassung

Die vorliegende Arbeit befaßt sich mit der Feinstruktur wachsender Blutcapillaren und deren Permeabilität für kolloidale Partikel. Untersucht wurden Blutgefäße der Ratte in einer Schnittwunde des quergestreiften Muskels, sowie Gefäße, die die geätzte Hornhaut vascularisieren. Die Stellen erhöhter vasculärer Durchlässigkeit wurden durch kolloidale Partikel (Kohle, Quecksilbersulfid und Gold), die kurz vor der Tötung intravenös eingespritzt wurden, markiert. Diese Partikel treten durch den Endothelverband leicht verletzter Blutgefäße und häuften sich zwischen Endothel und Basalmembran an.

Für *lichtmikroskopische* Untersuchungen wurden die Gewebe als Flachpräparate eingeschlossen. Es wurden zwei Formen der Gefäßneubildung beobachtet: In Muskelwunden ausschließlich ein System verästelter Sprossen, in der Hornhaut öfters außerdem eine Anordnung von Bogen und Schleifen. Es wird angenommen, daß Sprossen der morphologische Ausdruck eines raschen Wachstumsvorganges sind, während Schleifen durch ein allmähliches Verlängern der Blutgefäße zustande kommen.

Neugebildete Blutgefäße erwiesen sich als abnormal durchlässig. Der Ort der abnormalen Permeabilität liegt in oder nahe der sich verschiebenden Gefäßspitze.

Mit dem *Elektronenmikroskop* gesehen, geben die Endothelzellen der neugebildeten Blutgefäße den Eindruck sehr „aktiver" Zellen: zahlreiche Mitochondrien und freie Ribosomen, sowie ein gut entwickeltes endoplasmatisches Reticulum waren vorhanden. Die Verbindungen der Zellen waren, im Gegensatz

zu normalem Endothel in Muskel und Bindegewebe, sehr ungleichmäßig. Die Form der Zellverbindung variierte von langen dachziegelartigen Überdeckungen bis zu Stellen, wo sich die benachbarten Endothelzellen lediglich leicht oder nicht mehr berührten. Zwischen diesen nur losen Zellverbindungen oder Lücken traten die in den Blutstrom eingespritzten kolloidalen Partikel aus dem Gefäß aus. Die Fragilität der neugebildeten Capillaren kann durch dieselben morphologischen Eigenheiten, die der erhöhten Durchlässigkeit zugrunde liegen, erklärt werden.

Ein weiterer Befund war das Vorhandensein von cytoplasmatischen Fortsätzen, die von der Außenseite der Endothelzelle in das benachbarte Gewebe hineinragen und welche die Merkmale von Pseudopodien besitzen. Sie wurden als Zeichen von amöbenartiger Fortbewegung der Endothelzellen gedeutet.

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