STUDIES ON THE PATHOGENESIS OF ACUTE INFLAMMATION

I. CHANGES OF ENDOTHELIAL PERMEABILITY IN RABBIT EAR-CHAMBERS INJURED BY HEAT

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THE increased permeability induced by injury in vascular endothelium has been recognized as a key event in the evolution of acute inflammation. Escape of intravascular solute and of formed elements *via* altered endothelium has been held essential for a successful defence against noxious biological, chemical and physical agents. Despite the type of injury, increases of endothelial permeability were remarkably uniform qualitatively. The magnitude of permeability change was, however, apparently directly proportional to the intensity of trauma. As a result of many experimental studies it has been suggested that derangement of endothelial integrity during acute inflammation was causally related to material released from injured tissue ; substances considered as potential mediators of permeability alteration have been histamine, (Lewis and Grant, 1924), leukotaxine, (Menkin, 1955), certain polypeptides, (Duthie and Chain, 1939), 5-hydroxytryptamine, (Spector and Willoughby, 1957) and proteolytic enzymes found in plasma, (Miles and Wilhelm, 1955). Since study of such agents depended eventually upon an *in vivo* characterization by bioassay, variable behaviour in different species resulted in confusion as to the ultimate significance of each material in the genesis of increased vascular permeability. Additionally, different methods used for extraction and preparation of these substances caused uncertainty as to their actual existence in inflamed tissue. As a result, the exact biochemical mechanism responsible for endothelial alteration remains unknown.

Along somewhat different lines, study of experimental bacterial skin infections revealed vascular permeability to be increased maximally 1–6 hr. after lesions were established ; thereafter permeability returned toward pre-injury levels (Burke and Miles, 1958). Significant enhancement of infection in similar lesions was accomplished by one of several manoeuvres ; by inducing peripheral vascular collapse, by administering either adrenalin locally or Liquoid systemically 2–4 hr. after infection was initiated (Miles, Miles and Burke, 1957). Modification of peripheral blood flow in a like manner either before or after this critical time did not materially alter the evolution of infection. Since this decisive period coincided with the time when the exudative phenomena were most prominent, it was interpreted as evidence of the need for patent vascular channels to supply opsonins and phagocytic cells from circulating blood required for early control of infection. It was not apparent, however, why such infections were not modifiable either before or after this narrow time zone.

Previous microcirculatory studies have shown injured blood vessels to be highly permeable to plasma containing vital dyes. Although capillaries have been regarded as the major site of fluid loss during inflammation, venules were also believed to be a significant source for the leakage of plasma. There has been, however, no systematic microcirculatory study of acutely injured tissue designed to determiue quantitatively either the general pattern of plasma exudation from all segments of the peripheral circulation or the exact anatomical site of major losses of fluid.

The present studies were concerned with permeability changes of blood vessels in rabbit ear-chambers injured by heat. This inflammatory reaction has been described in detail previously (Allison, Smith and Wood, 1955) and proved an ideal model for study with Evans Blue Dye (henceforth referred to as T–1824) as the indicator for increased permeability of endothelium. It was found that major leakage of dye occurred in lesions 1–3 hr. of age and was less prominent immediately after injury and in older lesions. Stasis of blood flow with occlusion of highly permeable patent vessels was the event most obviously responsible for halting leakage of dye stained plasma. Greatest staining of tissue was observed at definite anatomical portions of the vasculature ; principally about thoroughfare channels or around capillary-venous radicals near arteriovenous fistulae.

METHODS

The type of ear-chamber, animals, basic experimental conditions and technique for producing tissue injury by heat have been described previously (Allison, Smith, and Wood, 1955). The inflammatory reaction produced by heat injury in the rabbit ear-chamber has been studied microscopically many times and served as the control for experiments described herein.

Five ml. of a 4·0 per cent aqueous solution of T-1824 (W761–1 lot no. 002093, generously supplied by Warner-Chilcott Laboratories Division, New York) was diluted in 15·0 ml. pyrogen free isotonic saline for intravenous administration. More dilute solutions of dye suitable for clinical use were not satisfactory because tissue staining was not distinct at high magnifications. This volume of dye solution was administered intravenously over a period of 3 min. *via* a suitable vein in the ear not being used for microscopic studies. The procedure was well tolerated initially but after 10–14 days, rabbits often developed paralysis of the hind legs and were killed.

Concentration of dye in plasma was determined from blood obtained at appropriate intervals from suitable ear veins. Usually 2·0 ml. of blood was drawn into syringes containing 0·1 molar sodium oxalate to give a final ratio of blood to anticoagulant of 9 : 1. After thorough mixing by repeated inversion, samples were centrifuged for 10 min. at 2000 rpm and plasma removed by gentle suction. No evidence of haemolysis was detected. Optical density readings of plasma containing dye were made with a Beckman model B spectrophotometer at 615 mμ and compared to control plasma diluted in similar fashion (Markus and Feigen, 1955). Plasma samples obtained during the first 24 hr. were diluted 1 : 500 in distilled water to obtain satisfactory spectrophotometer readings. After 24 hr., plasma samples were diluted 1 : 100 since dye concentration was much lower. Colour photographic records using 35 mm. film were made with equipment identical to that described previously (Allison, Smith and Wood, 1955).

RESULTS

Clearance of T-1824 from the Blood Stream

It is known that plasma concentrations of T-1824 are influenced by several factors among which are : (a) mixing during circulation ; (b) binding by plasma proteins, principally albumin ; (c) leakage or filtration into tissue with binding

EXPLANATION OF PLATES

Figs. 2, 3, 4, 5.—This series exemplifies leakage of T-1824 in ear-chambers of rabbits injured either immediately before or immediately after administration of dye. The tissue was burned 11 min. before dye was given in this experiment.

FIG. 2.—Approximately 15 min. after T-1824 was injected. Note major leakage of dye was from venous channels closely associated with arterial connections. The shadow (S) over lower half was cast by external portion of platinum pin.

FIG. 3.—Same ear-chamber as Fig. 2 but 1 hr. after dye administration. A broad ring of dye extended peripherally but not into avascular central zone (C).

FIG. 4.—Same as Figs. 2 and 3 but 6 hr. following injection of dye. Note clearing of T-1824 from peripheral portion of chamber but persistence of colour about platinum pin (C). Stasis of arteries (A) had begun and early perivascular haemorrhage was present.

FIG. 5.—Same as Figs. 2, 3 and 4 but 24 hr. after dye had been given. Tissues other than central portion (C) appear free of dye. Arteries noted previously (A) were completely static and surrounded by haemorrhage.

Figs. 6, 7, 8 and 9 depict intense staining observed when dye was given 1–3 hr. after thermal injury (compare with previous series). The ear-chamber was injured 2 hr. before was injected T-1824 in this series.

FIG. 6.—About 15 min. after dye was given. The intense ring of blue stain is obvious and has extended into central zone (C).

FIG. 7.—Same preparation as Fig. 6 but 1 hr. after giving dye. Blue colouration was greatly intensified and had begun to extend peripherally.

FIG. 8.—Continuation of Figs. 6 and 7 but 6 hr. after dye was given. Note almost complete clearing of tissues containing vessels.

FIG. 9.—Same series as Figs. 6, 7 and 8 but 24 hr. following T-1824 administration. The only visible dye was in the central zone (C).

Fig. 10, 11, 12 and 13 are representative of endothelial permeability found 5 or 6 hr. after heat injury. This particular preparation was burned 6 hr. prior to T-1824 administration.

FIG. 10.—Although slightly larger, little leakage of dye was noted in this lesion 15 min. after T-1824 had been given. Supernatant cells (SC) in upper right and lower half washed in from periphery after burn.

FIG. 11.—Same as Fig. 10 but 1 hr. after injection of dye. Note only traces of colour in contrast to that seen in Fig. 7.

FIG. 12.—Same as Fig. 10 and 11 but 6 hr. after loading with T-1824. Continuous leakage of dye resulted in a blue ring in the central avascular area (C) but little elsewhere. Dye was concentrated by supernatant phagocytic cells (SC).

FIG. 13.—Same as Fig. 10, 11 and 12 but 24 hr. after injection of dye. Peripheral tissues appeared clear of dye except where large numbers of supernatant phagocytes (SC) were present. Persistence of dye in the central zone (C) can be seen.

Fig. 14, 15,16 and 17 are representative of dye leakage in ear-chambers injured 24 hr. before T-1824 was given.

FIG. 14.—No staining was visible 15 min. after dye had been given. Haemorrhage arising from static vessels about the central platinum pin may be seen.

FIG. 15.—Same as Fig. 14 but 1 hr. after intravenous loading with dye. Again, no leakage into tissue was detected.

FIG. 16.—Same as Fig. 14 and 15 but 6 hr. after dye was given. Still no evidence of major escape of dye into tissue. Fresh haemorrhages (H) noted in upper half developed after animal struggled.

FIG. 17.—Same as Fig. 14, 15 and 16 but 24 hr. following injection of T-1824. For the first time, slight staining with dye was noted at lower left side of platinum pin.

FIG. 18.—T-1824 was given 3 hr. after heat injury. Perivascular staining occurred only about vessels (PV) and not about static channels (SV).

FIG. 19.—Same as Fig. 18 but higher magnification. Note lack of staining about static vessels (SV) as contrasted to intense colour adjacent to patent venules (PV). Blue colour of plasma in partially filled vessels (V) was due to plasma skimming.

FIG. 20.—Leakage of dye from artery (A) 2 hr. after injury and 10 min. after dye was given. This artery near central area (C) was subsequently occluded by static blood.

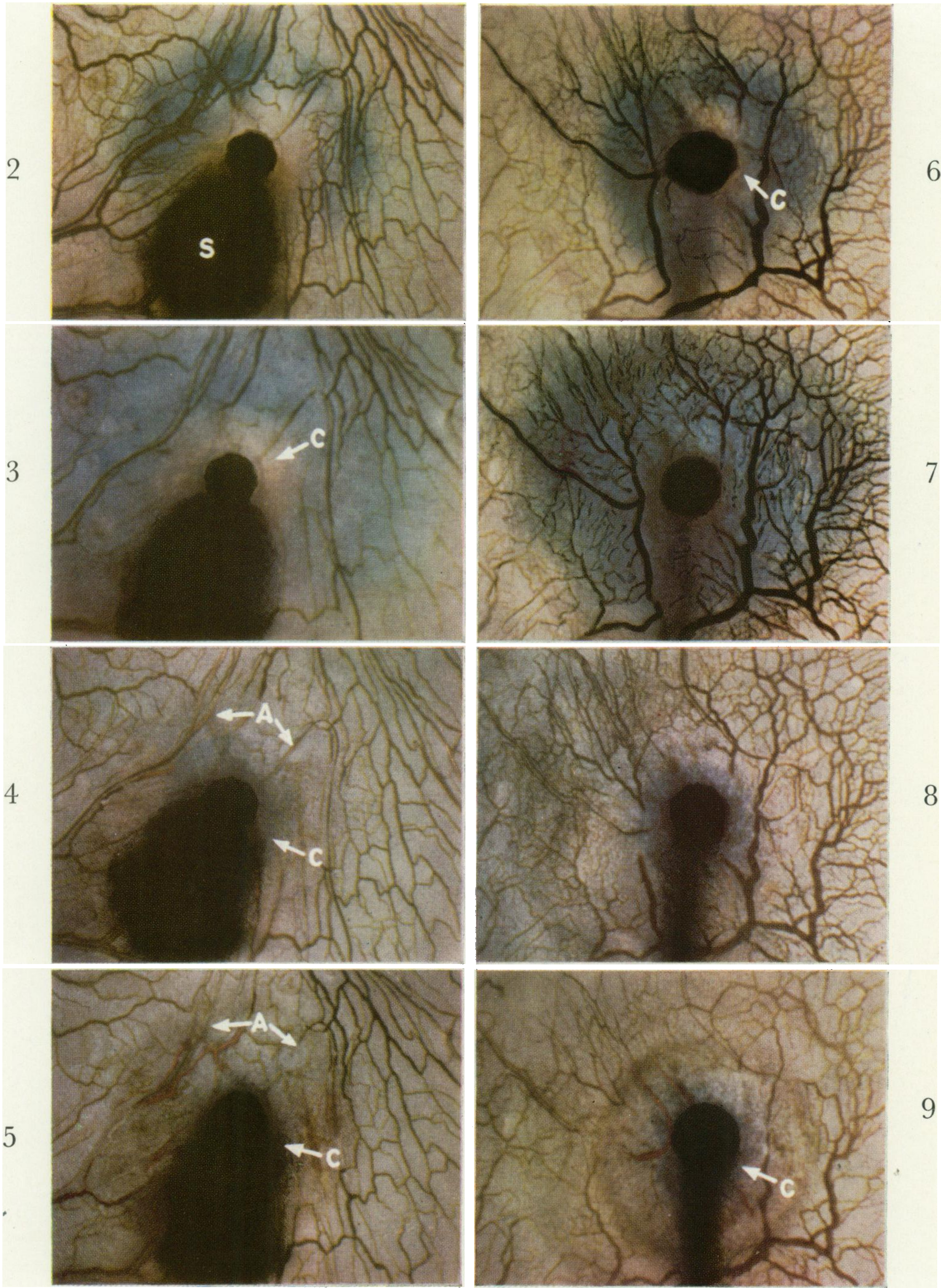
FIG. 21.—Concentration of T-1824 within granules of perivascular cells (PC) 24 hr. after injury and administration of dye. These cells, presumably macrophages, were next to wall of artery (A). Note shunting of flow caused by injury.

FIG. 22 and 23 illustrate that vessels most permeable after injury eventually became static. As in Fig. 22 taken 1 hr. after injury, two tributaries of a draining vein permitted a large quantity of dye to escape within 15 min. after dye had been given. The small static vessel (SV) extending into central zone did not allow extensive loss of dye. When Fig. 23 was taken 3 hr. after injury, one vein (V) on left was static and the other vein (V) on right eventually became occluded. In meantime, much dye migrated into central zone.

FIG. 24.—Two hours after T-1824 had been given 2 distinct rings of blue dye (1 and 2) were visible in an ear-chamber injured 3 hr. earlier. A portion of a third ring had been present on left side but was obscured by rapid spreading of dye.

FIG. 25.—Another example of ring phenomenon ; 1 hr. after T-1824 had been given and 3 hr. after injury. Note width of outer ring (1) as it spread to cover almost all of periphery. The inner ring (2) migrated in toward central avascular zone.

Fig. 22 and 23 were from Type A Kodachrome film ; all others were from Ektachrome film. Prints were made by Eastman Kodak Company as Kodachromes.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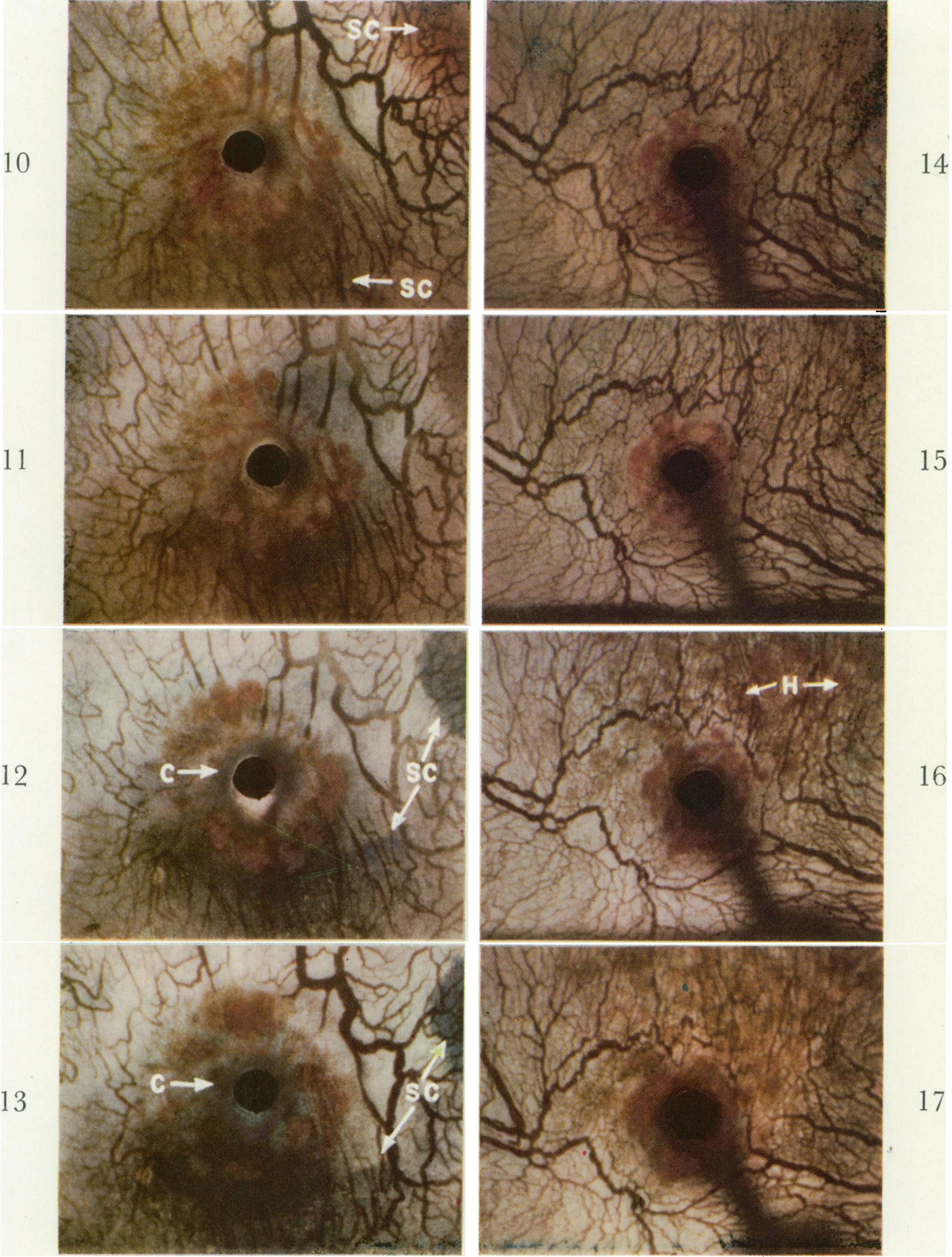

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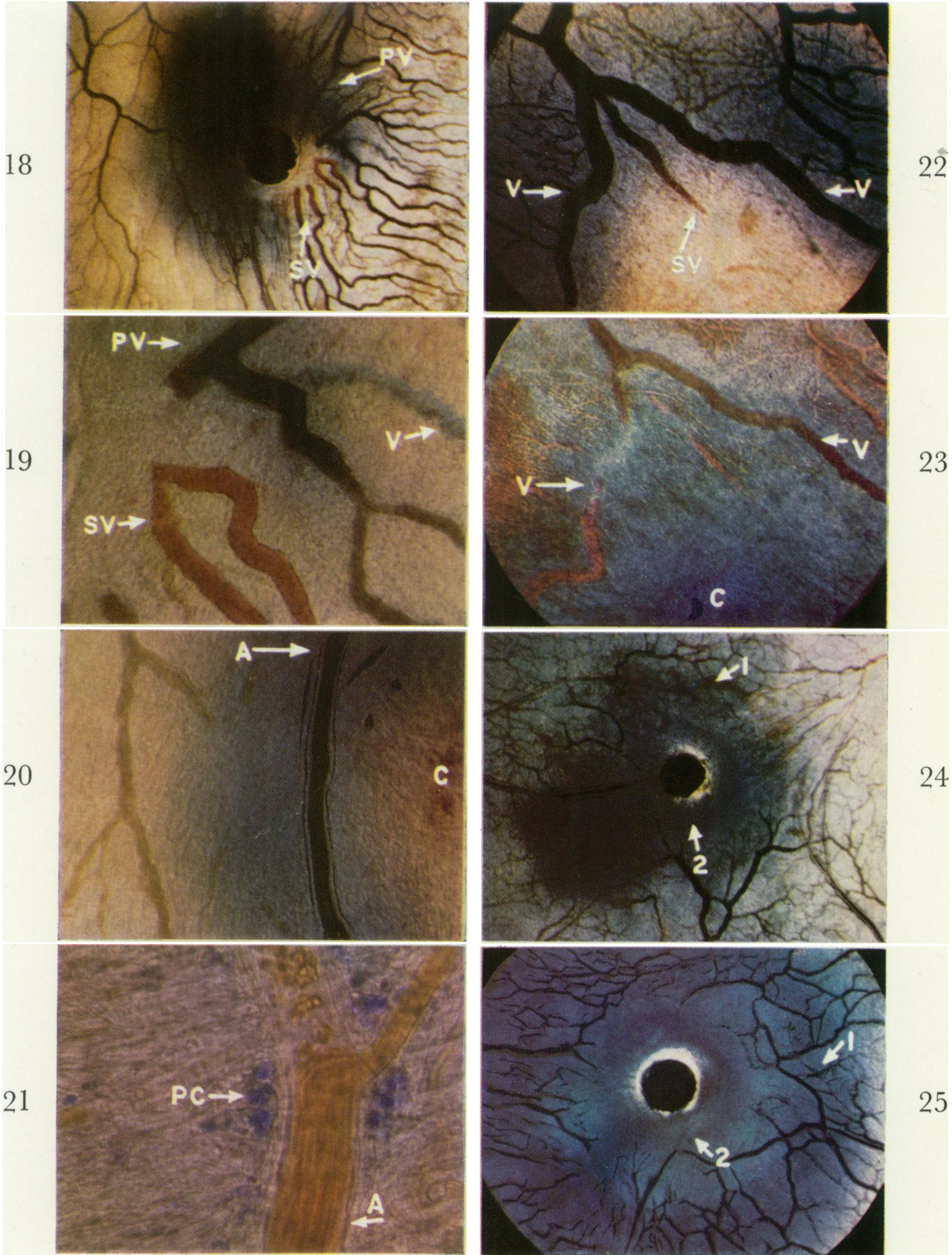
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by cells ; and (d) recirculation *via* tissue lymph (Markus and Feigen, 1955). The half life for T-1824 has been determined for a number of species including rabbits but never at concentrations used in these experiments. Accordingly, it was necessary to determine persistence of dye intravascularly during the times microscopic studies were to be performed since the magnitude of leakage would be directly proportional to the plasma concentration.

Rabbits were bled for control samples and then given 20 ml. of 1 per cent solution of T-1824. Samples of venous blood were obtained 5 min., 1, 2, 3, 4, 5, 6, 24, 48, and 72 hr. after loading with dye. By the time administration of dye was completed, a deep blue colour was visible in the conjunctivae, nose, gums,

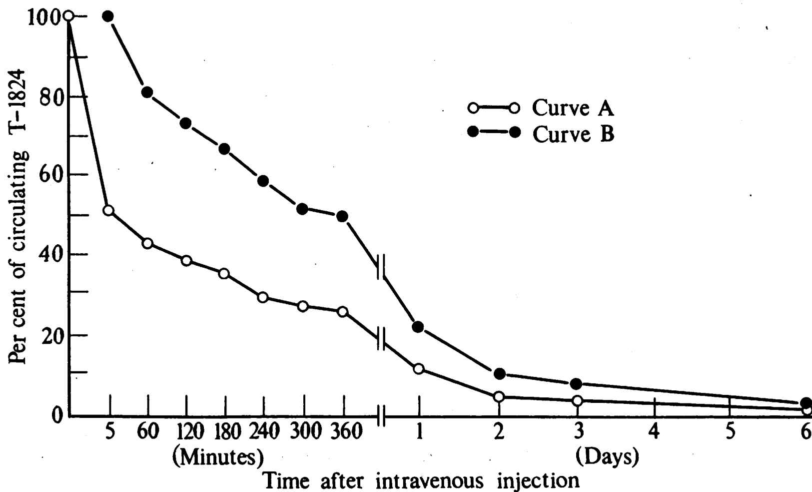

FIG. 1.—Mean disappearance curves of intravenously administered T-1824 in 4 rabbits. The first point on curve A, zero time, represents predicted plasma concentration ; obtained by multiplying total quantity of dye administered (0·2 gm.) × total plasma volume (38 ml./kg. body weight). Other points on curve A were obtained by comparing actual concentration with 100 per cent value. The initial point on curve B, 5 min. after injection, was determined and assigned value of 100 per cent for comparison with all subsequent samples.

and ears. Little change in colour was seen after 10–14 days when most animals were killed ; however, gradual loss of blue colour was observed in animals surviving 3 months or longer.

As may be seen in curve A of Fig. 1, concentration of dye in venous blood 5–11 min. after intravenous loading was only 50 per cent of anticipated levels based on calculations of total plasma volume of rabbits. Thereafter, plasma levels declined gradually over the ensuing week. The fact that plasma values were not as predicted probably was a result of rapid leakage before mixing was complete of unbound T-1824. Binding of this quantity of dye to plasma proteins required several minutes *in vitro* after thorough mixing. Thus it was conceivable that a large amount of free T-1824 did escape into tissue or was phagocytosed during the first minutes of mixing *in vivo*. Binding of dye by plasma proteins was complete *in vivo* 5 min. after loading when the first sample of blood was obtained since precipitation of protein with 5 per cent sodium tungstate completely decolourized the supernatant. When the value of the 5 min. specimen was used as a reference

point, a curve was obtained showing decline of available dye in circulation during times of microscopic study (Curve B, Fig. 1). From curve B, Fig. 1 it may be seen that only 50 per cent of dye was still in the circulation 6 hr. after loading. Preliminary electrophoretic studies indicated that saturation of albumin was not complete at concentrations of T-1824 used and no binding by globulins was detected.

From these results two facts were apparent. First, binding of dye by plasma insured identification of major changes of permeability induced by injury since rapid leakage of intravascular protein occurs during inflammation. Second, rapid clearance of dye from the circulation within a few hours after administration limited valid microscopic quantitation of tissue staining to a relatively short period. To obtain data of permeability changes throughout the first 24 hr. of acute inflammation it was necessary, therefore, to administer dye to rabbits with ear-chamber lesions of various ages. The following account is a description of these studies.

Behavior of T-1824 in Uninjured Rabbit Ear-chambers

No local staining of tissue about arterioles, capillaries and venules was seen in fully mature ear-chambers free of all evidence of inflammation. Vessels containing blood appeared dark bluish brown but vascular tone, rate and volume of blood flow were not altered. Vascular channels devoid of erythrocytes but filled with plasma appeared bright blue. The colour was less intense within 6 hr. and only a faint blue cast was visible in plasma 24 hr. after administration of dye. Vessels containing red blood cells regained their usual brownish red hue the following day.

Within 30 min. a faint diffuse blue tinge was noted in connective tissue. Lymph also had a light blue colour but in no instance did it equal the intense hue seen in plasma. Connective tissue of uninjured chambers never became intensely stained even when followed for 10–14 days. Within 24 hr., however, scattered cells located perivascularly, within lymphatics, and on the surface of ear-chamber tissue were intensely stained. The dye in these cells, presumably macrophages, was concentrated within cytoplasmic granules (Fig. 21). Persistence of T-1824 stained cells was noted for as long as 8 weeks after administration when observations were discontinued.

In no instance was there evidence of altered intravascular haemostasis since platelets, erythrocytes and leucocytes did not become adhesive or appear changed in number. Platelet thrombi were not encountered nor were conglomerations or sludges of red blood cells. Similarly there was no evidence of significant leucopenia or leucocytosis after administration of T-1824. Vascular tone, rate and volume of blood flow were not detectably modified by loading with dye. No effort was made to record blood pressure or temperature changes when T-1824 was given; however, lack of notable systemic response indicated little if any contamination with bacterial pyrogens.

Behavior of T-1824 in Injured Rabbit Ear-chambers

General pattern of tissue staining after thermal damage.—Since leakage of dye was unpredictable in thick ear-chambers composed of several layers of super-

imposed blood vessels, experiments were limited to thin preparations containing only one vascular stratum. Lesions under such circumstances were more consistent as to staining, size, and intensity of inflammatory response. Extravascular dye was most often seen in injured preparations as early as 3 min. after administration of T-1824 whereas in a few ear-chambers little or no staining was seen until 30 min. after injection. No T-1824 was noted about vessels filled with static blood at the outer rim of the lesion (Fig. 18, 19, 22). Staining of tissue developed only about blood vessels still patent at the periphery of injured tissue. It was further noted that tissue staining was most prominent either, about thoroughfare channels and arteriovenous shunts opened when other channels of blood flow were occluded, or along arterioles bordering on injured tissue (Fig. 20). Unless near an arteriovenous communication capillaries did not permit escape of dye as freely as vessels in which hydrostatic pressures were near that of the arterial circulation. In the next 3–6 hr. stasis of circulation extended peripherally to occlude highly permeable patent vessels. Thus, vessels permitting greatest escape of dye early in the reaction could be predicted almost with certainty to become static later (Fig. 22, 23). Sufficient dye to cause microscopic staining locally did not appear about remote vessels in mid-chamber and peripheral tissue.

As perivascular dye reached maximum concentrations, a narrow blue ring formed along the outer edge of injured tissue (Fig. 2, 6, 18). The inner margin of the blue ring slowly enveloped blood vessels within the zone of stasis whereas the outer edge lay over patent vessels (Fig. 7). Colouration of tissue was sharpest approximately 1 hr. after dye had been injected; thereafter the ring became wider, more diffuse and less intense (Fig. 4, 8). The quantity of stain appearing in extravascular tissue, as shall be discussed in subsequent sections, was directly related to the time that had elapsed between injury and administration of dye.

In an occassional preparation during the first hour after loading, T-1824 appeared to concentrate in distinct lines within several zones of the lesion (Fig. 24, 25). The concentric distribution of the lines as a series of rings imparted the appearance of a target to the ear-chamber. The first or outermost ring was peripheral to the static rim of the lesion and appeared to migrate away from the centre before becoming indistinct. The second or middle ring lay over the static zone and tended to migrate centrally to join dye in the third or inner ring. This last formation was immediately adjacent to the platinum pin and was the only part of the phenomenon that persisted past the second or third hour after dye administration. Staining of tissue between the lines of dye was less intense yet stain was obviously present in small quantity. Distribution of T-1824 in this fashion was transient since continuous migration obliterated the pattern.

Within 3 hr. after T-1824 had been administered, the blue ring began to fade as dye diffused both peripherally and through the static zone into the central ischaemic portion of the lesion (Fig. 4, 8). Escape of T-1824 from the centre, zone of greatest damage, was retarded since this area continued to be sharply stained 6 and 24 hr. later. All tissue of the ear chamber except the central zone appeared light blue 24 hr. after administration of dye (Fig. 5, 9, 13, 17). Phagocytic cells accumulated sufficient dye to stand out distinctly by this time (Fig. 12, 13, 21). Apparently the large number of these cells, in this instance predominantly leucocytes, accounted for persistence of dye about the platinum pin long after undamaged connective tissue was cleared of dye. Thereafter, little change in intensity of staining was observed within the next 7–14 days. Clearance of

dye *via* ear-chamber lymphatics was of minor significance in many preparations since these structures were rudimentary and often without anastomosis to the greater lymphatic system of the ear.

*Tissue staining by T-*1824 *given immediately before or immediately after injury.*—Little difference in staining intensity was noted when T-1824 was injected either immediately before or after ear-chamber tissue had been damaged by heat. As may be seen in Fig. 2, 3, 4, and 5 which are representative of ear-chambers studied shortly after injury, definite staining of perivascular tissue was present 15 min. after injection of dye (Fig. 2). One hour after administration (Fig. 3) slight intensification of colour developed and the width of dye-containing tissue was increased. After 6 hr. (Fig. 4) the discolouration was much less distinct at the periphery of the lesion but concentration in the central zone was definite. Little change was noted 24 hr. after injection although by this time tissue phagocytic cells contained granules deeply stained with blue dye (Fig. 5).

*Tissue staining by T-*1824 *injected* 2 *hr. after injury.*—Approximately 1–3 hr. after heat injury endothelial permeability was greatly enhanced. No sharp or abrupt increase occurred at this time since the change was gradual and varied slightly in each animal. For purposes of illustration, ear-chambers 2 hr. after damage were selected for documentation since this time fell within the period of maximum change. From Fig. 6, an ear-chamber injured 2 hr. earlier, it can be seen that a large amount of dye escaped into perivascular tissue 15 min. after T-1824 was given. One hour after injection of dye (Fig. 7) a bright blue ring had formed which was much more intense than when dye was administered immediately after injury (compare with Fig. 3). Within 3–6 hr. after dye was given, 5–8 hr. after injury, staining was diffuse with spread both centrally and peripherally (Fig. 8). The following day much fading had occurred with persistence of dye only in the region of the platinum pin (Fig. 9).

Tissue staining by T-1824 *injected* 6 *hr. after injury.*—Patent blood vessels located nearest to injured tissue began to be occluded by stasis 3 hr. after injury. The process was usually advanced by the sixth hour and accompanied by early perivascular haemorrhage. Thereafter, haemorrhage became much more intense about these obstructed blood vessels. A progressive fall in tissue staining by T-1824 was noted after inflammation had been in progress 3 hr. or longer regardless of the size of the lesion. Even though minor variations in diameter occurred, the same changes in tissue staining were noted even in exceptionally large burns. As an example, 15 min. after intravenous loading very little perivascular dye was seen in ear-chambers injured 6 hr. previously (Fig. 10) and colour intensity (Fig. 11, 12, 13) never did approach that seen when T-1824 was injected 2 hr. after injury (compare with Fig. 6, 7, 8, 9).

*Tissue staining by T-*1824 *injected* 24 *hr. after injury.*—Dye administered 24 hr. after thermal injury resulted in a staining pattern similar to that when T-1824 was given 6 hr. after heat damage. Dye escaped only about patent vessels and not in the vicinity of either empty vascular channels in the central zone or vessels filled with static blood in the lesion edge (Fig. 14, 15, 16, 17). Distribution of stain was usually spotty; notably about venous channels near arteriovenous shunts. Within 6–8 hr. after injection a small quantity of dye accumulated within phagocytic cells of the central zone and persisted throughout the remainder of observation. Little discolouration of peripheral tissue developed during the ensuing 24 hr. and a distinct ring of blue was never visible about the lesion.

Influence of T-1824 on other Aspects of Inflammation

Vascular tone.—There was no evidence intravenous loading with T-1824 caused significant alteration of either volume or rate of blood flow in thermally damaged ear-chambers of rabbits when compared with control. Vasodilatation accompanied by increased amplitude of arterial pulsations developed as expected ; furthermore, dilatation of the circulation persisted through the 24 hr. period of observation.

Perivascular haemorrhage.—Extrusion of erythrocytes from static blood vessels was not impaired by T-1824 as may be seen in Fig. 4 which depicts early haemorrhage 6 hr. after injury and intravenous administration of dye. Likewise, there was no evidence T-1824 enhanced red blood cell diapedesis either by increasing vascular fragility or by depressing the blood clotting mechanism. Although not a prominent phenomenon, intravascular thrombi were as common as usual in injured ear chambers.

Intravascular formed elements.—Formation of rouleaux by erythrocytes was not prevented or accentuated by T-1824 and circulatory stasis with agglutination of red blood cells either singly or in clumps progressed in the usual manner. No coating of blue dye was seen on the surface of these sticky cells. Adhesiveness of platelets to injured endothelium and to marginated leucocytes was not altered by T-1824. As mentioned previously, platelets formed occlusive thrombi and mixed emboli as in unstained controls.

Sticking of white blood cells to segments of injured endothelium was not influenced by loading with Evans Blue Dye. Within 15–30 min. after injury unilateral sticking was the earliest evidence of leucocytic response to acute injury (Allison, Smith and Wood, 1955). The sticking reaction became progressively heavier and was fully developed within 1–2 hr. after trauma despite previous administration of dye. Between 6–9 hr. after thermal damage and administration of dye, sticking of white cells diminished in intensity. By 24 hr., only light sticking persisted despite vasodilatation otherwise consistent with acute inflammation. Diapedesis of white blood cells began shortly after sticking developed and was not impaired as to rate and intensity during the period of observation. Leucocytes gradually accumulated within the central zone of greatest injury eventuating in a dense infiltration 24 hr. after injury and administration of dye. Many such cells with intracytoplasmic dye stained granules remained near the platinum pin for many days and were responsible for persistence of colour in this region.

The formation of the peculiar intravascular globular structures after heat injury noted in previous studies was not influenced by T-1824 loading. It was also of interest that these globular bodies did not accumulate sufficient dye to stain blue.

Effect of T-1824 on Repair of Ear-chambers Injured by Heat

Repair of damaged tissue progressed gradually 2–3 weeks following acute insult. Restoration was chiefly distinguished by revascularization of injured tissue either by recanalization of static and collapsed vessels or by ingrowth of completely new endothelial buds ; the latter event was less common. There was no evidence T-1824 impaired replacement of damaged connective tissue.

DISCUSSION

These microcirculatory studies demonstrated a distinct and relatively brief time after acute heat injury when endothelium was most permeable. This was in agreement with findings derived by others from study of inflammation produced by bacterial infections and chemical injury (Rigdon, 1940; Burke and Miles, 1958). During the 24 hr. after injury in our experiments, leakage of fluid continued at a reduced rate and, although not as dramatic as oedema formed after macroscopic burns, this late alteration of permeability was believed consistent with larger lesions.

Although no attempt was made to investigate the basic mechanism responsible for increased permeability, certain of our observations deserve comment since they bear directly on the experimental studies of other investigators. Exudation of fluid within the first few minutes after injury may have resulted from several events. First, any trauma could induce sufficient physical structural change to modify endothelial continuity and integrity; however, in these ear-chamber experiments mechanical disturbance was kept at a minimum. Nevertheless, distortion of tissue undoubtedly occurred and, although probably slight, must have caused some escape of dye. Second, liberation of histamine and/or serotonin from perivascular mast cells may have contributed to the immediate staining visible after injury. Histamine reputedly participates prominently in the early burst of vascular permeability after injury but its influence is transient and probably exhausted after 30–60 min. (Spector and Willoughby, 1957). Serotonin has also been suggested as responsible for enhancing endothelial permeability, particularly in rats (Rowley and Benditt, 1956), but in rabbits it had little effect on the integrity of blood vessels (Sparrow and Wilhelm, 1957). Third, the permeability promoting substances demonstrable in dilute plasma of several species and associated with β-globulins have also been found in rabbit plasma. Careful study revealed it to behave as an enzyme polypeptide that was antagonized by certain proteolytic enzyme inhibitors yet it was quite distinctly not endogenous plasmin (Wilhelm, Mill, Sparrow, Mackay, and Miles, 1958). Fourth, the degradation of cellular matter by the immediate surge of proteolytic activity from tissue injured by heat (Ungar and Damgaard, 1954) may result in other substances capable of modifying endothelial integrity. The precise importance of these various permeability promoting substances in the evolution of acute inflammation remains to be defined; yet in view of their biological activity, they most probably are of some significance. It is possible, therefore, that early increases of endothelial permeability noted in these experiments could have been caused by any one if not all of the factors discussed above.

The exaggerated leakage of T-1824 observed 1–3 hr. after heat injury suggested either gradual accumulation of material capable of increasing permeability of undamaged endothelium or progressive deterioration of injured endothelial cells. Selective antagonism by pharmacological agents has been accepted as strong circumstantial evidence for the existence of substances capable of increasing permeability within inflammed tissue. Problems related to their demonstration and quantitation have been reviewed recently by Spector (1958). Previous experimental data indicated that enzymatic breakdown of injured tissue released active substances, primarily peptides, capable of enhancing vascular permeability of recipient animals. Although peptides of various length were studied, short

chain structures were found to be most active (Spector, 1951) yet their participation in the pathogenesis of inflammation has not been exactly delineated. Leukotaxine, as described by Menkin (1955), is another substance of considerable biological activity yet of unknown significance with reference to late alterations of endothelium *in vivo*. In a similar way, the permeability promoting factors found in diluted plasma (Wilhelm, *et al.* 1958) have not been implicated precisely in the modification of endothelium that develops 1–3 hr. after injury.

An alternate explanation for the delayed increase of permeability need not entail participation of any of the biologically active materials discussed above. Rapid diffusion of dye through the lesions (Fig. 3, 7) suggested that the viscosity of injured ear-chamber tissue was quite low. In a fluid environment of this type it seemed unlikely that molecular substances smaller than plasma proteins such as short chain polypeptides had achieved high concentrations locally. If this were the case it would seem as reasonable to relate endothelial alteration to the original damage as to relate it to traces of material of questionable existence and activity. Thus it could be postulated further that as these damaged cells gradually deteriorated they slowly lost their functional integrity as a semipermeable membrane. Sticking of white blood cells and platelets, deposition of fibrin, and eventually circulatory stasis were visual proof of the extreme injury these segments of the vasculature had sustained. Since these latter events are generally associated with the blood clotting mechanism it did indicate in this instance at least the release of thromboplastic substances either from damaged endothelium or from perivascular connective tissue. Eventually, as the most permeable patent vessels were occluded by stasis, staining of connective tissue was sharply curtailed. It seemed probable therefore that obstruction of blood flow by stasis partially explained the reduced permeability noted in our ear-chamber experiments 3 hr. or more after injury. For example, when dye was administered 6 hr. or more after injury, stasis presumably prevented extensive staining of tissue since the most permeable vessels had already been occluded. Similar conclusions were reached by Sevitt (1949) in the study of larger burns with T-1824 used as an indicator of endothelial integrity.

It is now justifiable to compare these results with those obtained by Rigdon, (1940) and Burke and Miles (1958). First, their description of a period of greatest permeability induced by both chemical and bacterial trauma coincided with our finding of a similar time of greatest leakage of dye in ear-chambers damaged by heat. Disappearance of the enhanced permeability phase in ear-chambers could be correlated with occlusion of previously patent porous blood vessels. Stasis of blood flow was a consistent feature of such ear-chambers injured by heat and appeared responsible for arresting excessive exudation of intravascular T-1824. In the skin infections studies by Burke and Miles (1958) stasis of blood flow was not believed responsible for decreasing leakage of dye after 4 hr. had elapsed but this particular phase of the reaction was not looked for. It is highly probable that stasis did occur commonly in their studies, especially in infections resulting in tissue necrosis. since cessation of blood flow always accompanies macroscopic death of tissue. That this was so in their type of experiment will require additional documentation. Their experiments resembled ours, however, in that infection or injury was limited as to magnitude and duration and was not self perpetuating with progressive involvement of tissue. It is thus tempting to conclude that any injury sufficiently intense to produce death and necrosis of

tissue should result in permeability changes of the type described in this paper, whereas less intense injury, *i.e.* not causing death of cells, should not cause the same sequence of events since occlusion of circulation by stasis is not an expected event. As expected after milder degrees of trauma, dye escaped into tissue for a brief time shortly after injury and in much smaller quantities. Significant late seepage 2–6 hr. after injury was not expected and did not occur.

A second point worthy of comment was the obvious relation between enhancement of experimental bacterial infections by modifying local blood flow (Miles, Miles, Burke, 1957) and the time when endothelium was most permeable. Reduction of blood flow about infected tissue during a critical time amounted to an anatomical blockade of the lesion with protection of noxious agents from blood borne elements of the host defence. As a result bacteria could then proliferate with little restriction until the circulation was re-established. Control of infection was thus delayed until blood flow returned ; the result was damage to a greater area of tissue. If on the other hand circulation in the vicinity of the lesion was not restricted until 4 hr. after inception of infection, no enhancement of disease occurred. It was thus apparent that not only had something already happened locally which enabled the host to control the infection but that this something responsible for containment was related to the previous circulatory adequacy. From our studies it is apparent that considerable exudation occurred before the sixth hour and that stasis of blood flow with vascular occlusion was the prime limiting factor of the reaction. Thus, any event limiting flow of blood during this critical period of high permeability will seriously compromise the ability of the host to contain infectious agents.

After Landis (1927) showed a direct relation between stasis and rapid leakage of fluid from injured blood vessels. it was reasoned that increased permeability was the major cause of stasis. The finding in our experiments that little dye leaked from vessels found to be static immediately after injury was therefore of interest (Fig. 2). Presumably, the time of greatest loss of fluid was so short that too little escaped to be detected even though T-1824 had been given before injury. This type of stasis developed only in channels immediately occluded by injury and although totally devoid of active blood flow were in communication with patent channels. Of additional note was the fact that the agglutinated erythrocytes did not adhere to endothelium. Stasis developing later in the reaction, however, was accompanied by considerable leakage of fluid and there was a distinct tendency for the agglutinated erythrocytes to stick to endothelium. Major escape of fluid continued only so long as movement of the blood continued and occasionally this covered a span of several hours. Obstruction of previously patent channels in this instance was apparently related to change of cellular surfaces since development of stasis was usually associated with intense sticking of the formed elements to each other and to endothelium. This type of stasis differed from that seen immediately after injury since interruption of flow in patent channels occurred only after a pronounced change of all cellular membranes. It, like leucocytic sticking, may possibly be related to a phase of blood clotting.

Without fail, it was the patent vessel bordering heat injured tissue that was the major site for exudation of intravascular solute (Fig. 22). In most instances these vessels were venules but less intense leakage of dye was noted about capillaries and arterioles (Fig. 20). Staining of tissue was most prominent about those portions of the vasculature receiving arterial blood *via* shunts opened after

injury had occluded normal channels of flow. Segments of vessel bordering the lesion but some distance from arterial connections permitted much less dye to escape. The importance of intravascular hydrostatic pressures in the exudation of fluid as emphasized by Landis (1927) was thus confirmed. It is understandable therefore why leakage of fluid from capillaries was not prominent since they did not commonly participate in forming arterial shunts. In no instance was there evidence of a permeability gradient along various segments of vessels as described by Rous and Smith (1931). The thickness of arterial walls seemed to limit escape of dye although marked perivascular staining was noted when an artery was extensively injured (Fig. 20).

Differential staining of connective tissue with vital dyes absorbed to plasma proteins as depicted in Fig. 24, 25 has not been reported previously. While studying the circulatory phenomena of experimental macroscopic burns in guinea pigs, Sevitt (1949) encountered the formation of a single ring of blue after T-1824 had been injected. Secondary rings were not recorded although in less severely burned animals rapid migration of dye into the centre of the lesion was noted. In our experiments this curious target configuration observed 1–2 hr. after administration of dye was a transient event since the lines fused or faded after 15–30 min. No explanation was immediately available but several possibilities warrant consideration. Separations of the lines suggested migration of different plasma proteins labeled with T-1824 as might occur in an electrophoretic system. It is known that T-1824 migrates primarily with plasma albumin although if present in high enough concentration movement with the α-, β-, and γ-globulins can occur (Rawson, 1943). The electromotive force or injury potential required to exert a directional influence on movement of extravascular plasma proteins labeled with dye conceivably could exist between injured and normal ear-chamber tissue. Actually the conditions that obtain within both normal and injured ear-chambers as to pH, oxygen and carbon dioxide tensions are not known although previous studies (Allison, Sleator, and Wood, unpublished) revealed marked changes of electrical potential during vasoconstriction between 2 platinum electrodes installed within uninjured ear-chambers. Efforts to demonstrate a current of injury after thermal damage in such ear-chambers, however, were never successful due to insoluble problems of design. Nonetheless an electrical field induced by injury might modify the behaviour of plasma proteins in damaged tissue and result in the production of our ring phenomenon.

Another explanation of the rings may be one of diffusion of plasma proteins through the colloid of connective tissue and ground substance in a manner analagous to a chromatographic column. Ordinarily, as in other chromatography systems, the molecular configuration and size of the various proteins would influence the rapidity of movement. Thus one would not expect such rapid movement of proteins as seen in our ear-chambers but changes of tissue pH and lowered viscosity of ground substance could influence rates of movement in the direction we recorded. Otherwise, the lines could represent simple diffusion or flow of protein molecules through a semiliquid medium.

As a third and less likely explanation one might liken the blue circles to the precipitation of inorganic salts in certain colloidal systems as described by Liesegang, whereby a series of permanent lines or rings may be formed. The rings observed in our studies were evanescent and did not resemble the deposit of insoluble salts. Although an explanation was not available for this curious finding,

it is an interesting possibility that plasma proteins may in fact diffuse or migrate through injured tissue at different rates. Such a phenomenon might be of real physiological importance in instances where ground substance has been altered by disease. As an example an abnormally viscous ground substance could materially impede transport of plasma protein fractions needed for preservation of host defences, essential for metabolic equilibrium or perhaps required for repair of damaged structures. Similarly, abnormal plasma proteins might not migrate as expected in normal ground substance and thus not participate effectively in preserving homeostasis within the host's tissues.

SUMMARY

In these studies, rabbit ear-chambers injured by heat proved a highly satisfactory model for investigating changes of endothelial permeability demonstrable *in vivo* by intravenously administered T-1824. The following deductions could be made from our findings :

Injury by heat caused an immediate but modest increase of vascular permeability. A gradual but transient secondary increase of permeability ensued over the next 3–4 hr. but slowly subsided by the sixth hour. Thereafter, a slight increase of permeability persisted through the subsequent 24 hr. The possible significance of these findings was discussed.

Leakage of dye stained plasma was noted from all visible segments of the vasculature but was most prominent about vessels near an arterial communication. This association was believed to substantiate the important role of intravascular hydrostatic pressure in the transendothelial transport of fluid. For this reason, venous channels, the commonest site for arteriovenous shunts, were found to afford greatest loss of fluid.

Although stasis of blood flow was the event most prominently associated with enhanced vascular permeability, it could not be causally connected to the phenomenon. For example, vessels that became static immediately after injury were not responsible for major leaks of fluid. Yet, channels most permeable to T-1824 almost invariably became static although there was no set time for this to occur since some highly porous injured vessels remained patent for 4–6 hr. whereas others were occluded within 2–3 hr. It was thus concluded that enhanced endothelial permeability was not the lone factor responsible for stasis.

An unexpected finding was the remarkable blue rings that formed in 1–2 hr. after T-1824 administration about burns 2–3 hr. old. Although a precise explanation for their occurrence was not available, it was thought they might represent a distribution peculiarity of extravascular plasma proteins labeled with T-1824 under conditions of acute injury.

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