STUDIES ON INFLAMMATION

II. The Site of Action of Histamine and Serotonin along the Vascular Tree: A Topographic Study

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ABSTRACT

While it is an established fact that histamine and serotonin increase the permeability of blood vessels, the exact portion of the vascular tree which is so affected has not been conclusively demonstrated. The present study was undertaken to clarify this point.

Our experiments were based on a method to which we refer as "vascular labeling," and which permits one to identify leaking vessels by means of visible accumulations of foreign particles within their walls. The mechanism of the labeling, elucidated by previous electron microscopic studies, is the following. Histamine and serotonin cause the endothelial cells of certain vessels to separate, and thus to create discrete intercellular gaps. Plasma escapes through these gaps, and filters through the basement membrane. If the plasma has been previously loaded (by intravenous injection) with colloidal particles of a black material such as carbon or mercuric sulfide, these particles—too large to pass through the basement membrane—will be retained and accumulate in visible amounts within the wall of the leaking vessel. This method is used to maximal advantage if the tissue is cleared and examined by transillumination *in toto*, so that leaking vessels can be accurately identified in their relationship to the vascular tree.

As a test tissue we used the rat cremaster, a laminar striated muscle which can be easily excised with its vascular supply virtually intact. The rats were prepared with an intravenous injection of carbon or HgS, and a subcutaneous injection into the scrotum of histamine, serotonin, or NaCl (as a control). The injected drug diffused into the underlying cremaster and the vessels became labeled. One hour later, when the carbon had been cleared from the blood stream, the animal was killed. The cremaster was excised, stretched, fixed in formalin, cleared in glycerin, and examined by transillumination under a light microscope.

The lesions induced by histamine and serotonin were identical. The leaking vessels, as indicated by the carbon deposits, always belonged to the venous side of the circulation. The heaviest deposits were found in venules 20 to 30 micra in diameter. The deposits decreased towards larger venules up to a maximum diameter of 75 to 80 micra, and towards the finer vessels until the caliber reached approximately 7 micra. Essentially spared by the deposits were the finest vessels, 4 to 7 micra in diameter, and constituting an extensive network oriented along the muscular fibers.

By killing animals at varying intervals after the injections, it was found that the carbon particles were slowly removed from the vascular walls by the action of phagocytic cells. After 10 months there was still enough carbon locally to be recognized by the naked eye.

The process of acute inflammation, according to one of the basic principles of general pathology, is in large measure the result of an increased permeability of blood capillaries (1, 2). This concept is so fundamental that it may appear to lie beyond the range of challenge. To be sure, it would scarcely be reasonable to question the existence of an increased permeability of the blood vessels, an obvious fact which was firmly established by Cohnheim as early as 1873 (3). On the other hand, the actual site of the leak has never been exactly identified. It could conceivably appear in the capillaries, in the arterioles, in the venules, or in a combination of these; perhaps it could even vary according to the injurious agent. Surprising as it may seem, the current belief that the *capillaries* become more permeable is founded not on direct evidence, but rather on tradition born of cursory observations. This is true not only for the process of acute inflammation, but also for the mechanism of action of histamine, serotonin, and the other substances known to affect the permeability of blood vessels.

The lack of precise information on this point is easily explained by three major technical difficulties which are encountered, at the level of the light microscope, when a change as subtle as increased permeability is to be recognized and correctly located in the maze of the vascular network. In the first place, the primary alteration of the endothelium lies beyond the resolution of the light microscope (4, 5), as Cohnheim himself implied when he speculated about a "molecular change" (6). Furthermore, histological sections are singularly unsuited for a study of this kind; the terminal ramifications of the arterial and venous trees, and the capillary network, are difficult or impossible to distinguish from each other when seen in cross-section, and even more so, of course, when the vessels have become abnormal in structure and caliber. Finally, the traditional experiment, currently used to demonstrate an increased vascular permeability, does not lend itself to detailed observations. A dye such as trypan blue is injected into the blood stream; the dye will leak out more readily at the site of vascular damage (1, 2). This procedure is very demonstrative to the naked eye, but disappointing under the microscope; colored plasma escapes freely out of the damaged vessels, and the result is a diffuse blue haze. Hence there is little hope to pinpoint the actual site of the lesion along any single type of vessel.

The first of these difficulties has been overcome by means of the electron microscope: it is now clear that histamine and serotonin cause leaks to appear between endothelial cells, whereas the basement membrane remains intact and literally filters the plasma which escapes through the opening (4, 5). On the basis of this observation it becomes a simple matter to label leaking vessels in such a manner that they may be identified with the light microscope. Instead of a dye, a suspension of suitable particulate matter, such as carbon black, should be injected into the blood stream. Then, wherever a leak is present in the endothelium, plasma will flow out; but the suspended particles, if of appropriate size, should be held back by the filtering effect of the basement membrane, and soon accumulate in amounts large enough to be visible with the light microscope. This effect should be put to maximal advantage by examining the tissue as a whole—with its vascular tree intact—rather than in tissue sections.

We have applied this procedure to the study of two agents which are typical representatives of the compounds known to increase "capillary" permeability: histamine and serotonin. These are the best known chemical mediators of vascular injury in acute inflammation (7, 8). Topographically the result was quite unexpected: both mediators, when studied in this manner, appear to exert their specific effect on the venous side of the vascular tree.

MATERIAL AND METHODS

Most of our observations were made on a laminar muscle of the albino rat, the cremaster. Briefly, the animal first received an intravenous injection of black colloidal particles, and immediately thereafter a local injection of the injurious agent into the subcutaneous tissue of the scrotum. Thus the agent diffused into the underlying muscle, which had not been subjected to direct mechanical trauma. After an appropriate time interval the animal was sacrificed, and the cremaster was excised, fixed, cleared and examined by transillumination.

The anatomical relationships of the cremaster muscle, and some of the reasons for selecting it, were dealt with in the first paper of this series (4). For the purpose of the studies presented herein, it will suffice to recall that each cremaster forms a pouch containing one testis; and that this pouch can be easily isolated

and stretched, to become a membrane 2 to 3 cm in diameter and 0.25 mm in thickness which can be mounted on a glass slide in the same fashion as a tissue section. The cremaster is almost ideally suited for topographic studies of the vascular tree; not only because of its thinness, but also and more especially because it can be isolated with its vascular system virtually intact. On the visceral side it presents a natural surface (the serosa) which need not be traumatized by dissection; and the overlying skin has a vascular supply which is relatively independent, as indicated by the fact that it can be cleaved off with very little hemorrhage even in the live anesthetized animal. The muscle itself consists of two very thin layers of striated fibers, and "sandwiched" between these layers run the major arteries and veins: an arrangement which makes it even easier to dissect out the muscle without injury to its main blood vessels.

The rats were of the Sprague-Dawley (Holtzman) strain and weighed 200 to 350 gm; within each experiment, uniform age and weight were maintained.

Preparation of the Cremaster for Microscopic Examination: Under ether anesthesia the thoracic cage is opened; the ensuing pneumothorax will kill the animal in about 1 minute. Before cardiac arrest, the root of the scrotum is rapidly clamped—on either side—with a large hemostat, applied deeply enough to include the vascular peduncle which leads from the abdominal cavity to the upper pole of the testis. The scrotal skin is rapidly removed, the testes with their envelopes are excised *in toto* and dipped for a few minutes, with the clamps still applied, in 10 per cent formalin. This procedure helps to minimize the loss of blood in subsequent handling and hence to preserve, as much as possible, the natural pattern of vascular injection (if the animal is killed by bleeding the number of visible vessels is sharply decreased). Thereafter the cremasters are dissected off under formalin, gently stretched, and pinned onto dental plate wax. After formalin fixation for 24 hours, the thin fascia on the subcutaneous side is carefully stripped off under the binocular microscope. The muscle is briefly rinsed in water and then cleared in two changes of glycerin (48 and 24 hours). Finally the preparation is trimmed, floated in two changes of warm glycerin jelly for a few minutes, and mounted under coverslips (a modification of the usual formula for glycerin jelly, which was found most suitable for these preparations, consists of a mixture of equal parts of glycerin and 10 per cent gelatin solution, with a few crystals of phenol). In this state the preparation is as permanent as a tissue section, and can be examined by transmitted light with good resolution at magnifications as high as 500.

Local and Intravenous Injections: With the animal under ether anesthesia, the hair covering the proximal part of the scrotum was gently removed with an electric clipper, and the colloidal suspension was injected intravenously. Immediately thereafter, one injection either of test substance or of a saline control was administered subcutaneously over the mid-ventral aspect of each cremaster, great care being taken to avoid stretching of the skin and all unnecessary trauma. The order of the intravenous and subcutaneous injections could be reversed with no apparent difference, as long as the two injections were administered in immediate sequence. The labeling of the vessels occurred very rapidly, but 1 hour was allowed for the particulate matter to be removed from the blood stream. After this time the animal was killed and the cremaster prepared as described. Variations in the timing of injections and death of the animal are indicated under Results.

Colloidal Suspensions: We used primarily carbon black especially prepared for experimental use by the Guenther-Wagner Pelikan-Werke, Hannover, Germany (Batch # C11/1431a). This preparation contains about 100 mg/cc of carbon with an indicated average particle size of 200 A; it is stabilized with 4.5 per cent fish glue and contains 1.3 per cent phenol as a preserving agent. This solution was injected into the saphenous vein in a dose of 0.1 cc/100 gm body weight (10 mg carbon/100 gm body weight). Doses five times greater are tolerated by the animals without apparent harm, but require a longer period to be cleared from the blood stream.

In some experiments we used a preparation of colloidal mercuric sulfide (black) prepared by Hille and Co., Chicago, and containing HgS, a stabilizer, and 0.2 per cent cresol. This preparation is supplied as a 2 per cent suspension ("Mersulfol") and also in the form of a dry material which contains approximately 25 per cent of HgS; this was dissolved in saline to a concentration of 4 per cent HgS, and injected intravenously in the dose of 0.5 cc/100 gm. Larger doses sometimes caused the animal to die with pulmonary edema.

Test Solutions: Subcutaneous injections in the skin of the scrotum were all administered in the same volume, 0.05 cc. This was accomplished with the use of a Hamilton microsyringe (made to deliver 0.05 cc with an accuracy of ± 0.1 per cent) fitted with a gauge 30 needle. *Histamine* diphosphate (Abbott Laboratories, North Chicago, Illinois, and Eli Lilly & Co., Indianapolis) was used in a concentration of 1 mg/cc; the dose hereafter referred to as standard, amounted to 18 μg of histamine base in 0.05 cc. *Serotonin* (5-hydroxy-tryptamine creatinine sulfate, Nutritional Biochemicals Corp., Cleveland) was employed as a solution of 0.0126 mg/cc freshly prepared before use. In this concentration, 0.05 cc (0.27 μg of serotonin base) produded a lesion approximately matching that of the histamine standard.

EXPERIMENTAL PROCEDURE AND RESULTS

A. CONTROLS

Effect of the Colloidal Suspension Alone

As soon as the suspension of carbon or HgS had been injected intravenously, the skin and visible mucosae of the rats took on a definite grey hue. Microscopically, preparations of cremaster taken during the first 10 minutes (in the absence of any local injection) were scarcely distinguishable from those of normal animals; there was a faint greyish discoloration of the plasma, together with a dust-like scattering of fine black particles, particularly with the carbon suspension. Whether these particles were all suspended in the blood stream, or sometimes adherent to the endothelial lining, could not be established in our fixed preparations; however, the grains appeared to be randomly and uniformly distributed in all the vessels of the cremaster, and the relevant point for our experiments is that larger aggregates or deposits did not form anywhere.

Controls for the Local Injection of Histamine and Serotonin

In most rats, the test substance (histamine or serotonin) was injected on one side of the scrotum, while the other side was injected with an equal amount of 0.85 per cent NaCl. On this control side, the cremaster usually developed a small lesion often scarcely visible (Fig. 2), but occasionally as large as 5 mm. Microscopically the pattern was identical with that induced by histamine or serotonin: that is, the blackening was limited to the venules (see below). In other animals saline only was injected on both sides, with no concomitant histamine administered to the same animal; the result was the same as with the previous series. The simple introduction of the needle, or the injection of 0.05 cc of air, caused no lesion at all.

B. EFFECT OF HISTAMINE

If histamine is injected locally, and the carbon suspension intravenously, a blackening of the

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Explanation of Plates

All figures represent rat cremaster muscles, excised, stretched, fixed in formalin, cleared in glycerin (see Methods), and mounted between a glass slide and a coverslip. No histological stains were applied. All figures except Figs. 1 and 2 were taken with a light microscope (Zeiss Ultraphot) at magnifications of 30 to 200. An opal glass 3 mm thick was interposed between the microscope stage and the preparation, as a diffusing filter. Figs. 1 and 2 were taken with a Leitz Aristophot assembly, by a combination of epi- and transillumination.

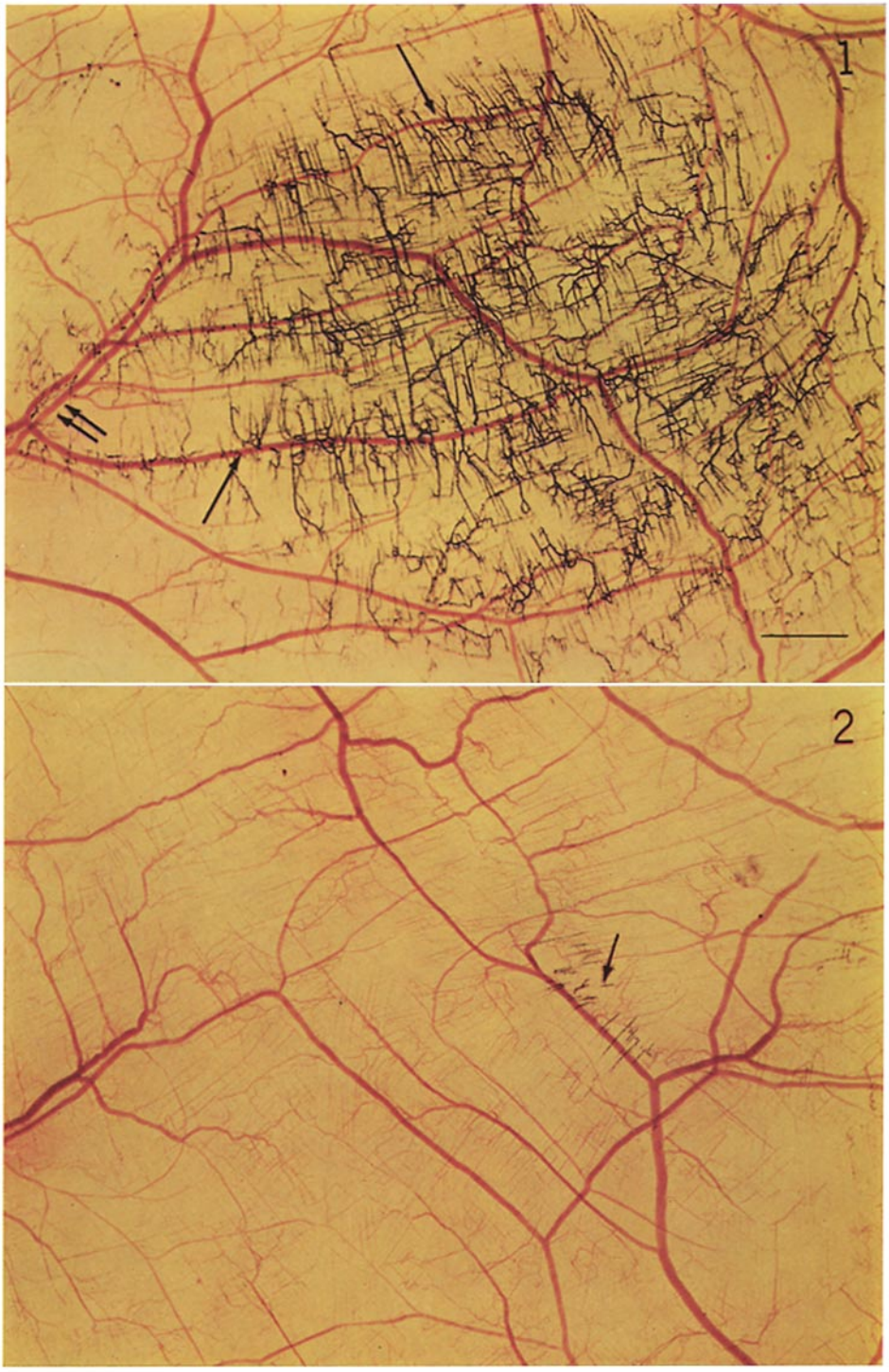
FIGURE 1

Topographic view of a histamine-induced lesion: a demonstration of the vascular labeling method, whereby leaking vessels are blackened with carbon deposits.

One hour prior to sacrifice, this rat received an i.v. injection of carbon suspension and a local injection of histamine (18 μg). The circulating carbon has now been cleared from the blood stream. *Double arrow:* arteriole. No blackened branches stem from this vessel. *Single arrows:* venules, about 100 micra in caliber. These larger venules appear to be unaffected, but inserted along them are black tree-like structures which represent leaking tributaries, with branches 2 or 3 orders. Scale = 1 mm.

FIGURE 2

Control, in which 0.05 cc of 0.85 per cent NaCl was substituted for histamine (all other conditions as in Fig. 1). There is no indication of vascular leakage, except for a very small region (right center) in which faint carbon deposits have appeared, again only in branches of a venule (*arrow*). Scale = 1 mm.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vessels within the cremaster occurs very rapidly. The characteristic effect, which will be referred to hereafter as *vascular labeling*, shows quantitative variations with the dose of histamine; the following description corresponds to the dose which we found to give optimal results (0.05 cc of 0.1 per cent histamine phosphate). If the animal is killed after 2 to 7 minutes, at the site of the subcutaneous injection the cremaster shows a dark patch 15 to 20 mm in diameter in which a faint black network can be detected grossly. In subsequent stages this network becomes more pronounced, and after 10 to 15 minutes it appears to have reached maximum intensity. On closer inspection this area shows a pattern which is sometimes more obvious but always apparent, even to the naked eye, particularly in the preparations cleared with glycerin: the blackened vessels are not uniformly distributed, but mostly grouped in many small, tree-like or feather-like arrangements, separated by narrow clear spaces (Fig. 1). Under a low-power enlargement it is easy to observe that this pattern corresponds to a definite portion of the vascular tree: each discrete unit represents a venule surrounded by its system of converging tributaries. Proximally and distally to this segment of the venous tree, the vessels (capillaries and larger veins) are not blackened (Figs. 3, 5, 6).

The blackening of the venous system is sharply restricted to the terminal portion of the tree, and within a relatively narrow range of calibers. In general, it is heaviest in the venules with an outer diameter of 20 to 30 micra, which at low enlargements appear as black cylinders (Fig. 3). Proceeding in the direction of the blood flow, the carbon deposit ends often very abruptly where the venule opens into a larger vessel (Fig. 7); other times it tapers off along the course of the vessel, and the last traces can be found up to a maximum caliber of 75 to 80 micra. Proceeding towards the capillary, the deposit breaks up into uneven patches, and ceases often quite abruptly where the lumen has narrowed to the point of allowing the passage of a single red blood cell (Figs. 8, 5, 6). Beyond this point there is a vast system of very fine vessels, 4 to 5 micra in diameter, which remain quite free of carbon deposits. These vessels form a three-dimensional network, with long meshes oriented along the muscular fibers (Fig. 8). Some of the meshes are visible because they contain red blood cells; however, the true extent of this network which remains free of deposits is better appreciated if the whole vascular system is injected with an opaque mass[1] (Fig. 4).

At enlargements of 100 to 400 times the mor-

[1] We used a mixture of our carbon suspension with a cold gelatin mass which has the advantage of being fluid at room temperature (5 gm of gelatin are dissolved in 100 cc of warm distilled water, then 5 gm of potassium iodide are added). Further details may be found in E. A. Bean, Animal Micrology, University of Chicago Press, 5th edition, 1953, 98.

FIGURES 3 AND 4

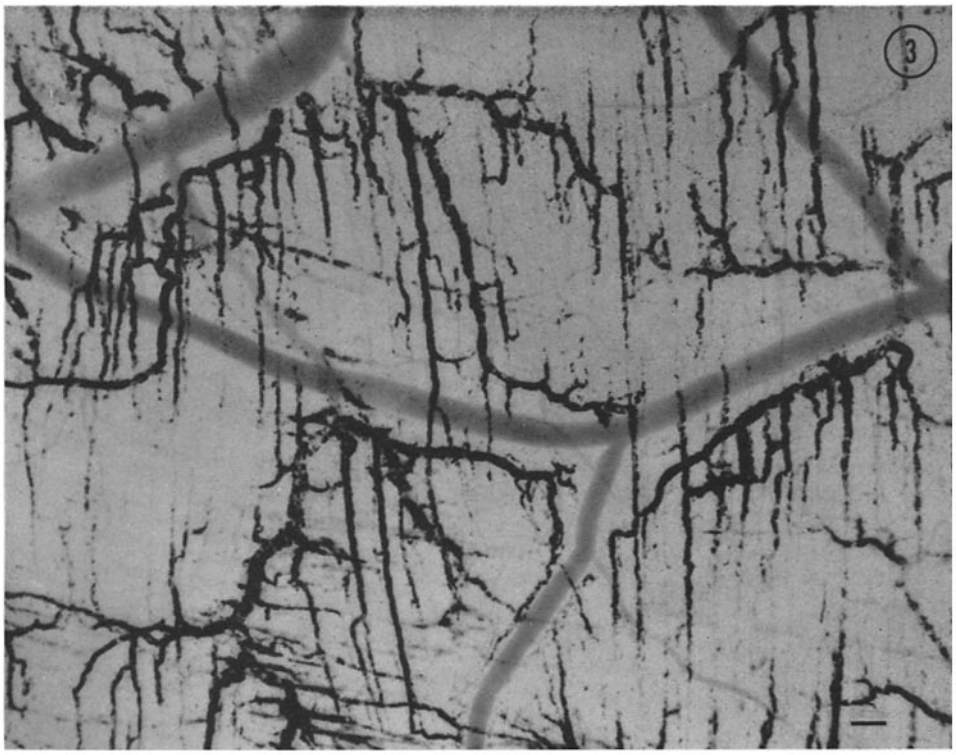
Two cremasters prepared in different ways and shown at the same enlargement (× 45) to demonstrate the fact that a vast number of very fine vessels are not caused to "leak" by the action of histamine or serotonin.

FIGURE 3

Typical example of vascular labeling induced, in this case, by serotonin. The black, branching structures are venules. Experimental procedures as indicated for Fig. 1. Scale = 100 micra.

FIGURE 4

Cremaster of a normal rat, in which the entire vascular system has been injected with a mixture of carbon and gelatin (see Methods). This preparation shows a large number of very fine vessels (capillaries, in the strictest sense) superimposed in different planes. By comparing this photograph with the one above, it becomes obvious that the great majority of these fine vessels are not blackened by the method of vascular labeling. Scale = 100 micra.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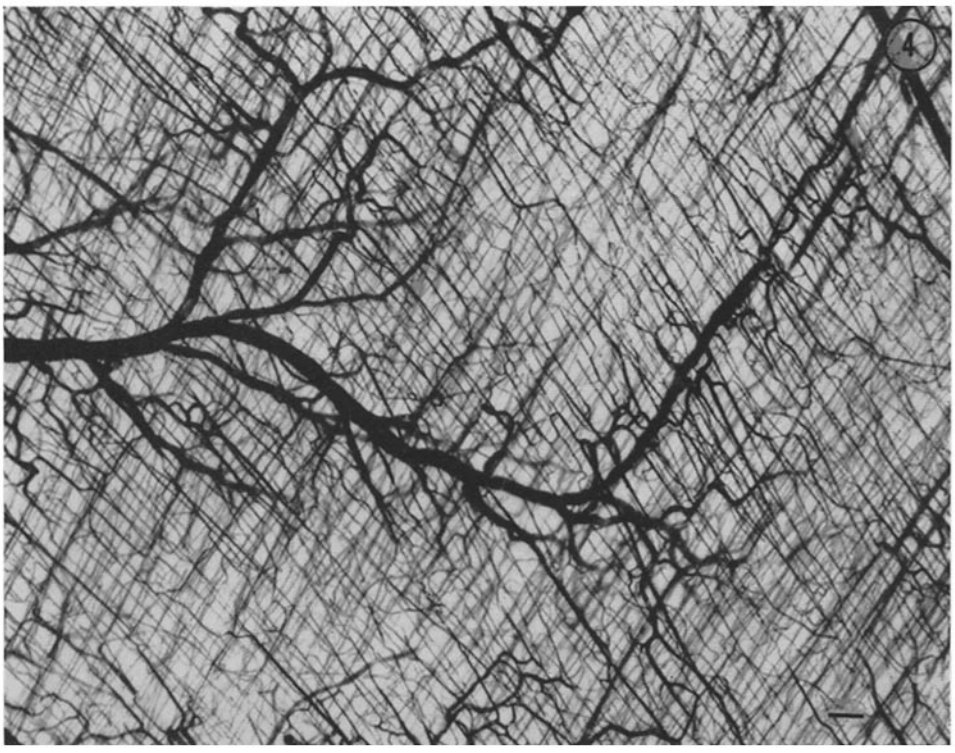
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phology of the carbon deposit can be studied in considerable detail. Even where the layer of deposit is heaviest it shows very fine fenestrations, and a ragged, moth-eaten aspect which indicates that the carbon is seeping into a very uneven system of clefts (4). The outer surface, corresponding to the adventitia, is relatively smooth, but occasional leaks can be found where groups of granules have reached as far as 10 to 15 micra into the extravascular spaces (Fig. 9). In the larger venules the deposit is occasionally broken up into irregular rings encircling the vessel, an orientation probably determined by the presence of smooth muscle cells (Fig. 12). More often, where the deposit is slight, it appears in the form of short, straight lines parallel to the axis of the vessel; Fig. 10 shows this pattern, which is frequently repeated along a segment of the venule. A complete flagstone pattern reminiscent of that obtained by perfusion with $AgNO_3$ was never observed.

The amount of carbon injected intravenously can be increased or decreased by a factor of 3 without any recognizable difference in the intensity of the blackening. The smallest dose of histamine phosphate which can bring about a significant blackening is 0.05 cc of a 0.01 per cent solution (1.8 μg of histamine base); with a 0.1 per cent solution which we adopted as a standard dose, the lesion has an average diameter of about 15 mm.

In rats injected with colloidal HgS rather than carbon, the black deposits have the same topographic distribution (Fig. 6) but they are more transparent, and smoother in appearance (Fig. 11), perhaps on account of the smaller dimension of the particles.

Individual variations in the intensity of the response were frequently observed. The variability affected the diameter of the over-all lesion, not the selective location of the carbon deposits along the venular tree. With a given standard dose of histamine the diameter of the blackened patch could vary between 10 and 20 mm. On the other hand, there was much less variation in the response of the two cremasters of a single rat.

Perfusions of the whole animal with horse serum were also accomplished, in order to remove the red blood cells and obtain an even sharper definition of the carbon deposits. Rats were given a local injection of histamine and an intravenous injection of carbon or HgS in the usual fashion. After 10 to 15 min. they were etherized, perfusion was started into the saphenous vein, and a jugular was cut (fluid: 50 to 90 cc. of warm heparinized horse serum, containing 0.3 per cent of trypan blue for a gross control of the success of the perfusion). The heart kept beating for a prolonged period. The preparations of cremaster appeared almost completely devoid of red blood cells. None of the carbon or HgS deposit appeared to have been removed; the blackened vessels stood out in sharper contrast against an almost empty background, but there was otherwise little to be learned from these preparations.

Later Fate of the Carbon Deposits

In a series of rats, a lesion was produced in the cremaster with the combined injections of histamine and carbon black, as usual. The cremasters from these animals were then examined after periods varying between 30 minutes and 10 months. It was found that the particles of carbon tend to emigrate rather rapidly across the wall of the vessel. After 3 hours (Fig. 14) the outer surface of the deposits is noticeably more ragged, and many fine granules appear which at 6 hours

FIGURES 5 AND 6

Cremasters after a local injection of histamine; the leaking vessels have been labeled with carbon (Fig. 5) or colloidal HgS (Fig. 6). The deposits of carbon are somewhat coarser than those of HgS, but the distribution is very similar.

The fields have been selected to demonstrate the sequence arteriole → capillary → venule, and thus the elective deposition of tracer particles on the venous side. *A* = arteriole, *V* = venule. Note that considerable lengths of the finest capillary segments (*C*) are spared. The relative number of these unlabeled capillaries is actually much greater than can be judged from these photographs, because many of these vessels lie above and below the plane of focus. Scale = 100 micra. Fig. 5, × 160. Fig. 6, × 140.


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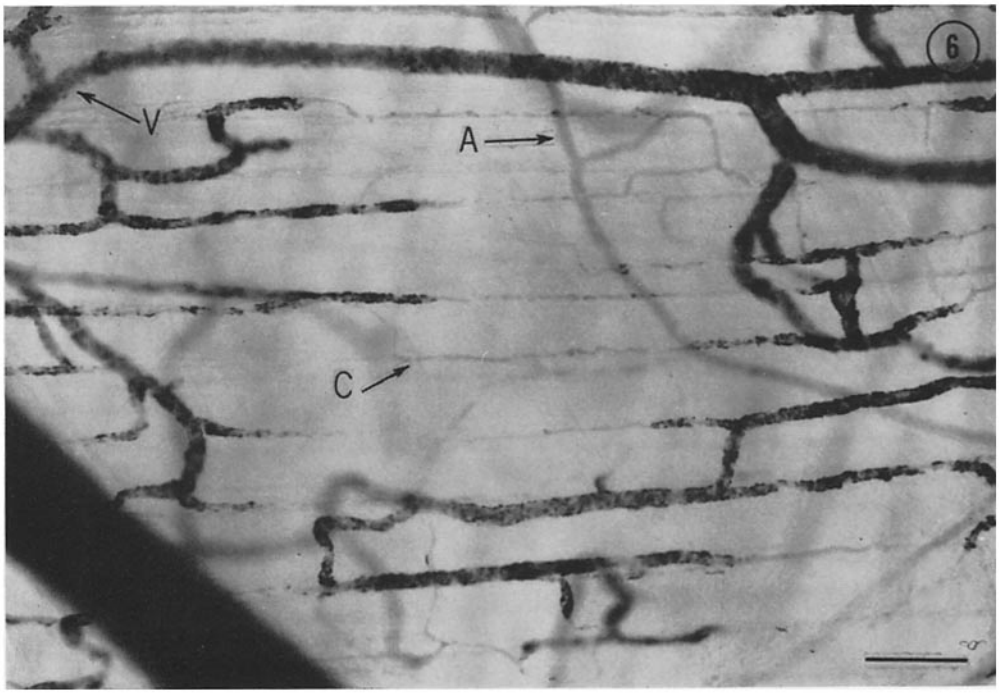
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V
A
C

form an almost continuous sprinkling along the adventitia. At 12 hours many of the granules are collected into lumps, suggestive of single macrophages, and at 24 hours (Fig. 15) even the continuous black layer which obscured the medium-sized venules begins to break up into lumps, probably because it becomes condensed within cells. At 7 days (Fig. 16) the black patch grossly visible on the cremaster begins to fade slightly, indicating that some of the carbon has been carried away; microscopically, the carbon now appears to be collected in macrophages, mostly aligned in rows along the vessels, while others are scattered around the tissue. The larger venules, rather oddly, have a distinct row of macrophages on two opposite sides, as if these cells avoided that portion of the circumference of the vessel which faces the peritoneal or the cutaneous surface. After 1 month (Fig. 17) the black deposits still suggest a vascular pattern, but now the venules are simply outlined by a succession of black, elongated macrophages seemingly flattened against the adventitia; at low powers it appears as if a black pencil had drawn a broken line along the venules. After 3 and 10 months (Figs. 18 and 19) the pattern is the same: a grey patch is still visible, though faintly, to the naked eye; microscopically only the larger venules are outlined, most of the carbon having by now disappeared. A progressive blackening of the lumbar lymph nodes was noticed from the 7th day onwards.

C. EFFECT OF SEROTONIN

When carbon was injected intravenously, and serotonin locally, carbon labeling developed in the venules in a manner identical to that already described for histamine. Quantitatively, however, serotonin was much more powerful on a mole-to-mole basis; a dose calculated to be equimolar with that of the standard amount of histamine (0.05 cc of a 0.126 per cent solution of serotonin creatinine sulfate) induced a lesion so large as to include one whole cremaster and half of that on the opposite side. Judging from the diameter of the lesion, an effect comparable to that of histamine was obtained with a dose approximately one hundredth as great on a molar basis.

DISCUSSION

A. THE PHENOMENON OF VASCULAR LABELING: EARLIER INTERPRETATIONS

All the experiments described in this paper represent an application of the phenomenon to which we referred earlier as vascular labeling. In short, if histamine or serotonin is injected locally, and a carbon suspension intravenously, there develops a blackening of certain vessels in the area of the local injection. On the strength of electron microscopic evidence (4, 5) it is safe to interpret this blackening as follows: the endothelium develops discrete leaks, and as plasma filters through the basement membrane, the suspended tracer particles are retained.

While this interpretation may be new, the actual phenomenon of vascular blackening in injured tissues, after an intravenous injection of India ink or carbon, has been described a number of times in the literature. These earlier studies

FIGURES 7 AND 8

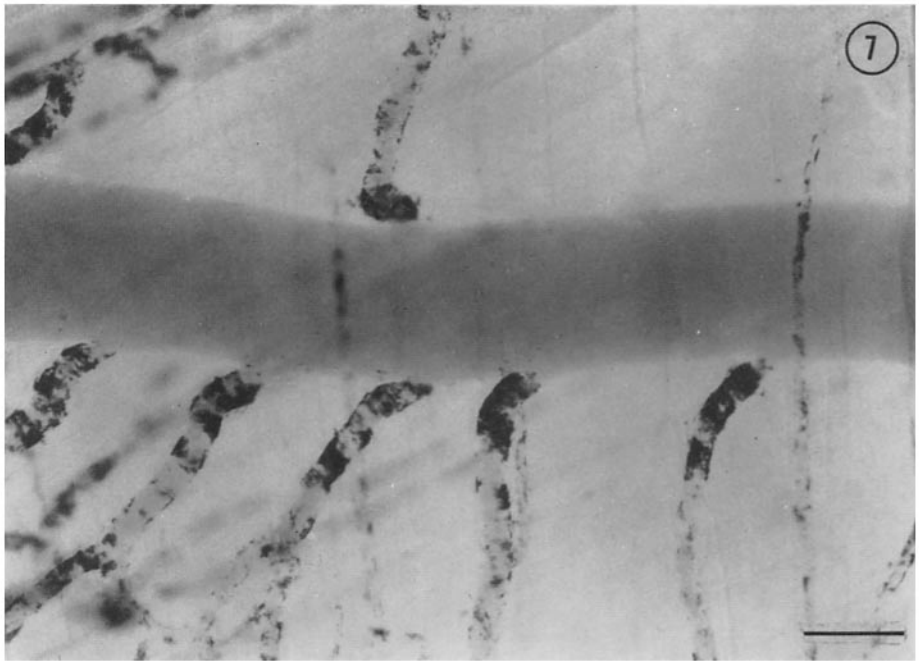
Vessels labeled with carbon after local injection of histamine. Examples of the largest (Fig. 7) and the smallest (Fig. 8) vessels which are affected.

FIGURE 7

The deposits cease sharply as the tributaries join the main venule. Scale = 100 micra. × 130.

FIGURE 8

Note the absence of deposits along the finest vessels. The caliber of these vessels (arrows) is indicated by the single row of red blood cells contained in thel umen. As soon as these vessels begin to enlarge (towards the venous end), granular deposits begin to appear. Scale = 100 micra. × 440.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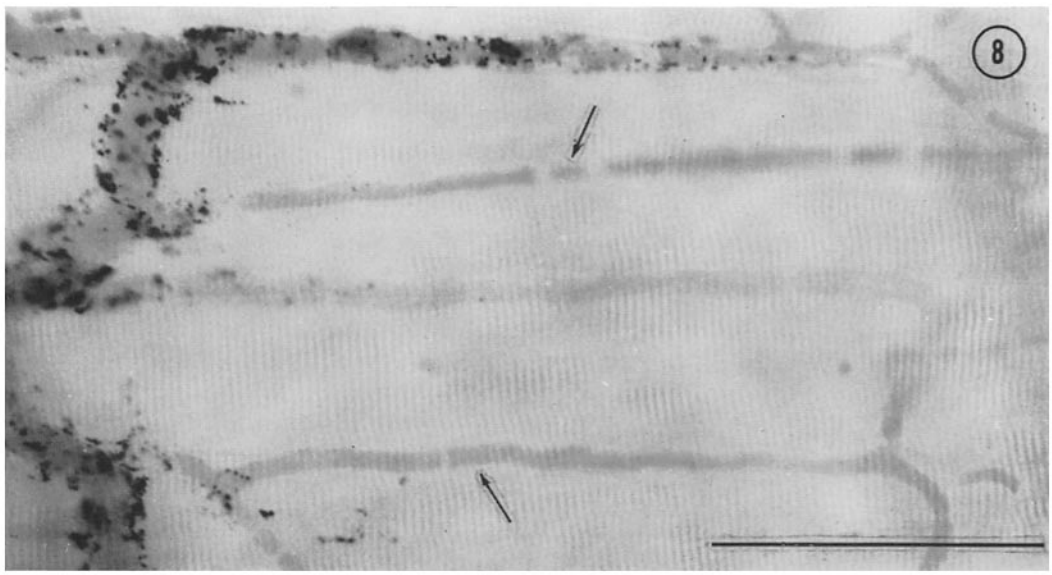
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were made on living tissues or on histological sections, techniques which did not allow one to distinguish clearly between intravascular, intramural and intracellular deposits of the carbon; hence many of the conclusions drawn by these authors can no longer be accepted.

Thus Herzog (9), Stilwell (10) and Schopper (11) who studied the tongue of the living frog after intravenous injection of India ink, interpreted the black deposit as a *superficial coating of the endothelium*, which slowly penetrates and crosses the vascular wall by phagocytosis and cellular transport. In point of fact, these papers include careful descriptions of early *reversible* deposits which leave little doubt that some superficial coating of the endothelium did actually occur. This should be expected, because the material injected was India ink; the latter contains shellac, which promotes intravascular clotting (12). This may well explain the intravascular "filaments" described by Schopper (11). The carbon suspension used in our experiments did not contain shellac. In our previous study we did observe occasional superficial deposits (4); this mechanism, however, may account for only a minor part of the blackening.

The case of Krogh (13) is rather unique, in that he formulated the correct hypothesis, performed our same experiment, and obtained our same results but considered them a failure. Krogh had actually entertained the idea that when the permeability of a blood vessel is increased, this might occur by the formation of "fissures between the endothelial cells, . . . especially at the points where the borders of three or more cells meet." He therefore injected dialyzed India ink intravenously into frogs, and expected that at a site of injury (by urethane) grey plasma would escape: ". . . but the result of the actual experiment was that the indian ink was held back quantitatively, while the clear plasma disappeared as before. . . ." Krogh did not consider the presence of a basement membrane, though it had been described previously (14). Hence, "discarding [the original hypothesis, he] came to the conclusion that mechanical stretching of the capillary wall must be responsible for the increase in permeability. . . ."

Chambers and Zweifach have repeatedly described the deposits of carbon in injured vessels, particularly in venules, and along the intercellular junctions (15, 16). They deduced from these observations that injury brings about a *swelling and stickiness of the intercellular cement* (15, 16).

FIGURES 9 TO 12

Carbon- or HgS-labeled venules after a local injection of histamine: structural details of the deposits. Scale = 100 micra.

FIGURE 9

One hour after the injection of carbon, a few particles (arrow) have found their way as far out as the adventitia. × 490.

FIGURE 10

Carbon deposits in the pattern of regular parallel lines (arrow). This image is interpreted as indicating—in all likelihood (4)—clefts between endothelial cells. × 340.

FIGURE 11

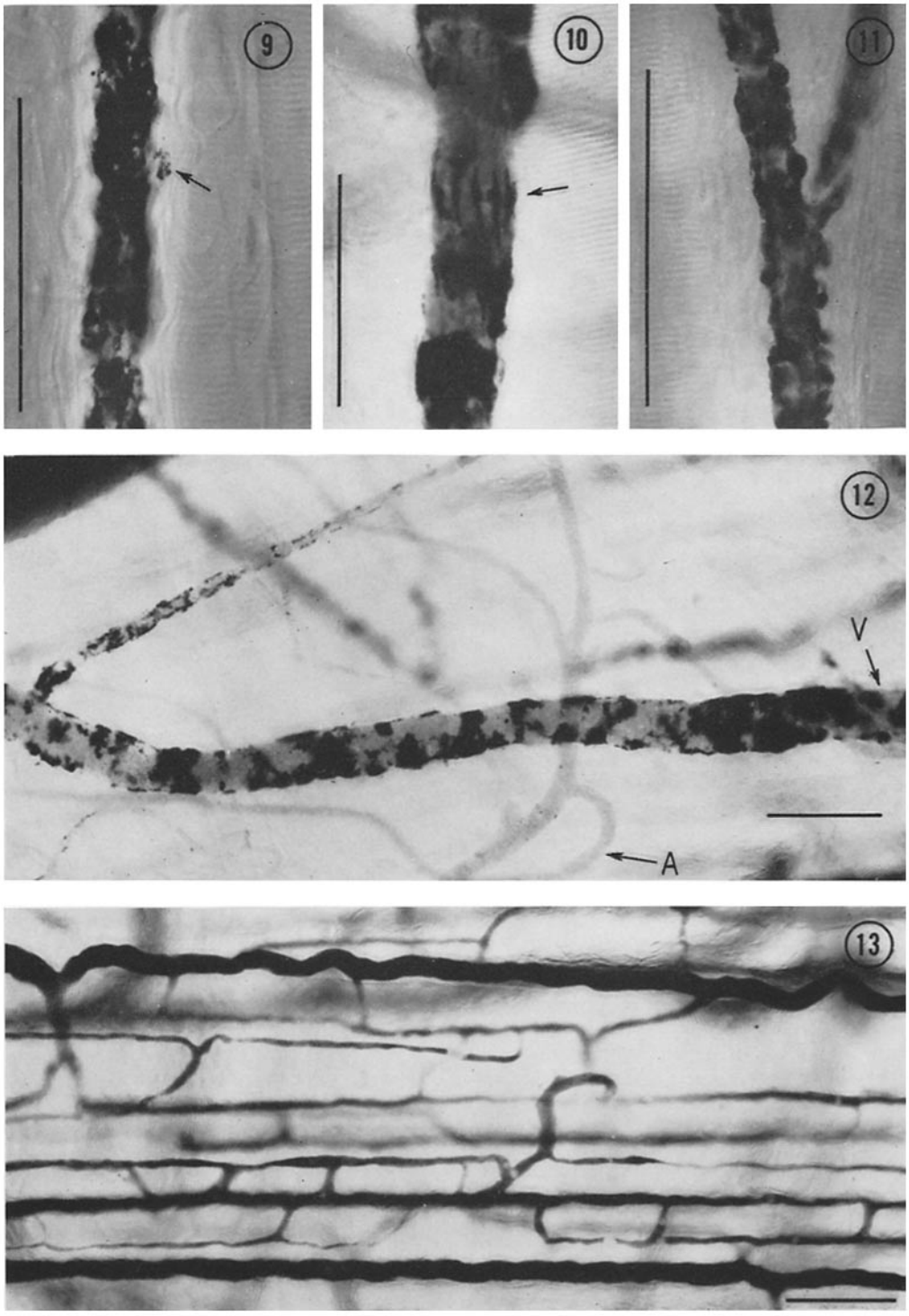
Venule labeled with colloidal HgS. The deposit appears less coarse than with carbon. × 460.

FIGURE 12

Carbon deposits in the pattern of incomplete rings surrounding a venule (*V*). This arrangement may be determined by smooth muscle cells wrapped around the vessel, perpendicular to its axis. *A* = an arteriole, devoid—as usual— of deposits. × 170.

FIGURE 13

Normal rat cremaster, in which the vessels have been injected with a mixture of carbon and gelatin (same preparation as for Fig. 4). Note the characteristic ladder pattern of the finest vessels, which run between the muscle fibers. Scale = 100 micra. × 170.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Another interpretation which can no longer be held is that which explains the blackening as an expression of *increased phagocytic activity of the endothelium.* This view originated with Jancsó (17–18), who observed that if histamine is painted on the skin of a white mouse, and India ink is injected intravenously, many small vessels in the painted area become black. This effect, later referred to as "Jancsó phenomenon" (19), became the starting point of a series of papers aimed at demonstrating that histamine enhances phagocytosis (20, 21); a summary of this work may be found in a recent monograph on phagocytic stimulation (22).

This is not to say that phagocytosis plays no part in the phenomenon of vascular labeling. It does occur (4, 5), but as a relatively late and probably secondary event, involving the endothelial cells, and more especially the pericytes.

It should be pointed out, of course, that the observations published by the above-named authors remain essentially valid; only the interpretation has changed.

B. LOCALIZATION OF THE VASCULAR DAMAGE IN THE VENULES

If one examines at low magnification a cremaster in which the leaking vessels have been labeled with carbon (Figs. 1, 3) it is not difficult to identify them as branches of the venous tree. The largest venules with traces of deposits have a caliber of 75 to 80 micra. Proceeding upstream, the deposits become heavier, and reach a maximum in venules with a diameter of 20 to 30 micra; then decrease, and finally stop when the caliber has reached about 7 micra: that is, approximately the diameter of a red blood cell. This distribution of carbon deposits clearly suggests that the venules 20 to 30 micra in diameter are the most sensitive vessels. In fact, when the injury is slight, as at the periphery of the injection site, these are the only vessels to show any blackening at all.

The number of blackened vessels is so great as to suggest, at first sight, that all of the capillary system is affected. However, if the same preparation is compared with another one in which all the blood vessels have been filled with an opaque mass (Figs. 3 and 4) it becomes obvious that there is a vast system of very fine vessels which are almost completely free of carbon deposits. These vessels, to which we will refer, for the time being, as "unlabeled capillaries," are not easily visualized in our ordinary preparations, because they are incompletely filled with blood. Their caliber is the same as—or less than—the diameter of a red blood cell, and ranges approximately from 4 to 7 micra. Their basic pattern is not that of a tree, as is the case for the blackened vessels, but rather that of a three-dimensional network, with oblong meshes running parallel to the muscle fibers and often aligned in a ladder-like arrangement (Figs. 5, 6, 8, 13).

These observations are in complete agreement with our previous electron microscopic study (4), in which a striking feature of the cremaster muscle after injection of histamine or serotonin had been the consistent lack of lesions in the vessels of the smallest caliber. In other words, the vessels which appeared to be the most likely candidates for the term "capillary" did not develop leaks. Their outer diameter (measured on electron micrographs, and taking the basement membrane as a boundary) was 3 to 5 micra. Leaks developed with great regularity in vessels

FIGURES 14 TO 16

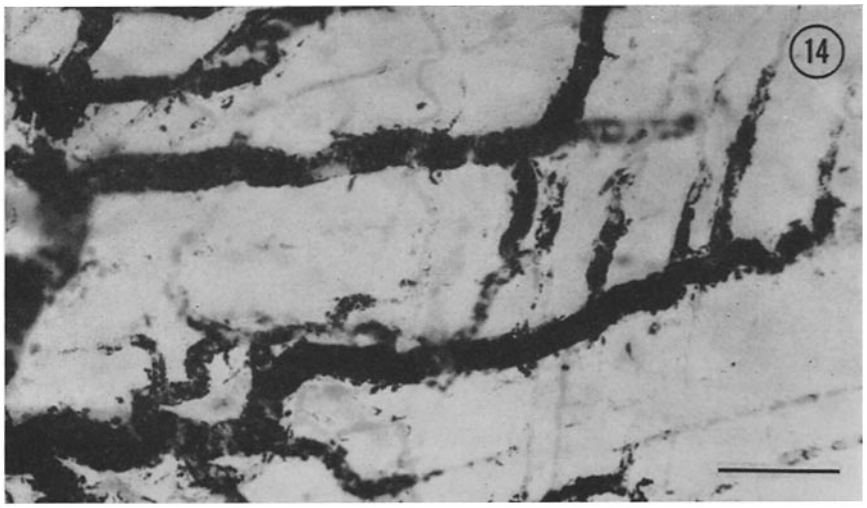
Aspect of carbon-labeled venules, at varying time intervals after the local injection of histamine. Scale = 100 micra. × 160.

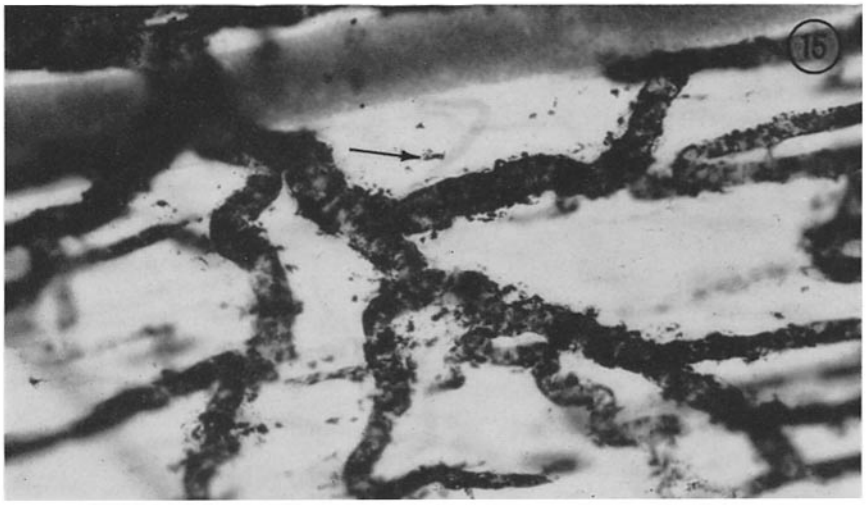
FIGURE 14

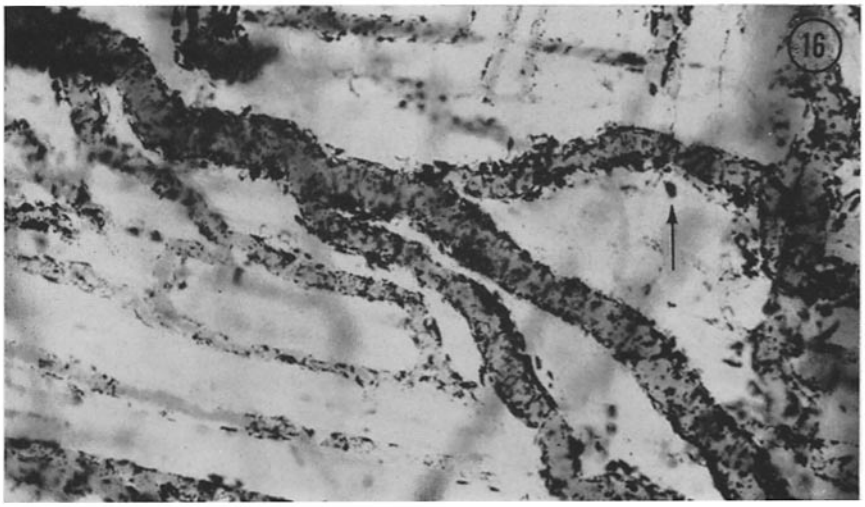
Stage of 6 hours. Small clusters of carbon (presumably contained within phagocytic cells) are beginning to appear along the contours of the venules.

FIGURES 15 AND 16

Stages of 1 and 7 days. The carbon is being concentrated in discrete granular masses, presumably intracellular, within and adjacent to the vascular wall. Some carbon also appears in perivascular clusters, probably within phagocytes (*arrows*).


14


15


16

about twice this diameter, but whether the vessels represented venules or the finest ramifications of arterioles could not be established by electron microscopy.

Our studies therefore indicate that histamine and serotonin bring out a fundamental difference in response between two types of blood vessels: the finest vessels, 4 to 7 micra in diameter (as measured in the fixed preparations) which remain practically free of demonstrable leaks, and the collecting vessels, from 7 or 8 to 75 or 80 micra in diameter, which develop extensive leaks. It would be tempting to identify these two kinds of vessels with capillaries and venules, respectively. At present, however, there is no general agreement as to the use of the word "capillary," as evidenced by the multiplicity of terms such as protocapillary, precapillary, postcapillary, true capillary, and the like (23). The end-point of the capillary on the venous side is particularly indefinite; it is often extended to include venous vessels (23).

In the past there has been little point in drawing a line between capillaries and venules, because the two types of vessels were generally assumed to be very similar, if not identical, in both structure and function (13). There is, of course, a structural similarity between capillaries and the finest venules: in either case the walls consist of endothelium without a muscular coat; the first smooth muscle cells begin to appear when the caliber reaches 45 to 50 micra (24, 25), and a continuous muscular media is not found until the caliber reaches about 200 micra (24, 25). Therefore, if one were to base the definition of capillary on the absence of smooth musculature, the diameter of the capillaries could reach at least 50 micra. This is a criterion often implied in histopathologic descriptions. Landis called capillaries (in mammals) vessels with a diameter of 5 to 20 micra (26). Among the textbooks of histology, there is a tendency to define the blood capillaries as those vessels which have a diameter "approximately equal to that of the red blood cells which must pass through them" (27). Thus Cowdry (28) states that the capillaries begin when the lumen is reduced to the minimum diameter which will let red blood cells pass freely: "this is considered in man to be about 8 microns. . . . The venous limit of the capillary bed commences where the capillaries run together to form vessels of larger diameter than 8 microns." For Maximow and Bloom (24) the caliber of the capillaries is closely related to the size of the red blood cells, and "in man it averages about 8 μ." Nonidez and Windle (29) follow the same criterion, and use the figure of 8 to 10 microns. Barrnett (25) states that capillaries "are the blood vessels having generally the smallest lumen and the simplest wall," their diameter varying "between 4 and 12 microns."

It would appear, then, that if we followed the definition of capillary indicated by the above-named textbooks, we could equate with good approximation our unlabeled capillaries with capillaries, and our labeled vessels with venules. In the literature, however, the term "capillary" is used more loosely (23). For the time being, therefore, it is safer to avoid strict reference to given vascular segments, and state that the endothelial leaks induced by histamine and serotonin are found on the venous side of the circulation, with a maximum effect on venules 20 to 30 micra in diameter, decreasing towards the larger veins, as well as towards the finest vessels.

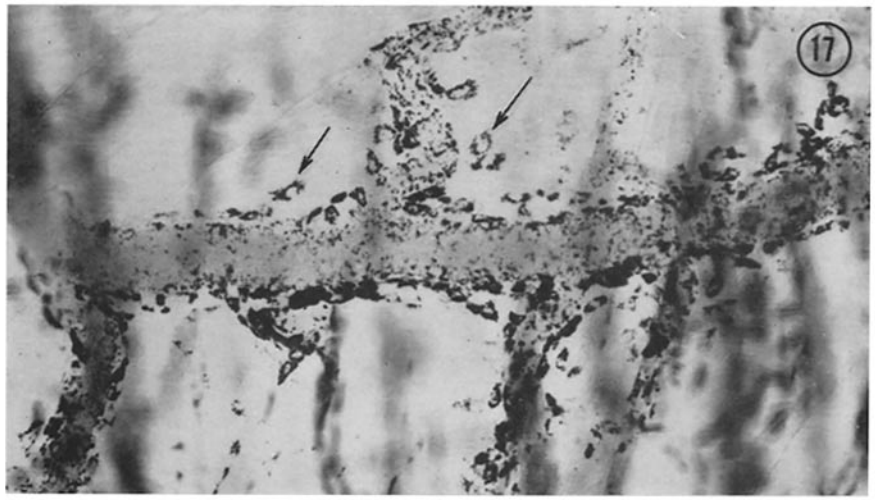
C. COMPARISON OF VASCULAR CHANGES INDUCED BY HISTAMINE AND SEROTONIN

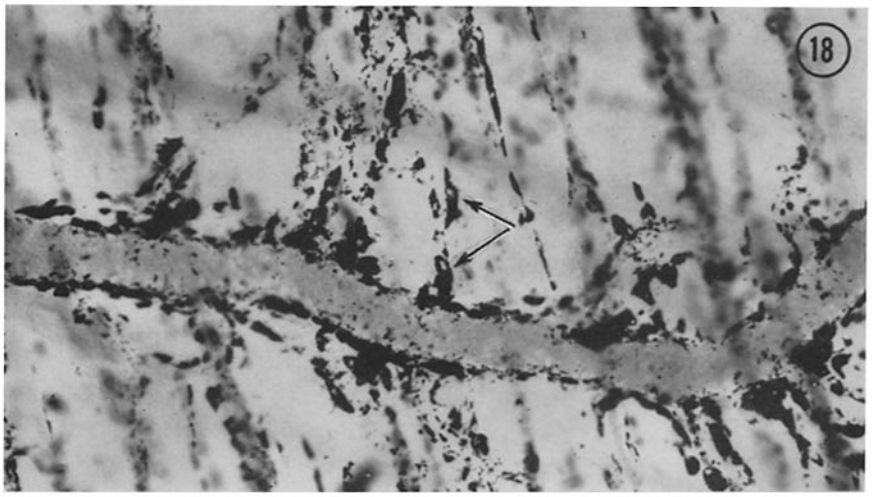
The vascular damage caused by histamine, when tested by the method of carbon labeling, was indistinguishable from that of serotonin. We were unable to convince ourselves that there was any difference either in the topographic distribution of the lesions along the venular tree, or in the actual structure of the carbon deposit.

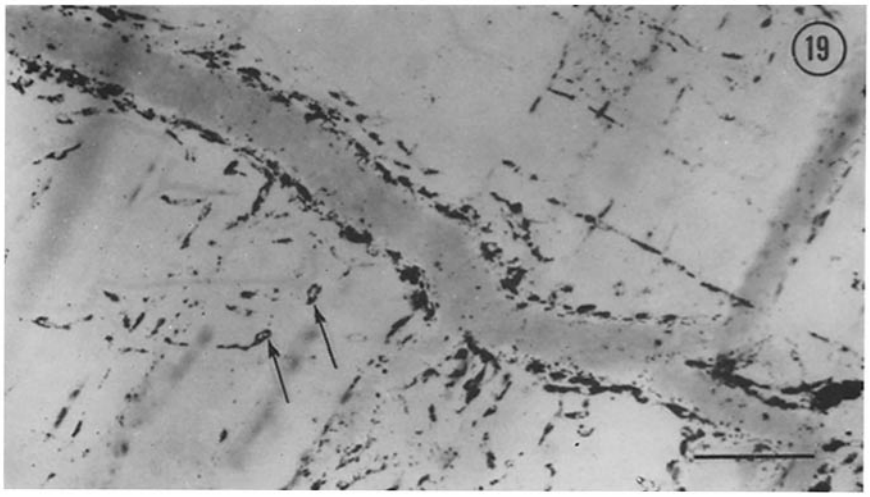
One cannot avoid some surprise at the observation that two dissimilar agents should bring about—in our experimental system—the same, very specific effect. This leads one, of course, to wonder whether there may not be some common underlying mechanism. According to Woolley, serotonin acts—at the cellular level—by combining with calcium ions and an acceptor substance (30, 31). At present there is little ground for further speculation.

FIGURES 17 TO 19

Further changes in the carbon deposits of the labeled venules; stages of 1, 3, and 10 months, respectively. The structures indicated by arrows represent phagocytic cells. Scale = 100 micra. × 160.


17


18


19

CONCLUDING REMARKS

An objection which could possibly be raised to our experiments is that the doses of histamine and serotonin, however small, could still be high in relation to the amount presumably released as a result of local injury. We have good reason to believe, however, that our results cannot be ascribed to "toxic" levels of the drugs. It is especially instructive to examine the periphery of the injection site, where the drugs are diluted to the minimum dose capable of inducing vascular leakage. Here the elective sensitivity of the venules is particularly obvious.

On the other hand, it should be expected that with very high, toxic concentrations, not only venules but also other vessels might be injured in a non-specific manner. We found evidence of this type of injury particularly after the injection of serotonin. In the very center of some preparations, where the cremaster had been exposed to the highest concentration of the drug, it was occasionally possible to observe very fine blackened vessels in the typical ladder pattern of the finest capillaries. It seems reasonable to assume that the effect of the drug, at this site, had been of a more drastic, less specific nature; the selective and more "physiological" effect being exerted in the surrounding area. This observation indicates that two types of vascular lesions should be considered; the specific ones, due to pharmacologic effects of mediators, and non-specific ones due to general cell damage. This problem will be dealt with in the next paper of this series.

We have applied the principle of vascular labeling, with carbon, to tissues other than the cremaster (fascia of the abdominal muscles, adipose tissue, mesentery, intestinal wall, testis, eyelid) and in animals other than the rat (mouse, hamster, guinea pig, rabbit, cat, and dog). Histamine was used in all these situations, serotonin in some. The elective sensitivity of the venular tree was confirmed without exception. We therefore have a certain degree of confidence in stating that this is, in all likelihood, a general phenomenon. This conclusion does not necessarily contradict the electron microscopic studies of Alksne (5), who also studied the effect of histamine on vascular permeability, using mouse skin as test tissue. Alksne described endothelial leaks in "capillaries," but did not specify the diameter of the leaking vessels. From three of his illustrations, however (nos. 3, 4, 10), the caliber of the leaking vessel can be estimated, and in each case it falls within the range of a collecting vessel or venule, rather than the terminal capillary network (*e.g.*, about 11 μ in Fig. 3). Alksne noticed, furthermore, that "not all capillaries" were affected, and suggested that this may have depended on a different functional state. It seems more likely that the "unaffected capillaries" may have been the finest. It is worth noting at this point that the vascularization of the skin represents a special case, in that the great majority of the small vessels are venules (32, 33, 13).

If then it is true that two of the major chemical mediators of inflammation, histamine and serotonin, spare the finest vessels (those which are undoubtedly capillaries, whatever definition one may follow) and act selectively on the venular tree, then one may legitimately wonder how these substances could have become almost synonymous with capillary poisons. This is particularly the case with histamine, the oldest and best established of the permeability-increasing factors. In his monograph on histamine, for instance, Rocha e Silva (34) states that histamine increases the permeability of the capillaries, and cites evidence in support. It is quite clear, however, that the arguments advanced do not allow one to distinguish capillaries from other vessels. The local *leakage of dye*, and the increase of *lymph flow*, are, of course, inadequate. The development of *stasis* in the capillaries and venules (stasis is here used in the sense of packing of red blood cells (35)) is often quoted as evidence of a capillary lesion (34). Actually, if stasis appeared first in the venules because of local leaks, it should necessarily develop as a secondary event in the capillaries. As for an *increase in diameter* (the "dilatation of capillaries" so often mentioned by pathologists), careful studies of intact tissues have failed to produce convincing evidence that histamine induces a significant dilatation of the capillary network. Vasodilatation does occur, but in vessels of larger caliber; the terminal capillary network is notable for its more complete injection with blood (36–39). The most logical conclusion seems to be that the dilated capillaries of traditional fame are actually venules filled with stagnant blood. This is quite apparent in some of the published illustrations which are labeled "dilated capillaries": the number of endothelial nuclei or of red blood cells in cross-

section clearly betray the vessel as a venule (*e.g.*, Fig. 2 in Menkin (40)).

In closing, it should be mentioned that all topographic studies of the finer vessels are hampered by lack of a rigorous terminology; that is, lack of generally recognized morphologic or functional definitions for each vascular segment. By accumulating more findings such as here presented—the elective sensitivity to chemical agents—it may become possible, in the future, to define more precisely those elusive entities which Lewis (33) felt obliged to group under the noncommittal term of "minute vessels."

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