THE GRADIENT OF VASCULAR PERMEABILITY

III. The Gradient Along the Capillaries and Venules of Frog Skin

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PLATES 11 TO 14

In previous papers (1, 2) experiments have been described which prove that the permeability of the muscle capillaries of mammals and frogs increases progressively along these vessels, being greatest in the region where they join the venules. The present communication deals with the conditions in frog skin.

The capillaries of the frog tongue, web, and mesentery have become classical objects of study while those of the skin of the body are employed but seldom. Yet in some species the skin offers opportunities beyond those of any of the structures mentioned. To watch tongue and mesentery they must of necessity be exposed and pinned out, subjected to more or less trauma, and kept wet with a drip, which differs from frog lymph (3). In the web two skin layers come together with result in complicated vascular pictures, and the circulation is far more frequently irregular than over the body. We have judged permeability by the escape of dyes from the blood. In the web and mesentery there is almost no interstitial tissue to stain, or to serve as a ground upon which the spread of dyes can be perceived. In the skin of the body on the other hand the capillaries ramify in a transparent tissue overlying a dense, grayish white substratum against which every least escape of dye is visible.

The Vessels Studied

The skin of *Rana pipiens* and *Rana clamitans* presents the general features (Figs. 1, 2, 3, and 9) described for European frogs (4). The transparent epithelium, pierced here and there by gland outlets, covers a loose-textured stratum spongiosum of corium wherein lie pear shaped glands. In *Rana clamitans* the spongiosum is so transparent, where it lacks granule cells, that the pitted surface of the underlying "sieve layer" of the stratum compactum can be closely inspected;

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but in *Rana pipiens* one sees it through a grayish mist. The compactum in both species is compact indeed, an opaque sheet with very numerous foramina, through which blood vessels, nerves, and lymphatics run vertically to reach the surface. Beneath it is a thin tela subcutanea, in which course the parent vessels. The tela is lined with endothelium on the side toward the lymph sac. The venules and arterioles emerging on the outer surface of the compactum connect with a continuous network of capillaries lying just beneath the skin epithelium (Figs. 1 and 3). It is with the escape of dye from this network that we have concerned ourselves; for deeper in the spongiosum there are no capillaries. The compactum is without blood or lymph vessels of its own (4) and it appears wholly unstained.

General Technique

The passage of dyes from the blood has been followed with the same apparatus as in previous work (1). Most of the frogs had been immobilized with curare, or etherized, or.pithed; but some few have been held and studied while unanesthetized. Etherization and pithing cause the circulation to become relatively sluggish, while, furthermore, reflex movements often complicate the observations; and hence we have relied largely on curarized frogs (0.1 cc. of 1 per cent curare into a dorsal lymph sac). As soon as the animal becomes flaccid it is laid upon wet filter paper and kept moist with water, or, more frequently, it is just submerged in a shallow dish with a paraffin bottom. Adolph (5) has shown that practically no fluid exchange takes place through the water-bathed skin of normal frogs. A region is selected for observation where the capillary rete is everywhere visible, by reason of the flowing blood, with its arterial and venous relationships clearly distinguishable. The blood stands out brilliantly in light passed through a methylene blue solution, and the spread of red dyes can be best followed under such conditions. With purple and blue dyes no such aid is needed. Prior to the injection the field is sketched, and while it is watched under the binocular microscope the dye is introduced into a vena dorsalis pedis. To immobilize the foot, it is pinned through the web upon a cork sunk at the corner of the dish.

The same dyes have been used as previously (2). All were carefully purified and tested upon mice for toxicity. They were in watery solution isotonic with frog blood (that is to say with 0.7 per cent NaCl), the amount given being most often 0.1–0.2 cc. for a frog of 30 gm.; though with substances of relatively poor tinctorial qualities, and larger frogs, as much as 0.35 cc. was sometimes required. The poorly diffusible dyes were injected slowly, in the course of a minute or slightly less, and highly diffusible ones within a few seconds.

The fact that frog capillaries let through a part of the plasma proteins (3, 6) led us to try erythrolitmin, the pigment of red cabbage, and methemoglobin (of the horse). The first two proved toxic intravenously, and methemoglobin failed to pass into the tissues in distinguishable amount. The ferrocyanide method of Weed yielded uninformative pictures owing to secondary extravascular spread of the reagents.

Vascularization of the Skin of the Belly of Rana pipiens

The smooth, white skin over the lower middle of the abdomen of a 30 gm. *Rana pipiens* is almost ideal for study. It is practically devoid of papillae, contains relatively few glands, and the capillary meshwork has a strikingly regular pattern (Fig. 1).

In the gross the belly skin appears shining white because of numerous "interference cells" just beneath the epithelium (Fig. 12), containing granules which reflect light. Occasionally they form an almost continuous sheet and the frog must be discarded; but usually they do not obscure the spread of dye from the vessels. While lying at the level of the capillary rete they have no evident relation to it; and they seldom encroach upon the outline of one of its meshes but serve instead to enhance its brilliancy by contrast. Occasional small areas are wholly free from them.

Venules and arterioles connect with the capillary network separately at fairly regular intervals. The meshes of the latter converge to the venules as in the liver, but they radiate correspondingly from the arterioles as well. The latter open directly into the rete, the result being that one sees the blood spray out in all directions, as from unseen fountains, in continuous non-pulsatile streams which pursue a zig-zag, helter-skelter course in the meshwork until they reach the venules. Some few of these are incorporated in the rete and course for a little distance at its level, receiving capillaries until they dip from sight (Fig. 1). The emergent arterioles are definitely narrower than the capillaries they feed. In vigorous frogs the blood current lags but little until the venules are reached.

The distance from venule to venule is usually 1 to 1½ mm., and from arteriole to venule somewhat more than half this distance. The lozenge-shaped meshes of the capillary network measure 1/16 to 1/8 mm. in greatest diameter.

The capillaries appear larger near the vein. This is mostly the result of the brighter hue of the blood as it becomes oxygenated during its progress within the rete (7). The influence of the factor can be readily appraised by injecting a dye which stains the plasma deeply and does not soon escape from it. Then the rete stands out like a drawing in colored ink and one perceives that the local differences in calibre are exceedingly slight, though some capillaries become a little wider near the venules (Fig. 1). It is not difficult to find regions where the difference is negligible, and ordinarily we have chosen such for study. Marked enlargement of the blood channels begins with the venules.

The Escape of Dyes into the Skin of the Belly

Our first observations were made in late April upon pithed or curarized frogs.

A 4 per cent trypan blue solution in twice distilled water was injected intravenously, 0.2 cc. for a frog of 23–31 gm., in 45 seconds to 1½ minutes. As the blood

became stained the vessels stood out sharply in blackish blue, and within a minute and a half, all told, the blue stain could be seen emerging, first where the capillaries entered the venules, and soon all along the further half of the capillary rete, in increasing amount as the venules were neared. Very quickly the meshes in this region became blurred with blue, the extravascular color spread laterally and became confluent, and within less than another minute the further half of the meshwork was wholly obscured by the diffuse, deep staining. While this happened the blood lost color, and in consequence the proximal portion of the capillary rete stood forth less sharply. Within 3 minutes after injection the distribution of stain from the skin vessels had to all intents and purposes ceased. The deepest color lay about the venous centers, shading off in the direction of the arterioles, rather abruptly ceasing about midway to them. In the gross the skin was now brilliantly speckled with blue dots on white. The dots were discrete, less than 1 mm. across, on a practically unstained, white ground. The fact was plain that the dye had got out so fast from the further capillary meshes that the blood was practically rid of it before any escape had taken place from the proximal ones.

These findings with trypan blue have been confirmed and extended with dyes of greater diffusibility (patent blue V, brom phenol blue, eosin, trypan red), and with the more poorly diffusible Chicago blue 6B, pontamine sky blue, and Congo red. The red dyes yield the best photographs (Figs. 4, 5, 6, 7, and 8). All escape much more rapidly from frog vessels than from those of rabbits. Phenol red, the most diffusible of our test substances, leaves frog vessels with such extraordinary swiftness, when the circulation is vigorous, that one cannot hope to perceive local differences in the rate of its distribution.

SPECIMEN PROTOCOLS

Phenol Red.—A curarized *Rana pipiens* of 23 gm. received 0.15 cc. of 2.9 per cent phenol red in 9 seconds. 1 second before the end of the injection the rete suddenly stood out in rose color,* and forthwith the dye escaped into the tissues everywhere along the vessels, the extravascular color rapidly becoming confluent by lateral spread.

Patent Blue V.—This dye is little less diffusible than phenol red (1). A *Rana pipiens* of 36 gm. was curarized and 0.1 cc. of 5.8 per cent patent blue V injected intravenously in 17 seconds. Only at the end of this time did the rete stand out in blue. The capillaries in the field drawn were somewhat larger in the neighborhood of the venules.

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* The tissues of the frog are distinctly alkaline, as compared with those of mammals.

32–42 sec.—The dye can be seen to pass out from the venules of the rete and the adjoining portion of the capillaries, from the venules first and most intensely. 1 min. 9 sec.—The stain has now escaped everywhere along the capillary network, grading off in the direction of the arterioles. Round where they join the rete the hue is notably pale; but the extravascular dye is spreading rapidly. In the pale regions "interference cells" are no more frequent than elsewhere. 2 min. 11 sec.— Circulation continues excellent. The rete can still be seen in the region of the arterioles, but everywhere further on it is blurred by stain. 2 min. 20 sec.—The coloration has become diffuse; local differences are no longer perceptible.

Brom phenol blue diffuses more slowly than patent blue but far faster than the trypan dyes. A *Rana pipiens* of 26 gm. was curarized and a spot selected for observation where the capillary meshes were everywhere of the same calibre. "Interference cells" were unusually few. An injection of 0.2 cc. of 2.7 per cent brom phenol blue was given in 29½ seconds.

Stained blood outlined the rete within the first 14 seconds; after 27 seconds the dye was observed to be coming out from the further portion of the network; and in another second from it everywhere. After 35 seconds altogether the dye was escaping abundantly along the entire course of the vessels, though still in greatest quantity near the venules. After 45 seconds the network was no longer visible, so deep was the coloration—a brilliant, dark, purply blue. The swift circulation could be discriminated with difficulty. After 2 minutes and 55 seconds the skin staining had become general.

Eosin.—A curarized *Rana pipiens* of 28 gm. was injected in 22 seconds with 0.25 cc. of 4.3 per cent eosin. The blood flow did not slacken locally, yet the rete only became outlined in color after 1 minute and 24 seconds in all. 25 seconds later the stain had appeared in the tissue about the further capillary meshwork. After 3 minutes in all the plasma was so far decolorized that moving corpuscles could be seen. There was still no staining round where the arterioles gave into the network, though elsewhere the color had spread laterally and was becoming diffuse. The hue was most intense where the collecting venules dipped down. After 4 minutes and 15 seconds the staining was diffuse, though still with pale spots where arteriolar blood entered the network. After 5 minutes and 15 seconds the skin was brilliantly and evenly red.

Trypan Red and Chicago Blue 6B.—The work with these substances finds place further on.

Congo red is the most indiffusible dye that has yielded positive findings.* A

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* The difficulties of ascertaining the molecular weight of Congo red have become classical (Freundlich, H., Kapillarchemie, Akademische Verlagsgesellschaft, Leipzig, 1923, 766). The results of freezing point determinations upon solutions of the dye in water give almost no indication of what its state will be when in plasma. There is much in the literature to suggest that the same holds true of many other dyes, if to a less extent.

The Congo red of the present work (Grübler, Dr. K. Hollborn) was dissolved in

curarized *Rana pipiens* of 34 gm. was injected with 0.2 cc. of 10 per cent Congo red in 1 minute and 6 seconds. The rete stood out in deep red after 22 seconds, but only after 5 minutes was extravascular dye noted, first in the tissue around the superficial venules, and after another minute about the adjoining capillary meshes. At the end of 13 minutes the staining was still pale and localized, none having occurred along the proximal half of the rete. The circulation was now sluggish, with contraction and emptying of some of the capillaries. Most of the dye had been lost from the blood, the integument of the thighs being deeply stained.

In numerous other experiments like phenomena were observed. Always, save in the case of phenol red, one could perceive that the rate at which dye escaped into the tissues, as well as the quantity, increased progressively along the capillary way from arteriole to superficial venule, reaching a maximum about the latter. Poorly diffusible dyes passed into the skin only from the venules and the adjoining capillary meshes during the considerable period before the blood was depleted of them.

Periglandular Staining

A distinctively great permeability of the capillaries which encircle the skin glands was noted frequently under conditions which suggested that functional activity might be its determining cause. The staining calls for description because of the complication it offered. Its physiological implications will be dealt with in a later paper.

The glands of the abdomen of *Rana pipiens* are pear-shaped structures in the stratum spongiosum (Figs. 11 and 13). Most are surrounded at the neck by a mesh of the capillary rete which differs from the others in being circular, slightly smaller, and with a somewhat narrower vascular lumen (Figs. 1 and 6). No glands are found immediately next the emergent arterioles or the superficial venules, but they are scattered throughout the rete elsewhere in no evident relation to the vascular pattern as a whole. Nearly all are of one sort (mucous glands). They consist of a single layer of epithelium which changes in appearance with the secretory changes, as in European frogs. In our first experiments, conducted in the early spring upon animals that had been kept in the ice box, periglandular

water, precipitated by the gradual addition of concentrated hydrochloric acid, washed on a filter paper, dissolved again by the very gradual addition of sodium hydroxide solution, using the least amount necessary for the purpose, and allowed to crystallize out. A 2 per cent solution of the material thus prepared had a Δ of 0.075°C.

staining did not attract attention. But in the later work, carried on in an especially warm May and June with frogs that had lived at room temperature, it frequently dominated the picture. Many individuals were still encountered, however, in which this was not the case. In frogs showing a special staining about the glands the outline of some or all of the encircling capillary rings became blurred by escaping dye before any could be seen elsewhere (Fig. 5); and the color, rapidly becoming confluent, gave rise to irregular patchings (Fig. 6) which in the gross differed strikingly from the ordinary dotting with color. The gland itself seldom stained, but was distinguishable by a collar of dye. Even with phenol red a special periglandular escape of dye was not infrequently perceptible during the instant before the skin flushed rose everywhere; and with poorly diffusible substances (Chicago blue 6B and Congo red) sometimes the only coloration prior to effective depletion of the blood occurred from the gland circlets. In not a few experiments the periglandular escape, like that from the meshwork generally, took place first and was greatest in the further capillary region.

Numerous tests have been made, with Chicago blue, Congo red, and trypan blue, upon the influence of the circulating amount of dye on periglandular staining. These leave no doubt that the smaller this amount the more marked is such staining as compared with that of the skin generally. For example, with large doses of Chicago blue 6B in frogs submerged at 16°C. we observed not merely a staining from the capillaries about glands but everywhere from the vessels of the further portion of the capillary rete, whereas with a small dose the only extravascular coloration was about the glands.

The experiments on periglandular staining illustrate a rule of practical importance in the study of regions where vascular permeability is relatively poor, as it is in the muscles, namely that a great deal of dye must be put in circulation, or one very highly diffusible, else all will get out of the blood into certain organs or regions before any has escaped elsewhere.

The Escape into the Skin of the Thighs

Near the crotch the thigh skin of *Rana pipiens* is rugose, with large papillae. Most of the arterial capillaries emerge at the summit of these latter—where "interference cells" are often so many as to mask any staining; and the blood streams in a close-meshed rete down their sides to reach venules that dip under in the valleys. Here and there, however, the situation is reversed, with result in an opportunity for enlightening comparisons. Under such circumstances the arterial capillaries emerge in the valley and the blood mounts the side of a papilla to vanish into a venule near its summit. Generally speaking the width of the capillaries increases considerably as the venule is neared.

The escape of highly diffusible dyes takes place so quickly into the skin of the upper, inner surface of the thighs that with them there is not time for local differences of distribution to be perceptible. However, with Chicago blue and Congo red a progressively increasing escape along the capillary way is readily to be noted. With the highly indiffusible Congo red it is attested in the gross by a mottling of ruddy dots on a white ground, each dot with a venule at its center.

The Skin Circulation of Rana clamitans

The rete on the abdomen of *Rana clamitans* is not plainly to be seen. On the upper, under surface of the thighs of females, however, there are patches like pellucid lagoons in a skin tinselled for the rest with "interference cells" or blackened by pigment cells. One peers into these lagoons as into water and sees the blood hurrying through the narrow meshes of a network of vessels that are themselves invisible. An especially large area of the sort is usually to be found just below the groin.

Through the skin the stratum compactum lies bare to view as an opaque, light gray sheet pitted by numerous funnel shaped orifices through some of which the vessels rise or disappear, not nearly filling them. Injections of gelatin-carmine and India ink masses into blood and lymph vessels, respectively, show most of the foramina to be pierced by a lymphatic only, the proportion with a blood vessel being the same as during life in warm weather. The compactum is often ridged slightly where the larger vessels run beneath it in the tela, and looking along the line of these ridges in the living frog one sees arterioles emerging at intervals or venules dipping out of sight. They pierce the compactum separately, as in European frogs (4). Only once have we seen venule and arteriole occupying the same foramen.

The arrangement of the superficial capillary network differs greatly from that in *pipiens* (Figs. 2 and 9). The meshes are larger, elongated or lozenge-shaped, 1/8 to 1/4 mm. in width, by several times this length often. Each encloses one or more skin glands, a fact which becomes evident only after introduction of a dye. There is less radiating from arterioles and converging upon venules than in *pipiens*, these vessels joining the network at very irregular intervals, often several of the same sort close together (Fig. 9). The blood may follow a tortuous course in the network or may flow almost straight, according to local conditions, the distance traversed to the venule being seldom as much as 1 mm., often far less because an arteriole emerges and venule dives down through adjacent foramina. Near the arterioles flow is relatively brisk. Not a few venules run for a little distance as part of the network, growing larger as new capillaries enter them, while others turn abruptly down. Those first mentioned may become three times as wide as capillaries while still a part of the rete. Under the circumstances of our experi-

ments, the entire capillary network seemed to be utilized by the blood stream. We never observed new channels to open even when considerable quantities of fluid had been given intravenously.

From this description it is plain that the almost diagrammatic distribution of dyes occurring in *pipiens* cannot be expected in *clamitans*. Nor is it found. But on the other hand the size of the vascular meshes and the transparency of the tissues enable one to watch closely the escape of dyes along individual channels. The course of flow shifts suddenly at times, but far less often than might be expected from the many pathways. Occasionally a circulation round and round a single mesh is observed, owing to tangential entrance of the current of blood. It is easy to select for observation capillary channels of the same width all the way to the venule (Fig. 9), and thus to rule out changes in area of permeable wall as affecting dye distribution. We have ordinarily chosen fields where the distance to be covered between arteriole and venule was a long one.

Escape of Dyes into the Skin of Rana clamitans

The findings in *Rana pipiens* have been confirmed and extended in *Rana clamitans*. In *clamitans* staining takes place somewhat the more slowly, a gradient of distribution being demonstrable even with phenol red. Only very exceptionally does a special escape of dye occur about individual glands.

SPECIMEN PROTOCOLS

Phenol Red.—A curarized *Rana clamitans* of 60 gm. was injected with 0.3 cc. of 4 per cent phenol red solution in 35 seconds. Several emergent arterioles were visible in the region selected and drawn, with distant venules that collected the blood from a capillary rete having no intermediate connections with the underlying tissue.

Within 16 seconds after the injection was begun the rete stood out brilliantly in red. After 52 seconds extravascular dye was noted around the surface venules, and in another 16 seconds about the neighboring meshes of the capillary rete itself. The circulation continued rapid. At the end of 2 minutes and 8 seconds in all, the dye was spreading laterally from next the vessels. After 2 minutes and 20 seconds some was present outside the walls of the capillary network everywhere, but the intensest staining was still about the venules, the color grading off in the direction of the arterioles. Even after 2 minutes and 38 seconds no diffusion had occurred around the latter. After 4 minutes and 12 seconds a general red staining had nearly obscured the vessels, yet after 7 minutes and 56 seconds the fact could still be ascertained that wherever there was a deeper patch of color a venule lay in its midst, and wherever an especially pale one arteriolar capillaries. The frog was now brilliant red wherever the corium could be seen.

In this experiment the phenol red was isotonic with mammalian blood; but its amount was so small that the hypertonicity for the frog could be neglected.

Patent Blue V.—A curarized *Rana clamitans* of 40 gm. was injected in 10 seconds with 0.15 cc. of 6 per cent patent blue V. The rete stood forth in blue, and within 25 seconds the dye could be seen emerging from venules and venous capillaries. After 50 seconds the tissue supplied from the proximal (arteriolar) half of the meshwork was alone free from stain; and after 2 minutes it was coming out here also, but in slighter quantity. A graded increase in intensity and amount of the staining now existed all along the capillary way. After 2½ minutes the whole spongiosum was diffusely blue because of secondary distribution of the dye, yet the regions where arterial blood entered the capillary web were paler than elsewhere. The glands were now plainly outlined because unstained.

4 min. 28 sec.—The diffuse coloration remains more intense in the further portion of the capillary rete. The color is deepest where the veins dip down. 5 min. 8 sec.—Circulation continues excellent. 6 min. 50 sec.—The whole spongiosum is like a blue jelly, least tinted around the emergent vessels.

Trypan Blue.—A curarized *Rana clamitans* of 62 gm. was injected with 0.3 cc. of 2.7 per cent trypan blue in 1 minute and 13 seconds. At the end of the first 38 seconds the rete was brilliantly blue, but not until after 2 minutes and 25 seconds in all did escaping dye begin to blur the vessels, first the superficial venules, then the neighboring meshes of the rete. At the end of 4 minutes and 30 seconds extravascular trypan blue was present everywhere along the latter except in the immediate neighborhood of the arterioles. Like other dyes it was seen to escape from the blood as a spreading fuzz of color, broadest and most pronounced next the superficial venules, thence gradually narrowing and paling in the direction of the arterioles.

5 min. 52 sec.—Still no staining where the arterioles join the rete. 6 min. 57 sec.—The dye has almost all left the blood, which is circulating briskly. 8 min. 12 sec.—Wherever one finds no staining in the region where a vessel sends blood into the rete, that vessel can be identified as an arteriole.

Chicago Blue 6B.—A curarized *Rana clamitans* of 56 gm. was injected with 0.3 cc. of 5.2 per cent Chicago blue 6B in the course of 1 minute and 5 seconds. After 28 seconds the vascular rete blued, and within 1 minute and 28 seconds the blue could be seen passing out into the tissue next the superficial venules, and in a few more seconds into that about the neighboring capillary meshes. During the first 2 minutes no spread took place from the portion of the rete nearer the arteriole.

3 min. 25 sec.—The dye has now passed out all along the capillary way except from those meshes into which the arteriolar blood directly pours, and it has obscured much of the network. The tissue supplied from the meshes nearest the arterioles attracts attention by reason of its lack of color. 8 min.—The picture is essentially unchanged. 11 min. 50 sec.—Still no staining where the arterioles join the network. A slow, secondary spread of color through the tissue is taking place. The glands begin to show brilliantly because unstained in a stained matrix.

The dyes regularly appeared first and in greatest quantity outside those collecting venules which formed part of the superficial vascular network, and later and in less amount from its capillary meshes. In the case of highly diffusible dyes staining occurred from the meshwork everywhere, though with progressively increasing rapidity and intensity along the way from arteriole to venule. The more indiffusible the dye the more did its escape tend to be limited to the venule and the furthest capillary region, and the slower was its spread both from the blood and through the tissues secondarily.

The Staining with Trypan Red

Landis has studied the escape of trypan red (vital red HR) from individual capillaries of the frog mesentery (8). He states that during the initial period of staining the dye frequently "did not come out equally along the entire length of any one capillary, but in greater amount outside the arterial portion of the vessel." Because of his findings we have made many tests with trypan red, which is excellently tolerated. Landis injected a saturated solution (quantity not mentioned). We find that 8.25 per cent of the pure Grübler preparation (Dr. Karl Hollborn) provides a saturated solution at room temperature, but that 2.8 per cent is isotonic with frog blood. A 2.8 per cent solution has been used in our work.

When 0.25 cc. of a 2.8 per cent solution is injected in the course of about a minute into a curarized 50 gm. *Rana clamitans*, the capillary rete stands out brilliantly; and by the time the injection is ended escape of the dye has begun. Always, when the circulation is brisk, it can be seen to emerge first from the collecting venules incorporated in the rete, and after a few seconds more from the further portion of the capillary meshwork everywhere, but in gradually lessening quantity toward the arterioles. The staining in the further capillary region becomes intense and confluent after a few minutes owing to lateral spread from the meshes, but round about where the arteriolar blood enters the network the tissue is still wholly devoid of color. The plasma has now paled so greatly that no further escape of dye can be expected here. Nor does it occur. For a long while the meshes receiving arterial blood can be discriminated by the pallor of the tissue in which they are situated. Even after an hour the skin is still stippled in the gross with red dots where lie those meshes adjacent to a venule.

The rate of this succession of events varied in the individual case. The dye escaped in a ragged fuzz or fringe, first about the superficial

venules, and then from the neighboring capillary meshes in an abundance that lessened progressively in the direction of the arterioles. The regions where these vessels joined the rete remained completely unstained. Similar findings were obtained in *Rana pipiens*, the distribution being even more regular (Figs. 4, 7, and 8). With large amounts of dye local differences in the staining were emphasized and the amount of unstained tissue reduced. With small amounts on the other hand, the coloration was an even one, for reasons already dealt with (1).

The findings were so contrary to those of Landis' that we have repeated his observations upon the mesentery.

This was done upon *Rana pipiens*, a species Landis employed. We have made several important deviations from his technique, while carefully following it in other respects. One was the utilization of isotonic dye solution, another the injection of the dye immediately after the field of study had been exposed and hastily sketched. The field was irrigated with oxygenated Ringer's solution at pH 8.2, and all animals were discarded in which the local circulation was not brisk. Landis did not inject the dye until after the mesentery had been in contact with Ringer's for an hour or two; and he noted that then the small vessels were in various stages of dilatation and secondary constriction. In view of his further observation that dyes pass with extreme rapidity through the capillary walls of dead or moribund animals such a wait seems hazardous. Landis observed the course of events along a single capillary whereas we have noted it everywhere in a low power field. Special care was taken to avoid pressure on the mesenteric veins.

Not only trypan red, but brom phenol blue, trypan blue, and Chicago blue 6B have been used. The findings were consistent. Save in some enlightening special instances, to be gone into later, the dyes have regularly escaped first from the distal portion of the capillaries and the smallest venules. Often none was seen to get out anywhere else. The extravascular dye was not washed away by the Ringer's as it emerged but was visible within the mesentery as a brilliant fuzz of color about the vessels, which spread away from these, not along them.

The escape of stain in rapid and disorderly fashion from isolated capillary segments was frequently noted; and when the mesentery had been irrigated for an hour prior to the injection this was especially frequent. The vascular conditions were then obviously abnormal. In an animal injected with brom phenol blue,—which escapes into the tissues with great ease,—a dense, blue staining took place from the arteriolar capillaries as these were entered and so much dye passed out here that the blood further on was never more than pale blue. Under such circumstances there was no chance for the usual gradient of distribution to assert itself. In other instances, as well, an escape of brom phenol blue took place from the proximal portion of the capillaries as the dye reached them but that from their further regions soon came to predominate.

The arrangement of the mesenteric capillaries is so irregular that in experiments such as the foregoing one could not hope to obtain a gross color pattern like that which in muscle and skin provides statistical evidence of a gradient of distribution along the capillaries; but the gradient was so marked that its existence was readily discernible under the microscope. From the venules the dyes seemed to get out a little more slowly than from the adjoining portions of the capillaries.

A relatively abundant escape from the arteriolar region of the capillaries was always traceable either to great diffusibility of the dye or to a damaged capillary system. Landis states that secondarily there occurred in his preparations "a rapid vital staining of the connective tissue around the smaller venous capillaries and particularly the smallest venules." Since this did not happen until 10 minutes after the introduction of dye, its relation to the gradient of distribution that we have studied is problematic. In view of Conklin's recent demonstration (9) that frog capillaries flushed with Ringer's solution become abnormally permeable, it has seemed unnecessary to repeat Landis' perfusion experiments with Ringer's containing various dyes.

Some irregular phenomena observed during the study of the skin have pertinence at this juncture.

In a thin *Rana pipiens* of 35 gm., the circulation over the belly was sluggish and irregular, with areas of stasis here and there. 0.25 cc. of trypan red solution was injected. It appeared in the tissues around some arterioles giving into the rete before the stained blood had had time to traverse its meshes, but after this had happened dye got out very rapidly from its further portion, especially from the venules, and by the end of 2½ minutes the characteristic, graded staining indicative of the gradient had developed. Here and there, however, were small, persisting patches of deeper red, testifying to an especially abundant escape of dye in certain arteriolar regions.

After the injection of eosin into a *Rana clamitans* with good circulation the dye was seen to escape from a few of the emergent arterioles and from the neighboring portions of the capillary meshwork when none had as yet passed out further on, though stained blood had reached the venules. The dye came out from one side of some vessels only, like an ecchymosis.

Similar abnormalities were sometimes encountered in the staining of frog muscle (2).

In a *Rana pipiens* having but a sluggish flow through the muscle the blood stained with pontamine sky blue could be seen to enter and pass through the exposed sartorius with extreme slowness, getting out as it did so from the arteriolar

portions of some capillaries and a little later escaping in a blue fringe here and there irregularly from certain segments only of these latter. Soon though, escape in the further capillary region preponderated over that elsewhere, and the colored barring developed which is characteristic of the gradient of distribution.

Influence of the Local Vascular State

On the way from skin arteriole to venule the blood passes through sections of a greater or less number of the meshes which make up the superficial rete. One can readily see in *Rana clamitans*, especially after the injection of a poorly diffusible dye, that some portions of the individual capillary mesh are not infrequently narrow whereas others are broad. Such dyes often escape from the broad portion of the mesh before they do from the narrow, although the blood flows briskly through both. This fact could sometimes be discerned even with trypan blue and trypan red, it frequently could with Chicago blue 6B, and most often and best with pontamine sky blue. The passage outwards of this last was limited almost entirely to the superficial venules and the adjoining meshes of the capillary network during the 15 or more minutes following its injection, and it spread very slowly from next these vessels; but the pattern was frequently confused by a deep staining here and there from capillary segments of distinctively large calibre.

Occasionally in *Rana clamitans* one saw dye passing out from certain portions of the linkage of meshes constituting the vascular way, and not from others, irrespective of their place with relation to arteriole and venule, and despite an active blood flow through them all, and a generally even calibre. The interpretation of such instances cannot be attempted.

All in all, the rule seemed to hold that wherever the capillary was wider, other things being equal, more dye escaped through its wall. There existed, of course, more surface to escape through, while furthermore dilatation is known to render capillaries especially permeable (10). The distribution of poorly diffusible dyes was far more evidently conditioned by local vascular differences than that of highly diffusible ones.

The Structure of the Skin

The tissue into which our dyes escaped has a simple histology.

The skin of *Rana clamitans* (Figs. 10, 14, and 15) is considerably less than 250μ in depth. The vessels coursing in the thin tela subcutanea pierce the stratum compactum perpendicularly. This dense and relatively thick layer of corium contains few cells. The spongiosum and epithelium overlying it are together only between 50 and 80μ deep in the regions where the escape of dyes is best watched. The epithelium occupies about 30μ of this total. The glands lying in the spongiosum are simple, flask shaped structures, with their bases in shallow depressions in the compactum. The spongiosum itself is a very loose connective tissue containing many star and spindle shaped cells and fibrillae.

The arterioles piercing the compactum to join the capillary rete have walls but one cell thick in cross-section (Figs. 14 and 15), like the capillary rete itself. Injections under pressure of carmine mass or India ink into a subcutaneous lymph sac of the leg disclose an abundant, closely anastomosing meshwork of broad lymphatics immediately beneath the rete, and paralleling it. The channels appear huge as compared with those for the blood, and their pattern has no evident relation to that of the capillary rete. Each of the foramina in the stratum compactum, including those occupied by a blood vessel, contains a lymphatic connecting with the meshwork. Langer long ago noted all this of European frogs (11).

The skin of the abdomen of *Rana pipiens* (Figs. 11, 12, and 13) is thicker, sometimes measuring 350μ, and it has a thicker epithelium and glands far more widely separated. The spongiosum is from $60–120\mu$ deep, on the average about 80μ. The layer of "interference cells" is situated at the same level as the capillary rete (Fig. 13), and the escape of dyes must be followed in regions where these cells are scattered evenly, else erroneous inferences may be drawn, since they do not stain. Where they are numerous the region remains white. The glands are of two sorts, ordinary mucous glands and "granule" glands, the latter sometimes as much as $1\tfrac{1}{2}$ mm. or more apart. The flask-shaped mucous glands are fairly numerous. They occupy the superficial regions of the spongiosum. The connective tissue of the spongiosum is loose and has the same general make-up as in *clamitans*, and the stratum compactum shows but a poorly defined "sieve layer." The tela subcutanea is thicker than in *clamitans*, with a dense stratum of "interference cells," and the walls of its arteries and veins show a muscle layer. This extends to the arterioles of the compactum, but the venules piercing the latter show only endothelium, like the superficial collecting venules with which they connect. The arterioles emerging into the spongiosum appear, in our preparations, to be walled by a single layer of cells only; but we have made no effort to demonstrate Rouget cells.

On the inner side of the thigh of *pipiens* near the crotch the skin is raised into papillae, the epithelium is thicker than on the abdomen, and large glands sit side by side, with the capillary rete running in a close, regular meshwork over their

shoulders. Here an occasional arteriole with a definite muscle layer has been observed in the spongiosum. The other vessels of this layer appear to be walled only by endothelium.

Scrutiny of the Findings

Do the dyes actually escape where first seen? Direct observation leaves no doubt of this. When an intense stain of appropriate diffusibility has been thrown into circulation one can watch it extend into the tissue from the blood of the least venules and the neighboring capillary meshes, at first emphasizing and broadening their outline (Figs. 4, 5, and 8) and then encasing them in a brilliant fringe or fuzzy sheath of color which, deepening, soon obscures them. Meanwhile no dye leaves the blood in the proximal capillary region. Its secondary, extravascular spread, often very rapid, is not along the course of the vessel but away from it. All this can be noted especially well in the pellucid skin of *Rana clamitans*.

As in mammals the local distribution of color depends to no slight extent upon the quantity of dye (1). When but little is brought by the blood the distribution taking place from the capillaries is an even one. Inequalities develop only when so much is brought, or a dye so indiffusible, that not enough is lost by the way to compensate for those influences which make for progressively greater staining as the blood advances along the capillary channel. When a highly diffusible dye is introduced into mammals a general staining rapidly ensues in the muscle, with an intenser staining superimposed thereon in the further capillary region. We have only exceptionally noted this in frog skin. The local color differences developing in it are so pronounced as to indicate that the opportunity for escape of the dyes must increase very greatly along the capillary.

There can be no doubt that many of the capillaries widen slightly as the venule is approached, with result in an increase in the surface through which exchange may take place. As already mentioned, we ordinarily selected for study regions in which the capillaries were of approximately the same size all along. Yet may not a generalized dilatation of the further portion of these vessels, occurring after the dye injection, have been responsible for the greater escape in this region? Scarcely. For the stained blood caused the vascular net-

work to stand out so sharply that any abnormal dilatation should have been clearly visible for a greater or less period; and none was seen. The influence of dilatation has been completely excluded by experiments with frogs submerged in water at 16°C. for comparative observations on periglandular staining as affected by dye quantity. The incidental cooling caused a contraction of all the skin capillaries with result that after the dye injection the rete appeared extremely narrow and of even calibre all along, the only vessels larger than the rest being venules. The circulation remained exceedingly brisk, and the color pattern due to the escape of dye differed no whit from the usual.

When curarized frogs are submerged in ice water the skin circulation ceases to all intents and purposes for a few minutes, the only visible flow being a dribble through the superficial venules. The amount of dye passing through the individual capillary under such circumstances is negligible; yet there is a constant if slow flow of it along the veins. Under these circumstances one might expect the tissue next the latter structures alone to stain, as actually happens.

Not improbably some of the dyes had an affinity for certain skin elements, though none came to attention during the experiments. We used many dyes in order to minimize the peculiarities of any one as a factor in the findings as a whole. One would have expected specific affinity to manifest itself most markedly by a color pattern when but little dye was given; yet only under these circumstances was the observed staining an even one. The spread from the vessels into the loose spongiosum as a fuzz of color did not in the least suggest a selective staining of cells and fibrils but instead an extension into the fluid of intercellular spaces.

DISCUSSION

The findings in frog skin accord with those in frog muscle (1) and in mammalian muscle as well (2). A mounting gradient of permeability exists along the capillaries of all three. Its presence in frog skin is the more enlightening because of local conditions which offer a check upon the influence of some that obtain in mammals.

The skin of the side and belly of the frog receives highly venous blood from a branch of the arteria pulmo-cutanea, and as this flows through the capillary rete it gives off carbon dioxide instead of taking the gas

up, with result that on entering the cutaneous veins it has the lowest CO_2 content to be found anywhere in the body (12). The course of events along the capillary way is in this respect the precise opposite of that in muscle. Since the gradient of permeability is similar in both tissues the conclusion seems justified that neither changes in the CO_2 content of the blood nor those of pH (as determined by CO_2) can be the cause of the gradient. Nor can changes in the oxygen content, since O_2 is taken up along the cutaneous capillaries, whereas it is given off along those of muscle.

The veins draining both the muscles and the portion of skin that we have studied in the frog connect with a portal system, and in consequence the fall in pressure along the capillaries is probably far less than in mammalian muscle, where resistance to flow is very marked (13). It may be recalled in this connection that dyes escape from the superficial cutaneous venules of frogs even more rapidly than from the adjoining capillaries, though from those of frog muscle a little the more slowly (2).

The highly permeable venules of the frog skin form part of a rete made up for the rest of capillaries. Their distance from the nearest capillary is the same as that from capillary to capillary, showing that they serve the tissue as do these latter. There is anatomical evidence that venules largely take the place of capillaries in the skin of man (13) and the mouse (14), but that in mammalian muscle they are somewhat little less effective than these vessels (1). In the pectoralis major of the pigeon and the diaphragm of the rabbit the capillaries enlarge toward their end and converge upon the transverse venule in so singular an arrangement as to suggest that special, eliminative tasks are laid upon them (2). Fröhlich and Zak (15) have witnessed an early escape of indigo carmine and potassium ferrocyanide in certain regions along the veins draining the frog's tongue; but the phenomenon was pronounced only when this member had been pulled from the mouth and pinned flat. They traced it to the local obstruction offered by valves to the transmission of back pressure when the veins nearer the heart contracted. The drawings of Frölich and Zak show the staining to have no resemblance whatever to the orderly escape of dyes that we have witnessed.

What can be the cause for the progressively increasing escape of dyes along the capillary way? Is it explainable in terms of the factors recognized as principally conditioning exchange with the tissues, namely hydrostatic pressure, osmosis, and diffusion? The possibility that these, severally or together, are responsible for the gradient will now be taken up.

Hydrostatic pressure drives the dye-laden blood along the capillaries, and, other things being equal, the amount that passes a given surface during a given period will depend on the local pressure differences,— all of which is to say that the rapidity with which vital staining occurs cannot but be conditioned in the large by the pressure factor. The view has been strongly held that substances tend to pass from the blood to the tissues from the first portion of the capillaries because the pressure is highest there, and from tissues to blood further on where the osmotic influence of the intravascular colloids supposedly becomes the dominant force. In a preceding paper we have dealt with this view in so far as it concerns the gradient along mammalian capillaries, and have shown that it is incompatible with the facts (1). In the case of the frog, however, the hypothesis has special claims to attention since the capillaries of this animal are far more permeable than mammalian ones. Lymph formation is an especially active process in the skin, where even a part of the proteins leave the blood (3, 16). There must be a continual, rapid passage of water from the vessels; and the question of where and how this passage takes place is highly relevant to the problem of dye escape, since the dyes are in watery solution. According to Landis (8), water filters from frog blood where the hydrostatic pressure is greatest, that is to say along the first part of capillaries, and it carries dyes with it, the result being that both the region and the rate of their escape into the tissue are directly determined by the pressure factor. Our repetition of Landis' observations on the mesentery do not support this view; for we found that the nearer conditions approximated the normal the more regularly was dye escape greatest where it should have been least if pressure changes along the capillary had been the controlling influence. In this respect the findings were identical with those in the skin. The possibility is far from being excluded that the spread of dyes from frog blood results in some part from a filtration of colored water under pressure; but such filtration, if it occurs, may be most abundant where the intracapillary pressure is lowest, that is to say at the further end of the capillary.

The important rôle of diffusion in vital staining has been acknowledged since Schulemann's basic contribution on the theme (17). Our findings fall in with his generalization that in the absence of specific

affinities the more diffusible a dye proves *in vitro* the more rapid is vital staining with it. Not only is the rate of coloration dependent upon the diffusibility of the coloring matter but, in our experience, the proportion of the capillary from which its escape takes place. Escape is regularly greatest, however, in the very region where the concentration in the plasma is least, owing to loss to the tissues along the capillary way.

Some of our test substances, as *e.g.* patent blue V, pass through the capillary wall with such swiftness that their osmotic influence, as affecting exchange with the tissues, must be considered negligible. Others, like Congo red, which might have exerted a significant influence, were introduced in isotonic solution, as was routine for that matter. According to the classical view the only osmotic influence worthy of note as determining normal exchange with the tissues is that of the blood colloids, which should attract fluid into the vessels in greater and greater proportion as the opposing force of hydrostatic pressure becomes less on the way to the veins. This osmotic influence should act, not to promote the passage of dyes into the tissues in greater and greater amount as the blood progresses along the capillary, but increasingly for a retention of these substances.

It is plain that if hydrostatic pressure, diffusion, and osmosis determined the local phenomena along the capillary way these should be precisely the opposite of the ones observed. In a previous paper we have described experiments which indicate the presence along the capillary of some stable arrangement which controls permeability (1). It may be that the perivascular tissue acts as a barrier about the capillary and becomes increasingly loose-textured along this vessel with result in an easier spread of dyes. Local differences in the vascular wall itself seem a more likely possibility. Differentiations of shape along certain capillaries have already attracted attention, as *e.g.* along those of the nail-fold. Sandison has recently reported (18) of capillaries new-formed in proliferating connective tissue of the rabbit's ear that toward the vein the cells are flatter, the channels wider, the current slower. Subtle modifications in structure may well be responsible for the gradient of permeability along the vessels that we have dealt with.

SUMMARY

A steeply mounting gradient of permeability is demonstrable along the meshwork of capillaries which connects the arterioles and venules of the skin of the frog. The venules incorporated in the meshwork are even more permeable than the capillary meshes giving into them.

The presence of the gradient under such differing conditions as exist along frog and mammalian capillaries enables one to rule out certain factors which might be invoked to explain it; and it is not explainable in terms of those influences generally recognized as conditioning exchange between the blood and tissues. Not improbably it results from a structural differentiation along the capillary.

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EXPLANATION OF PLATES

PLATE 11

FIG. 1. Superficial capillary meshwork in the skin of the abdomen of *Rana pipiens*, after injection of an ink-gelatin mass. Preparation mounted without clearing or staining. It has precisely the appearance noted prior to the escape of a deep-colored dye from the blood of the living animal. Where the lines of the arrows meet are some converging venules incorporated in the network. Other efferent and afferent vessels connect with it from beneath and hence are not visible. The capillary meshes which encircle glands are rounded and slightly smaller than the others. × 22.

FIG. 2. Superficial capillary network in a transparent region of the thigh skin of *Rana clamitans*. An India ink mass was followed by one of gelatin-carmine to differentiate venules and arterioles. The capillaries containing red mass are far less plainly visible than those holding black. (Fig. 9 shows the network in detail.) Everywhere foramina pit the stratum compactum. A group of contracted pigment cells obscures the vessels near the middle of the left edge of the preparation. The bright spots here and there are due to "interference cells." Specimen photographed while fresh. × 14.

FIG. 3. Cleared skin of the thigh of *Rana clamitans* viewed from beneath to show the distribution of vessels in the tela subcutanea. The small vessels ramifying against the dimly seen, superficial rete of the corium appear to end abruptly because they pierce the stratum compactum at right angles to supply this rete. × 22.

FIG. 4. Distribution of trypan red from a rete like that in Fig. 1. Curarized *Rana pipiens* with brain pithed, injected in the course of 38 seconds; photograph taken 4½ minutes later from the living animal. Already much dye has escaped from the blood into the tissue supplied from the further portion of the rete, and the plasma has become so far decolorized that the proximal capillary meshes are no longer plainly visible. The tissue supplied by them contains no stain. In the stained regions the capillaries and venules appear broadened and blurred by perivascular dye. See also Fig. 7. × 14.

PLATE 12

FIG. 5. Distribution of Congo red from the rete on the belly of a curarized *Rana pipiens*. The injection required 3/4 minute and the picture was not taken until 9¼ minutes later. Escape of dye into the tissues has just begun, from the further portion only of the capillary network and from gland circlets here and there. The arrows point to such circlets. The blood still contains so much Congo red that the network stands out as if injected, the meshes nearer the veins appearing relatively broad because of spread of the dye. × 14.

FIG. 6. Further stage in the staining of the same frog; photograph taken from the living animal 12 minutes after Fig. 5. The color pattern has now been rendered irregular by the escape of dye from gland circlets. Everywhere else the

Congo red has got out only from the further portion of the rete, the tissue about its proximal region being unstained. The rete here is lighter than in Fig. 5 because of a partial decolorization of the blood. × 14.

FIG. 7. Trypan red staining, in the gross. Photograph of the frog furnishing Fig. 4, taken from the living animal 10 minutes later. The distribution of the dye was essentially unchanged. The skin surface was peppered with red dots on a pale ground, numerous over the abdomen, where was some general staining also, and scattered over thorax, thigh, and throat. In the lower leg the circulation was poor and little staining had occurred. On the inner side of the thighs, on the other hand, it was confluent and so intense that the skin appears black in the photograph. Natural size.

FIG. 8. Distribution of trypan red to the skin of the upper thorax of *Rana pipiens*,—for comparison with Figs. 4 and 7; photograph taken 10 minutes after the dye injection. Less of the dye had got out than on the abdomen of the same animal, and into a smaller region. The capillaries appear broader in the stained skin than elsewhere, owing to stain in their walls. × 14.

PLATE 13

FIG. 9. A higher magnification of the skin of the preparation shown in Fig. 2. The big arrow points to an especially large opening in the "sieve layer" of the stratum compactum from which an artery (not seen) emerges to join a radiating capillary meshwork recognizable by the light hue of the red mass it contains. The capillaries can be traced in several directions to venules, distinguishable by their black content, which disappear into holes in the compactum. The small arrows point to emergent arterioles. × 46.

FIG. 10. Cross-section of the skin of the thigh of *Rana clamitans* in a region like that of Fig. 9; vessels injected with an India ink mass. The capillaries of the rete lie immediately beneath the epidermis, and are recognizable by the plugs of black in their lumen. Two vessels pierce the stratum compactum to reach the rete; like it they are walled by a single layer of cells. Only one of the glands lying in the loose spongiosum is cut through its lumen. The thin tela subcutanea and the dense, superficial "sieve layer" (*Siebschicht*) of the stratum compactum should be noted. Hematoxylin and eosin. × 192.

FIG. 11. Skin of the belly of *Rana pipiens*. Uninjected specimen showing the same layers as in *clamitans*. Three glands are shown cut across. The tela subcutanea is relatively thick. The capillaries cannot be discerned, but an artery pierces the stratum compactum. Hematoxylin and eosin. × 170.
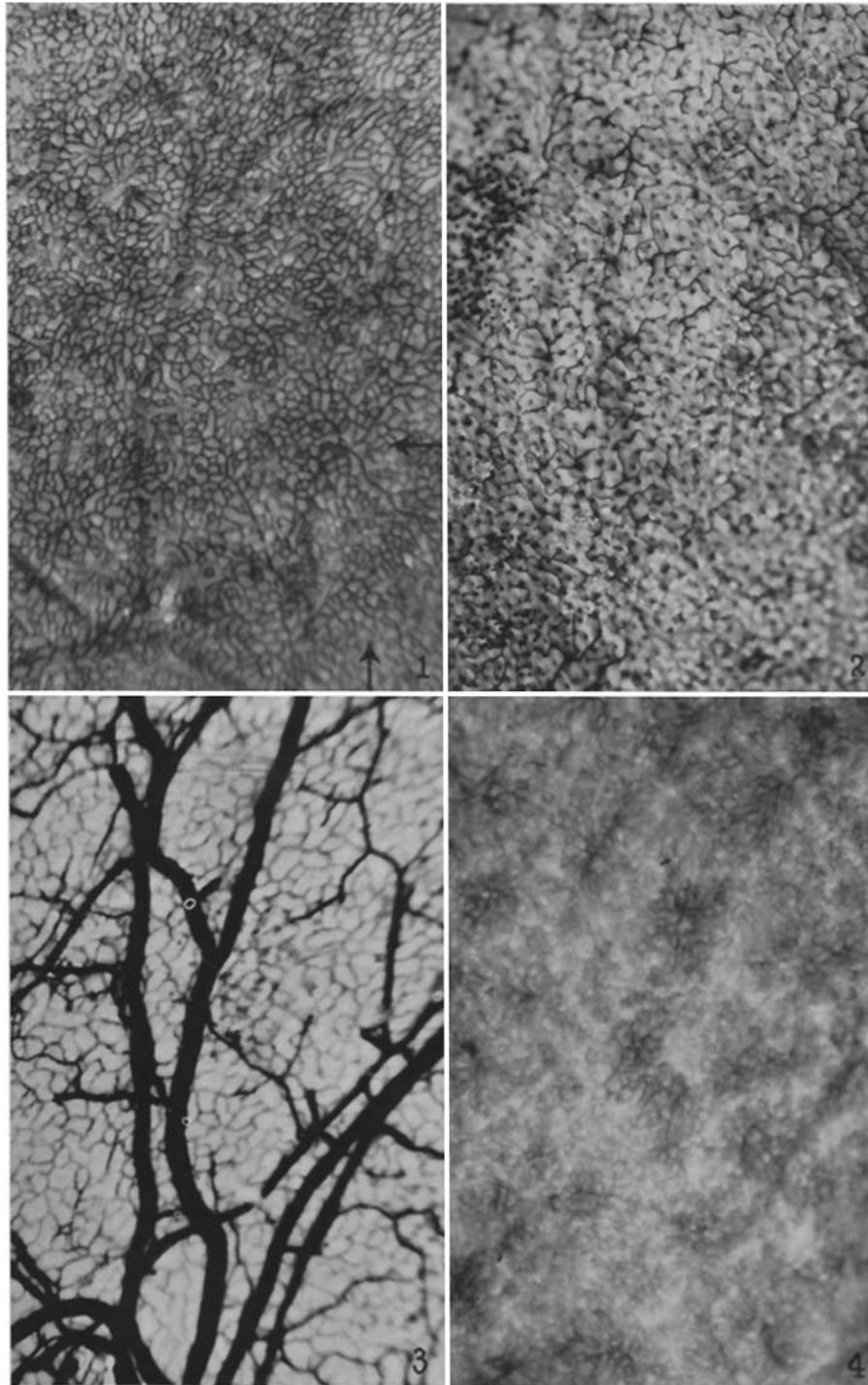
FIG. 12. Skin of the belly of *Rana pipiens*. Photographed to bring out in black the layers of "interference cells," just beneath the epidermis and in the tela subcutanea. The tissue is swollen as result of treatment with chemicals. Picric acid stain. × 170.
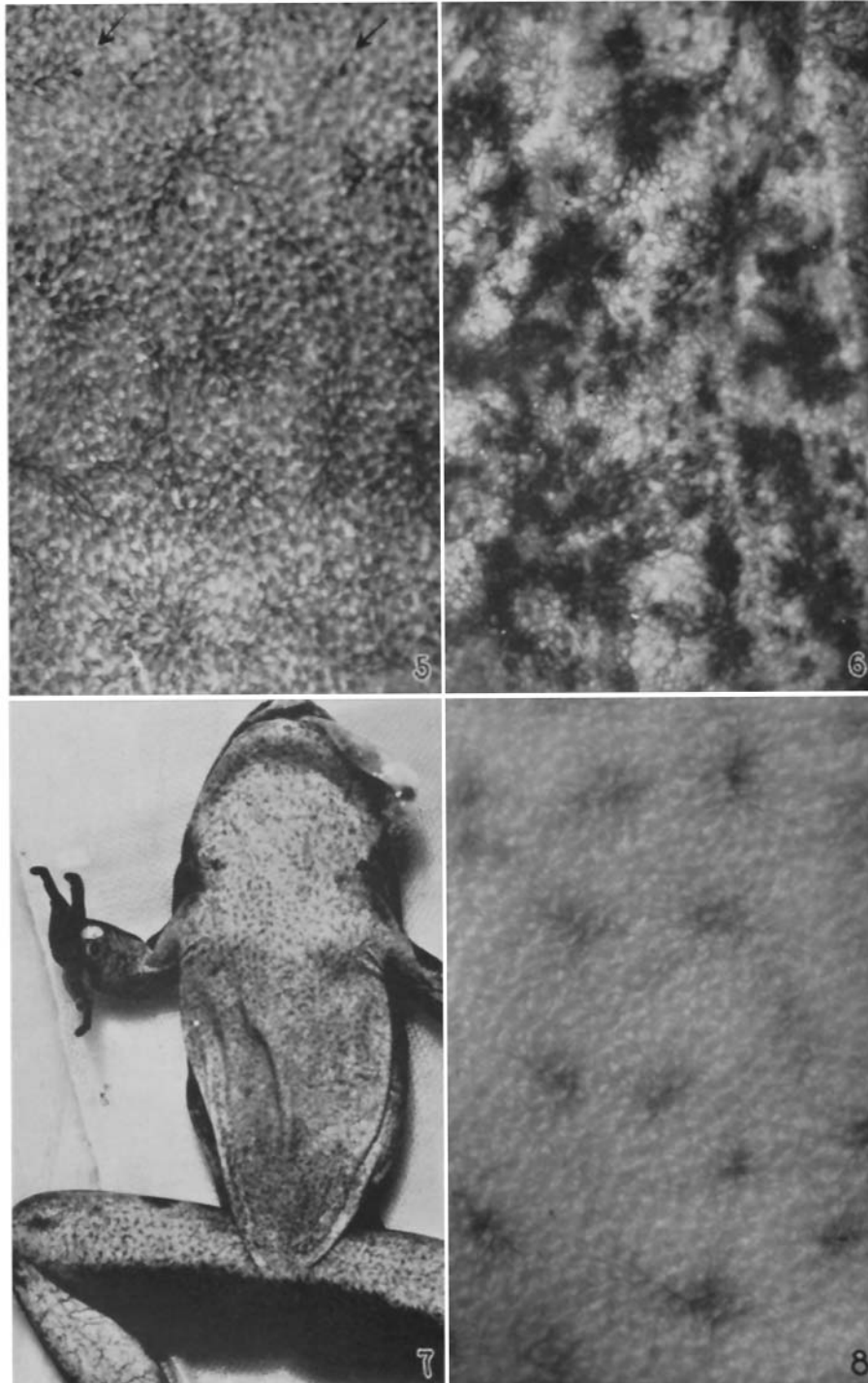
PLATE 14

FIG. 13. Skin of the belly of *Rana pipiens* after injection of the vessels with an India ink mass. The capillary rete lies immediately under the epidermis, with numerous "interference cells" at the same level, having gray granules. A large vessel is to be seen in the tela subcutanea. × 170.

FIG. 14. Injected skin of the thigh of *Rana climatans*, highly magnified, to show a venule piercing the stratum compactum and the cross-section of a capillary in the rete. The latter—recognizable by the black plug of India ink—is separated from the venule by the cross-section of a gland. Hematoxylin and eosin. × 360.
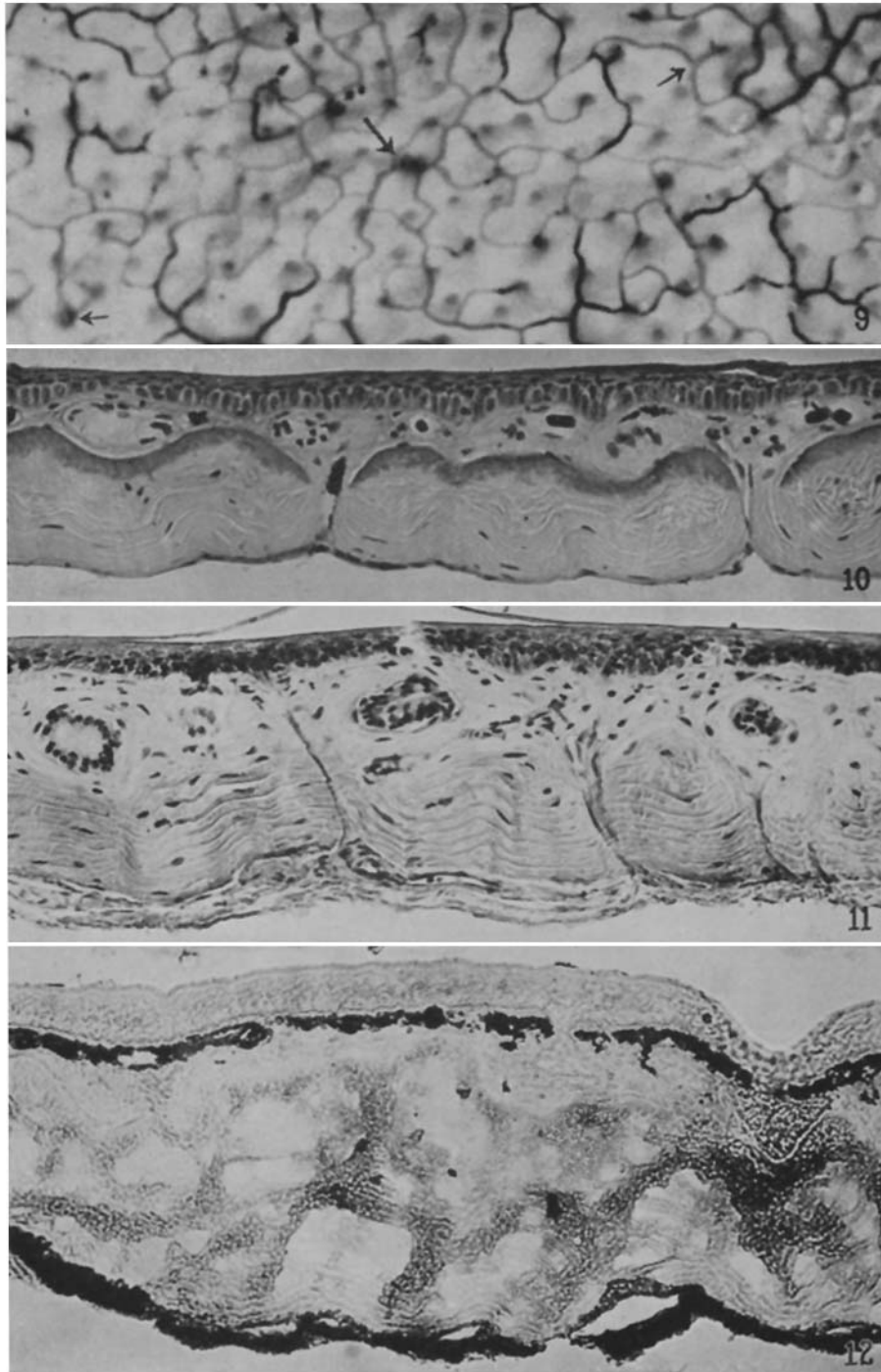
FIG. 15. Another part of the same preparation to show an emergent arteriole and a neighboring arteriolar capillary. Near the middle of the picture is a gland in cross-section and to its right another capillary. × 360.
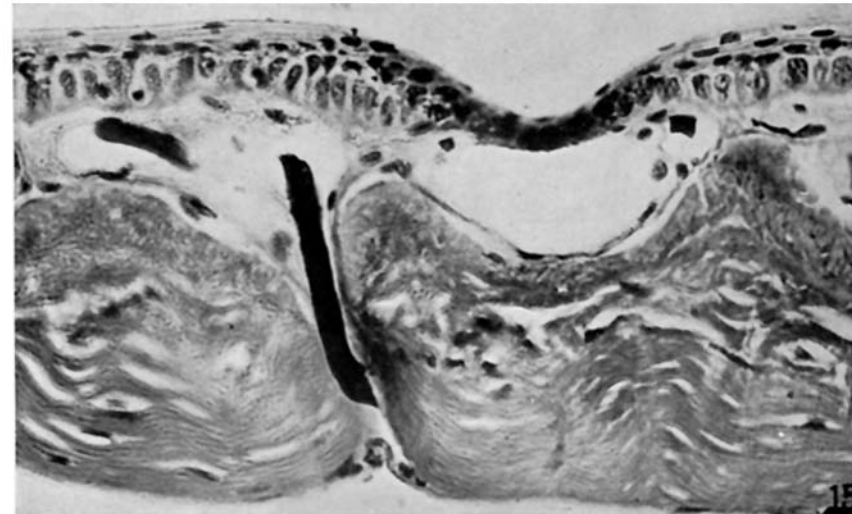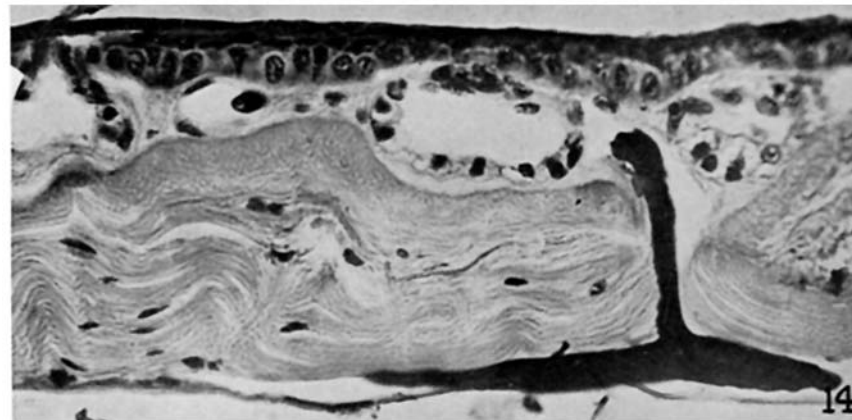
(Rous and Smith: Gradient of vascular permeability. III)

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