THE GRADIENT OF VASCULAR PERMEABILITY

II. The Conditions in Frog and Chicken Muscle, and in the Mammalian Diaphragm

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PLATES 8 TO 10

Experiments already reported (1) have shown that the permeability of the capillaries in the skeletal muscles of mammals increases progressively along their course and is greatest where they pass into the least venules. In the present paper we shall describe the state of affairs in frog and chicken muscle and in the mammalian diaphragm.

The Technique with Frogs

The same general methods were employed as previously. The spread of dyes from the blood was watched directly in muscle, with a binocular microscope, by the cooled light from an arc lamp. Ordinarily the tissue was exposed by cutting through the skin of the anesthetized animal from just above the knee to half way up the thigh on its anterior, outer aspect, and continuing the incision at right angles, to the inner side of the limb. On reflecting the triangular flap of skin thus loosened, portions of the sartorius, gracilis major, and crureus were laid bare, usually without the least bleeding. The muscles on the foreleg were occasionally studied; but those of the back and abdomen are so overlain with fascia and pigment cells as to be unsatisfactory. Owing to the large lymph spaces beneath the skin the mica windows used to protect mammalian muscle from drying would not stay in place. Occasionally the leg was submerged in frog Ringer's solution during the examination, but under such conditions less consistent results were obtained than when a thin sheet of cellophane (du Pont No. 350, 400, or 600) softened in Ringer's solution had been laid directly upon the exposed area. The sheet was kept moist with the solution, which also minimized reflection from its curved surface.

As control to the findings in anesthetized animals, numerous others were injected with dye and decapitated after various intervals. Summer frogs of two species were employed, *Rana pipiens* and *Rana clamitans*, the latter to but small extent

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since the arrangement of the vessels proved relatively unfavorable to the aim in view.

Despite many efforts we have been unable to find conditions under which a good circulation in the exposed tissue can be long maintained, or even assured to begin with. The practical absence of staining of the thigh muscle of unanesthetized frogs which behaved normally after the dye injection, leaping about when touched, proved that even in them the local circulation was insufficient to distribute the dyes in quantity. This can be attributed, in part, to posture,—for the muscles we examined bore much of the weight when the frog was quiet,—but mostly to the poor normal circulation. Krogh states that in resting frog muscle the capillary circulation is very variable and as a rule feeble, especially in the limbs (2). So often was the blood flow found to be highly irregular or nearly at a standstill on exposure of the muscle that we were forced to sacrifice frog after frog in order to obtain individuals in which, for reasons unknown, flow was good. Once it stopped in the exposed muscle, it practically never was resumed. Acting on the possibility that its cessation after pithing or anesthetization might be merely transient, we kept some animals for an hour or more thereafter before exposing the muscle. In them the circulation was more frequently at a standstill than in others opened at once.

The best preparations were obtained by injecting curare into a dorsal lymph sac (0.1 cc. of 1 per cent curare for a 30 gm. frog), pithing the brain immediately that the animal had become flaccid, and laying bare the muscle. The animal was placed on its back upon a moist cloth at the bottom of a shallow observation dish. Some frogs were pithed in both brain and cord; these in general had a poor local circulation which soon became worse irrespective of whether the tissue was exposed. Others were only curarized or etherized. The dye injection caused the muscle circulation to stop in some animals whereas in others receiving the same material it was unaffected. As a rule the skin circulation was brisk, no matter how poor that in the muscle.

The Vascular Arrangement in Frog Muscle

The arrangement of the muscle vessels is easily discerned in the sartorius (Fig. 1). It can be made out nearly as well during life as after the injection of colored masses.

In *Rana pipiens* the large vessels enter the muscle from beneath and ramify much more irregularly than in mammals. The larger venous tributaries connect with others of the same magnitude by collaterals, and the final arterioles and venules lie transverse to the muscle fibres, those in the same plane alternating at a distance of 1 to 2 mm., the gap being bridged by numerous capillaries in parallel. The venous twigs have an espaliered arrangement which appears slightly crowded as compared with the ramification from the corresponding arterioles.

The walls of all of the vessels are invisible in the living animal and, when these are empty, the tissue appears avascular. The capillaries are further apart and generally wider than in mammals; and their individual calibre as shown by their content varies greatly, a continuous column of blood several red cells thick flowing through some, while through others individual cells pass intermittently. All along their length the diameter is the same, but their number undergoes an increase in the further half of the region between arteriole to venule, owing to forkings that take place about midway (Fig. 1). In this respect there exists a striking similarity to mammalian muscle; but anastomoses between adjoining capillaries are somewhat more frequent, being met especially along their further portion, while furthermore some secondary forking may take place as the venules are neared, and these vessels are wider than the corresponding arterial twigs. Because of the resulting increase in the vascular bed, preparations injected with India ink and cleared appear slightly darker in the neighborhood of the transverse veins. It is frequently possible to find regions in which the capillarization is almost equally abundant everywhere between arteriole and venule, and such have been selected for our observations.

When the circulation is good, the whole field under the microscope appears at first view to be sliding rapidly in one direction or the other. Often, however, the blood barely creeps along. We have utilized many instances of both sorts. Stasis is readily discerned, and so too is unnatural congestion. In the crureus and adductor magnus, the vascularization, while more irregular than in the sartorius, has the same general arrangement; and in them circulation is especially well maintained after exposure. Their arterioles and venules lie relatively near together so that it is possible to watch several sets of capillary vessels at once under relatively high power.

Stained specimens show the capillaries to be walled by a single layer of flat cells with very flat nuclei. There are no obvious structural changes along their course. Direct arteriovenous anastomoses are occasionally seen; in fact the very large capillaries can almost be considered as such.

In *Rana clamitans* the transverse arterioles are far less regularly distributed than in *R. pipiens* and they break up into numerous twigs, each furnishing a few capillaries to the muscle. The venous trees are also relatively complicated. While the ultimate arterial or venous twigs from one transverse vessel usually lie near together, yet there is sufficient intrusion of those of the opposite sort from adjoining planes to render observations difficult, a difficulty added to by the shortness of the capillaries. The division into the latter takes place somewhat below the surface of the sartorius as a close arborization, and one sees only the final forks of the arteriole which go off at rather an obtuse angle. As in *pipiens* relatively few capillaries traverse the arteriolar tree from side to side, a fact which indicates, like our previous observations (1), that an effective exchange with the tissue takes place through the walls of the arterioles themselves. The number of capillaries increases considerably as the venules are approached.

Procedure with the Dyes

A more various set of dyes were employed than in the work with mammals. All had been freed of the extraneous matter which in not a few instances constituted a large proportion of their bulk. They were in watery solution isotonic with frog blood,—on the assumption that this itself has the tonicity of 0.7 per cent NaCl. The solutions had in some cases been brought to approximately the pH of blood but in others this precaution was not taken since the dye had no buffer value and the difference from the blood pH disclosed by the potentiometer was slight. The injections were made into the dorsalis pedis vein of the leg that was not to be studied. When the needle had been thrust for a little distance through the fascia before entering the vein at the junction of the venae tarseae lateralis and medialis, no bleeding followed. Magnifying spectacles or a watchmaker's glass were required if the vessel was to be entered regularly. Trial showed that unanesthetized animals yielded the most informative muscle staining if they were killed as soon as the rugose skin on the inner thigh surface next the crotch had stained deeply.

The Passage of Dyes from the Blood

Far less dye passed into the muscle than into the skin and some other organs; and the more poorly diffusible the material the greater this difference proved to be. Only by giving large amounts of pont-amine sky blue could one obtain an informative muscle staining before escape of the dye elsewhere had reduced the concentration in the blood below the effective level; while in the case of Congo red this end could not be achieved with the largest tolerable dose.

As one might expect from the abundant lymph formation in the frog (3) the passage of dyes from the blood is far more rapid than in the rabbit or guinea pig (1). Patent blue V and phenol red escaped at once all along the capillaries as the stained blood advanced through them, and local differences in rate of escape were not discernible with their aid. Such differences were readily brought out with less diffusible stains (Fig. 2).

SPECIMEN PROTOCOLS

Eosin (*Yellow*).—This dye belongs to a group not utilized in our previous work. The Grübler material (Dr. Karl Hollborn) was purified by extraction with absolute alcohol in the cold and evaporation on the steam bath in a strong current of air at 55°–60°. A 4.3 per cent solution in water was found by freezing point determinations to be isotonic with frog blood. The progress of the staining was most readily followed in light filtered through water tinted with methylene blue.

In animals with a brisk blood flow, as indicated by the almost instantaneous passage of the stained blood from arterioles to venules, the escape of eosin was so rapid everywhere along the little vessels that often the tissue next them appeared to color everywhere at the same rate. Resort was had for this reason to frogs in which a sluggish though continuous circulation was found, as when both brain and cord had been pithed. In these a series of phenomena were noted like those in rabbits injected with the very highly diffusible patent blue V after a large bleeding to lower the blood pressure and slow the local circulation (1). No doubt exists that in the frog the sluggish circulation was the result of a low pressure, for the signs of venous obstruction, capillary contraction, or developing stasis were wholly lacking. Some of the capillaries were empty, that is to say invisible, but many had the normal breadth, and flow in them was continuous.

0.2 cc. of eosin solution was injected for 40 gm. of frog in the course of from 10 seconds to a minute. After about half a minute deeply stained blood appeared in the terminal arterioles and dye could be seen to pass out at once from these vessels into the surrounding tissue and then from the capillaries as the stained blood flowed along them. Most of the eosin left the blood during the short journey, appearing almost to pour from the slender channels, and an interval elapsed,— of more than 20 seconds in one animal,—before enough of the stain entering the capillary reached its further end to color the plasma there definitely. At the end of about a minute and a half the muscle had become evenly pink everywhere. But now a transient, superimposed barring with deeper color developed, which was lost within another 2 or 3 minutes as the diffuse coloration intensified. The bars had venules at their center and they extended with diminishing intensity half way back to the arterioles.

There could be no doubt that eosin got out from arterioles as well as from capillaries, these latter proving highly permeable everywhere along their course. The tissue first reached by the stained blood was first served with dye by it, yet nevertheless an even staining soon developed, and secondarily there was superimposed upon this an especially intense coloration of the region traversed by the further portion of the capillaries and supplied with blood which was relatively poor in dye, owing to the continued escape of it along the proximal portion of the little vessels.

Brom Phenol Blue.—The spread of this substance in the tissues, as of other blue or purple dyes, could be followed in greater detail than that of red ones. No color filter was needed.

Usually 0.25 cc. of 2.7 per cent solution for 35 gm. of frog was injected in 20 to 30 seconds. When the local circulation was brisk the dye reached the muscle venules an instant after it appeared in the arterioles, and practically at once was

seen to escape everywhere from the capillaries, blurring them. Escape from the smallest venules took place at the same time. The coloration rapidly became diffuse. Within a minute, however, a barring with deeper color of the same general extent, situation, and graded character as that with eosin was superimposed upon the general color. After 90 seconds in all a special zone of color had formed just outside the transverse collecting venules, manifestly as result of the direct escape of dye through their walls. After little more than 2 minutes the staining in the barred region had completely obscured the capillaries there. By now the circulating quantity of brom phenol blue had so greatly diminished that the individual corpuscles could again be discerned. Nevertheless the bluish purple, diffuse staining underwent some further intensification, and in the next few minutes the bars were gradually lost in it. There was no zone of special staining along the large veins into which the transverse collecting venules gave.

The findings with brom phenol blue were like those with eosin except for a special staining along the venules, which may well have taken place with eosin but not have been discernible.

Trypan violet has been used but little.

In a curarized *Rana pipiens* of 46 gm. given 0.4 cc. of 2.9 per cent trypan violet in 57 seconds, the dye appeared in the muscle vessels within half a minute, and at the conclusion of the injection had begun to escape all along the further half of the capillaries, in increasing amount as the venules were neared. After 2½ minutes altogether a narrow zone of especially deep color could be seen just outside these latter. A violet barring in the distal capillary region was now very pronounced in the gross. Under the microscope the tissue appeared striated with color because of the stain outside individual capillaries, which extended further back along some than along others, with result that the margins of the bars had a step-like irregularity. The circulation continued excellent.

Trypan Red (*Vital Red HR*).—This proved highly satisfactory save in that it tended to do away with the paralysis of curare. It was well tolerated, readily followed in its extravascular spread with the aid of the methylene blue filter, and it did not obscure the blood corpuscles to such extent but what all changes in flow could be perceived.

Practically at once after the injection—0.3 cc. of 2.8 per cent dye for 50 gm. of frog, in 30 to 45 seconds—the further portion of each patent capillary became surrounded by a fuzz of color that, rapidly deepening, encased and blurred it. Extravascular dye was first visible in the region of the venocapillary junction, and then further back along the capillary. The distribution of stain, unlike a dye ecchymosis, was symmetrical, taking the form of a colored sheath which widened toward the venule. Along the proximal half of the capillary no staining occurred nor was there any about the arterioles. By the end of 2½ minutes the extravascular dye had spread laterally and become confluent. The muscle now was red everywhere

throughout the further capillary region, whereas nearer the arteriole there was still no staining. Thus there developed transverse bars (Fig. 2) which persisted for a considerable time. After a few minutes a narrow zone of intenser color developed next the small venules as if by direct escape of dye from them; but nothing of the sort was to be seen outside of the larger veins. Not infrequently situations could be found in which capillaries entered a venule on but one side; and only the tissue on this side was colored. Frequently when the circulation was vigorous, the red bars were superimposed upon a pink, generalized staining. They then appeared narrower, their limits being lost in the coloration of the rest of the tissue. In the gracilis major, where branching venules and arterioles are almost superimposed in adjoining planes, the color soon spread from one plane to another, confusing the picture.

The variations from the results with eosin and brom phenol blue were such as might have been expected, on the basis of previous work, from the slighter diffusibility of trypan red (1). Its escape from the further portion of individual capillaries as a fuzzy sheath or fringe, increasing in intensity as the venule was approached, duplicates observations frequently made with eosin and trypan blue, but not so readily to be interpreted in their case since they obscured the blood flow to such extent that intercurrent venous obstruction could not be ruled out as a possible cause for the phenomenon.

Trypan Blue (Grübler-Hollborn).—Trypan blue has a very different formula from trypan red, but nearly the same diffusibility (1). According to Schulemann (4), it kills when in Ringer's solution but is well tolerated in distilled water, as our preparation was. Large doses cause hemolysis (5). Dye ecchymoses were more often noted with it than with any other of our test materials, while furthermore the circulation in exposed muscle stopped with especial frequency after its injection. In the amount used by us it elicited no symptoms in unanesthetized frogs.

0.25 cc. of a 4 per cent solution was injected for 35 gm. of frog, in from 1/2 to 3/4 minute, rendering the blood so dark that the circulation seemed at a standstill. Well pronounced blue bars had developed ordinarily within 1½ to 3 minutes in the region traversed by the further third of the capillaries. The dye was seen to escape directly from the vessels in this region before it did from the venules into which they gave. In frogs with active circulation the barring was frequently superimposed upon some pale diffuse staining which had a pinkish cast referable to the presence in the dyestuff of a ruddy component more diffusible than is the blue one (6).

The findings closely resembled those with trypan red and trypan violet.

Chicago Blue 6B.—The escape from the vessels of this poorly diffusible dye was so gradual that under the microscope one saw only a very gradually developing extravascular mist in the region of the further portion of the capillaries.

Frogs of 35 gm. were given 0.1–0.2 cc. of 5.8 per cent solution in the course of a minute. Soon gradually deepening bars of blue were noted in the gross. Each had a transverse venule as its axis. All were extremely narrow, and there was but little opportunity for them to widen secondarily, since by the time they had developed the blood was largely depleted of the dye by its escape elsewhere. In unanesthetized frogs pithed for examination 5–8 minutes after dye injection, the barring was found to be superimposed upon a pale, diffuse staining. There was no localized zone of color immediately next the venules to suggest passage through their walls.

As already mentioned, dark dyes tint the plasma so deeply as to obscure the corpuscles. The blood flow can no longer be perceived except when cells containing black pigment are in circulation, as occasionally happens. The hopping course of these through the capillaries then attests to a general movement. Ordinarily, though, all the vessels appear as if rigid with stained injection mass, and only after considerable decolorization of the plasma has occurred can one see that circulation is going on. For this reason we have felt obliged to carry out many experiments with Chicago blue 6B. As might have been expected from its poor diffusibility (4) it escaped into the tissues to a very limited extent, and almost entirely from the far ends of the capillaries.

Pontamine sky blue 6B (du Pont de Nemours) is listed in Rowe's Colour Index (7), as identical with Chicago blue 6B (General Dyestuff Corporation). The crude dye stained mice far more slowly than Chicago blue 6B and the animals took on a blue-green color instead of a clear blue. This was true as well of the purified dye.

The crude material was purified like Chicago blue, by dialysis through Reeve Angell paper ("diphtheria parchment") against water at room temperature; but its salt content caused such an increase in bulk that reconcentration had to be carried out after about 20 hours, before purification was finished. Since no preservative had been added, the concentrate was heated at this time to just below the boiling point for 15 minutes to sterilize it. After a second dialysis of 24 hours, first against running water and then distilled, the material was evaporated on the steam bath.* Tests now showed it to be free from extraneous matter.

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* Needless to say, the process may have polymerized the dye. Such a change, if it occurred, was advantageous as broadening the conditions of experimentation.

Pontamine sky blue thus purified stands at the extreme limit of those dyes with which a visible vital staining of frog muscle can be accomplished. A 16.5 per cent solution is required for isotonicity with frog blood,—as compared with 5.8 per cent for our preparation of Chicago blue. Not a little dye deposits out of this solution during the routine filtration just prior to injection, and hence we have ordinarily used the material in half strength, an 8.25 per cent solution in 0.35 per cent saline. 0.2 cc. of this was well tolerated by a 35 gm. frog when injected in about a minute. The muscle vessels stood forth immediately as in a line drawing with deep blue ink, and after the lapse of several minutes the dye appeared in the tissue about the further end of the capillaries, and intensifying formed sharply defined, very narrow blue bars. Meanwhile the blood lost color. No diffuse coloration occurred, and no zone of special staining appeared next the transverse venules lying in the axis of the bars.

Pontamine blue gets out into muscle only from the further end of the capillaries.

Other Dyes.—Congo red and methemoglobin (of the horse) failed to escape in perceptible quantity. We were led to try the latter because of the permeability of frog capillaries for blood proteins (3).

Findings confirmatory of the foregoing were obtained in the few experiments upon *Rana clamitans*.

Analysis of the Results in Frogs

A gradient of dye distribution along the course of the small vessels proved readily demonstrable in frog muscle. The findings depended to no inconsiderable extent, however, on the amount of dye injected. When this was small and the substance readily diffusible (eosin, brom phenol blue) an even staining took place along the capillary way; while with more of the same material a barring of intenser color, in the region traversed by the further portion of the capillaries, was superimposed upon the diffuse coloration. When small quantities of poorly diffusible dyes (Chicago blue, pontamine sky blue) were injected none escaped into the muscle, though a great deal passed into the skin and some other organs; but with more a narrow barring of the muscle developed, one localized to the furthest capillary region; and when much had been thrown into the blood some pale, general staining of the muscle took place besides the barring, save in the case of the almost indiffusible pontamine sky blue. We have as a rule employed the least amount

of dye that would disclose the local differences. Whether the muscle fibres themselves took the stain was an immaterial matter. It was enough for our purposes to be able to perceive where and when the coloring matter got out into the tissues.

The findings were consistent despite the highly various circumstances of the work, and were nearly identical with those in mammals (1). They appear to provide evidence that there exists as in these latter a mounting gradient of permeability along the course of the capillaries, which reaches its height at the junction with the venules and falls away rather slowly along the latter. Yet this evidence must be scrutinized further before such a conclusion is justified.

That the coloration resulted from passage of dye into the tissues is certain since the mere filling of the vessels with stained blood did not produce it. Where capillaries carrying a rather poorly diffusible dye entered but one side of a transverse venule the staining of the tissue was limited to this side, a fact which indicates that the escape of such dyes occurred from the capillaries only.

As already mentioned, the capillaries usually undergo some numerical increase as the venule is approached; and the question arises of whether the intenser staining hereabouts may not have been due merely to a larger vascular surface through which exchange might take place, irrespective of any local difference in permeability. And there is still another possibility to account for the localized coloration in the case of easily diffusible dyes. These escape in abundance through the walls of the smallest venules, a fact attested by the development of a narrow zone of deep color next them. Often when the circulation is sluggish almost no blood flows through the individual capillary, yet enough in the aggregate for a slow current in the venule. Under such circumstances a considerable staining may take place from it while that from the capillaries will be negligible. We have frequently observed this occurrence; and though the dye escapes only from the venule it spreads secondarily with surprising ease. Indeed even in animals in which the circulation has stopped immediately after the dye-stained blood has entered the muscle, and the capillaries have contracted later, squeezing the blood into the venules, the tissue next the latter colors and the color soon extends backward toward the arterioles.

We have been able to appraise the importance of the increase in wall surface as the venule is neared by the selection of regions for observation in which this increase was practically absent. Here the same graded extravascular coloration took place along the further course of the capillaries as elsewhere in the muscle. Such coloration could not have arisen by a secondary spread backwards of poorly diffusible dyes, since to them the venules proved impermeable; and in the case of dyes more diffusible it developed by the lateral extension and coalescence of a colored mist which formed around each capillary through which the stained blood passed.

Final evidence of the existence of a gradient of permeability along the capillaries was obtained by observing directly the escape of dye through their walls. The fact that their individual calibre, be it large or small, is nearly the same all along should be emphasized in this connection. Some dyes (eosin, trypan red, trypan blue) could be seen to spread rapidly outwards from the blood, surrounding the wall through which they passed with a fuzzy sheath of deep color and soon hiding it completely. This sheath appeared first and was always most pronounced at the further end of the slender vessel, thence tapering and fading in the direction of the arteriole. Needless to say, the possible influence of intercurrent passive congestion and of abnormal capillary dilatation to account for the phenomenon had to be kept in mind. Sometimes its absence from muscle regions newly laid bare attested to abnormal vascular conditions in the tissue already exposed; and comparison showed that the capillaries of the latter were dilated. Abnormal capillary dilatation is known to increase permeability (8).* The fact deserves remark that the greatest escape was localized to the distal portion of the dilated vessels, though pronounced elsewhere. When all pathological instances had been excluded there remained a sufficiency in which a graded escape of dyes along the capillaries was directly noted. It took place deep in the transparent muscle as well as superficially. Trypan red yielded especially convincing results because any abnormalities of the blood flow could be readily detected despite the staining of the plasma.

The conclusion seems justified that the walls of the capillaries of frog muscle become increasingly permeable along their further portion. Whether a graded permeability extends back all the way to the arteriole our experiments have not disclosed, owing to the circumstance that the dyes which would be most affected by differences in the proximal capillary region get out so quickly everywhere that differences in the time at which they reach this point and that overwhelmingly condition the color phenomena. The fact that when such dyes are given in small amount their distribution along the capillaries is an even one, despite the lessening amount in the blood as it advances through the little vessels, indicates that permeability increases all along the latter to an extent that offsets the diminution just mentioned. This conception has already been brought forward in a paper on vascular permeability in mammalian muscle (1).

* Ecchymoses of dye are sometimes encountered in muscles that have been clumsily exposed. They are easily recognized, the dye escaping irregularly here and there, often from one side only of a vessel and in such great quantity as is never seen on ordinary occasion.

Character of the Staining in Chicken Muscle

Very different were the findings in chicken muscle.

Well nourished young Plymouth Rock pullets of 1100–1300 gm. were used for the work. They were etherized; a circular piece of skin was snipped out of a featherless area with careful avoidance of large vessels; and an oval, mica slip was inserted which fitted the opening snugly and lay against the muscle sheath. The skin is so loose in fowls that such a window can be pushed about with it over a large area, exposing many fields successively. Thus, for example, when it is placed at the lower edge of the thorax in the axillary line, either the pectoralis major or the external oblique can be observed at will. If the window is introduced further down over the oblique the entrance of air into the subcutaneous tissue is difficult to avoid. Immediately after the insertion the dye was injected, usually into a wing vein; and sometimes new windows were put in whilst the staining was going on. When a pectoral was to be watched the wing was let rest on a board, to avoid stretching the muscle. Staining was checked by decapitation, and layers of the muscle were rapidly separated out and inspected between glass plates under the binocular microscope. The thin sheet of the external oblique, removed *in toto*, proved especially suited to study, the relationship of arterioles, capillaries, and venules being almost diagrammatically visible (Fig. 4).

Chicago blue 6B, trypan red, trypan blue, brom phenol blue, and patent blue V were injected, in the proportions for body weight which had given clear cut results with mammals. The fowls were killed at times which seemed most favorable to the disclosure of local differences in vascular permeability.

The muscles always stained much less than those of rabbits. With trypan blue, for instance, which colors rabbit muscle brilliantly and promptly, almost no staining occurred in 20 minutes, so little dye passing from the blood that the pectorals appeared only pale green and the hemoglobin-containing muscles green-brown. By this time some of the other tissues (skin, gastro-intestinal tract) were deep blue, and the quantity of dye in circulation had so far diminished that but slight further coloration of the muscles could be hoped for. With trypan red the staining was also relatively slow and slight. Chicago blue 6B, a far less diffusible material (1), caused only the slightest muscle staining in half an hour; but not enough escaped elsewhere to reduce the concentration in the blood below the effective level and in the course of 2 hours the muscle became gradually and diffusely dark blue. Chicago blue yields in etherized rabbits a deep blue barring after 15 to 20 minutes. Brom phenol blue, which is fairly diffusible, gave a definite coloration of chicken muscle after 5 minutes; but the

color was pale as compared with that in the rabbit. Only with the very highly diffusible patent blue V was there intense, prompt staining.

These findings in fowls cannot be explained by circulatory difficulties or by lack of circulating dye. For the rapid passage of the latter into and through the muscle vessels was plainly visible; and intense staining of the skin and gastro-intestinal tract soon took place, with elimination into the bile. Furthermore there occurred a staining of the perivascular connective tissue within the muscle, accentuating the vessels.

Some typical findings will be given.

SPECIMEN PROTOCOLS

Chicago Blue 6B.—An 1100 gm. chicken was etherized at 10:40 a.m., and at 11:05 a mica window was inserted over the pectoralis major. The muscle appeared almost colorless under the binocular and beautifully transparent, with the circulation going on as if through channels in jelly. 11:15—There has been no local vascular dilatation since the muscle was exposed. 11:16½—Injection begun into a wing vein of 3.3 cc. 8 per cent Chicago blue 6B, in 3 minutes and 47 seconds.

20 sec.—The dye has reached the muscle and practically at once all its vessels stand out sharply in dark blue. 4 min.—The exposed tissue appears very pale blue in the gross. 7 min. 10 sec.—Muscle now evenly tinted, between *artemesia green* and *lily green* (9). 12 min.—The colored muscle is still transparent; the capillaries can be only dimly made out.

11:34—Skin green-blue, darkening gradually; 11:45—Muscle color approaching *lily green*; 11:48—Voided much dark blue, semi-fluid material. 11:51—Pectoralis darker and bluer than *lily green*. There is blue staining of the tissue surrounding a large artery and vein which emerge from the pectoralis to run on its superficial surface, as further some coloring of the intramuscular perivascular fascia. 12:08—Muscle evenly colored, darker than *dark Medici blue*. 12:33—Muscle between *dark Medici blue* and *dark green-blue slate*. The vessels are still brilliantly outlined. 12:45—The intramuscular connective tissue septae have gradually taken on a brilliant blue color. 1:15—Muscle everywhere of the same hue, between *Saccardo slate* and *dark green-blue slate*. The blood is less stained than before.

1:22—Decapitated. At autopsy the exposed pectoral and its control had the same even coloration, the lungs were unstained, the liver deep purply blue, the bile deep blue, and the intestines themselves deep blue and full of deep blue fluid. The gizzard was purply blue.

Brom Phenol Blue.—A 1300 gm. pullet was etherized at 3:30; between 3:45–47 two windows were inserted over the pectoralis major; and at 3:50 the injection was begun of 4 cc. of 4 per cent brom phenol blue solution. The dye was given in 7 seconds. It reached the muscle arteries 2 seconds before the conclusion of the injection and appeared in the veins immediately after.

3:51—No muscle staining; vessels brilliantly demarcated, without abnormal dilatation or contraction. 3:52—Skin has turned blue. The muscle capillaries are easily to be seen. 3:53—There is a medium blue, perivascular staining about the large vessels emerging through the sheath of the pectoralis. 3:53½—Eyelids deeply blue. 3:54½—Muscle staining blue evenly. 3:59—The skin is rapidly becoming a darker blue. Interior of mouth brilliant purply blue. 4:00—The larger muscle vessels are now emphasized by a blue staining of the perivascular connective tissues. 4:04—Muscle itself undoubtedly darker blue. 4:13—Skin deep blue. The muscle is darker and the thick fascial septae and the perivascular connective tissue stand out in darker blue. 4:18½—The muscle appears evenly stained, between *deep Dutch blue* and *slate blue*.

4:20—Killed. The external oblique, spread between plates and examined under the binocular microscope, shows everywhere a brilliant staining of the connective tissue about the larger vessels, and staining of their walls as well. Within the muscle itself the hue is even. So too in the pectoralis major. The alimentary tract is a deep, uniform purplish blue and contains a small amount of intensely stained mucus. The lymph is moderately blue, the liver reddish purple. The bladder bile is nearly if not quite as deeply colored as the solution used for injection.

Patent Blue V.—A Plymouth Rock chicken of 1100 gm. was etherized at 2:10; between 2:33–37 windows inserted; and at 2:47 the injection was begun of 3.3 cc. of patent blue V solution isotonic with the blood. The injection took 9 seconds. Meantime the external oblique was watched. It showed the vascular arrangement diagrammatically. 5 seconds after beginning the injection the blood in an arteriole under observation had become brilliantly blue, whereas it was still bright red in the accompanying venules. Immediately thereafter the muscle began to color diffusely everywhere, the dye leaving the plasma so completely that the venous blood continued to be ruddy. The hue of the muscle darkened fast, and 11 seconds after the end of the injection the animal was decapitated. By this time the venous blood had become perceptibly blue; but it was much less deeply colored than that in the arteries.

Both the pectoralis and the external oblique were now an intense and even blue-green. The latter muscle was rapidly removed and inspected between slides. The pectoralis major showed suggestions of a colored barring in the gross, but this proved referable merely to intense staining of the connective tissue surrounding the larger vessels. The intestines were deeply stained, and so too with the skin, the latter being more intensely colored than with Chicago blue 6B after several hours. The bladder bile was deep blue, and the liver dark with stain.

Other instances need not be given. Always the muscle tissue appeared diffusely colored in the gross. There was a complete absence of the barring which in mammals and frogs is expressive of a mounting gradient of permeability along the muscle capillaries.

The Vascular Arrangement in Chicken Muscle

A study of the arrangement of the vessels from which the dyes were distributed to chicken muscle showed why the findings were at variance with those in mammals and the frog. We used both fresh material and tissue fixed after injection with colored masses (Figs. 4 and 5).

The vessels of chicken muscle are much more abundant and minute than those of even the mouse. The slenderness of the final arterioles and venules at once attracts attention. In mammals and the frog these lie separate, in regular alternation transverse to the muscle fibres, the gaps between them being bridged by parallel capillaries. The latter are from 0.75 to 1.5 mm. long in the frog and rabbit, and their graded permeability results in a barred staining, the center of each bar being separated from the next by the distance between two venules, by 1.5 mm. to 3 mm. that is to say. In the chicken on the other hand the final arterioles and venules, while lying transverse to the fibres, do not alternate at considerable distances but run in parallel next each other, often two venules with an arteriole (Figs. 4 and 5). Each of the latter breaks up into a very small group of capillaries, and these, instead of emptying into the adjacent venule, course to another at a distance of 1/3 mm. or less. Immediately next this venule, however, is an arteriole giving off capillaries which run to the venule situated next the arteriole first mentioned. Thus it comes about that throughout the muscle small adjoining groups of parallel capillaries carry the dye-containing blood in opposite directions. These details can be studied in the living fowl injected with a dye which is long retained in the blood stream (Chicago blue 6B). Extremely thin venules and arterioles are then seen to rise side by side toward the surface of the beautifully transparent pectoralis major, the arterioles being remarkably slender; and they so interlace, while branching, that with difficulty one perceives them to be connected with the capillaries of different small groups of muscle fibres. The capillaries appear as the finest threads, even when the blood is deep colored.

In fixed and cleared specimens having red mass in the arterial system and black in the venous, the picture is like that which would be found if the muscle were possessed of a duplicate set of vessels, with one set shifted the length of a single group of capillaries so that venule and arteriole, not two arterioles or two venules, run side by side. By teasing fixed preparations under the microscope, or rubbing them thin, the ultimate venules and arterioles can be proved to ramify in slightly different planes, the result being that while small groups of fibres have the benefit of capillaries coursing in opposite directions, this is not true of them individually, or is true only where those of neighboring capillary groups adjoin (Fig. 5). A more detailed study of the state of affairs is desirable.

Not only are the capillaries of fowl muscle extremely short but the opposite direction of flow in those lying next each other should mask the influence of any local differences in permeability.

The Vascularization of Pigeon Muscle

The presence of two identical, very abundant and close-knit vascularizations, providing flow in opposite directions amidst the fibres of chicken muscle, attests to the necessities of the working organ, perhaps in the past when the fowl was a flying creature. However this may be, it is interesting to inquire into the vascularization of muscles responsible for long sustained, rapid flight. We have studied therefore the pectorals of the pigeon. The arrangement in them bespeaks functional urgencies very clearly.

Pigeon muscles are too dark for the distribution of dyes in them to be readily seen. Our preparations were injected with colored mass and cleared. As in the chicken the final transverse venules and arterioles appear to pass to the tissue side by side in parallel; but by careful teasing and abrasion of fixed material, it is possible to isolate individual very shallow layers of muscle with vessels intact, and then one sees that venules and arterioles really alternate (Fig. 7). The distance to be bridged by the capillaries is exceedingly short, not over 0.2 mm. at most, but it is increased in the case of about half of these vessels by an abrupt change in their course so that they converge upon the venule in a long, narrow fan, enlarging slightly while so doing (Fig. 7). The capillary fans alternate with arteriolar trees which have the same simple structure as those in the muscle of other animals.

The Gradient in the Mammalian Diaphragm

The continuous functioning of the mammalian diaphragm suggested an examination of this muscle.

As might be expected from the demands upon the diaphragm, its blood supply is especially rich, and it is always succulent with lymph. Early investigators noted that it stains far sooner and deeper with vital dyes than most skeletal muscles. Open capillaries are more numerous than in the latter, under ordinary conditions (2), and the circulation is excellently maintained and vital staining intense even after bleedings which result in so great a contraction of the vessels in other muscles that these fail to color at all (10).

The vascularization of the diaphragm has been described by Spalteholz (11), but into its minute features he did not go. It shows the same alternation of transverse arterioles and venules as other mammalian muscles but the vessels are nearer together, being little more than 1/2 mm. apart in the rabbit as compared with more than 1 mm. in the gracilis. A significant feature is a sudden, large increase in vascular area near the venules. Many of the capillaries fork about half way from the arteriole, as is usual in skeletal muscles, and thus their wall area is expanded; but in addition some of them enlarge near their end and turn and course alongside

the transverse venule before entering it, an arrangement suggestive of that in the pigeon pectoral (Fig. 3).

The capillaries which traverse the arteriolar tree are, as in other skeletal muscles, remarkably few, a fact which attests to efficient exchange through the arteriolar wall itself (1).

So rapid is the distribution of stain to the diaphragm that no local differences are ordinarily met in rabbits sacrificed a minute or so after an injection of the highly diffusible patent blue V or brom phenol blue. The organ has then an even, diffuse, deep blue hue, even when the animal has been bled beforehand in the attempt to cut down the circulation. But if Chicago blue 6B is injected and the diaphragm inspected immediately one finds "mackerel sky" barrings of color indicative of a gradient of distribution of the dye (Fig. 6).

Into an ear vein of an etherized rabbit 3 cc. per kilo of a standard, isotonic solution of Chicago blue 6B is run in the course of 2 minutes. The carotids are immediately severed, the abdominal wall slit from symphysis pubis to ensiform, and the under side of the diaphragm inspected *in situ*. Everywhere the muscle is seen to be barred with blue. The chest is now opened along the sternum, the large vessels clamped off below and above the diaphragm, in the order mentioned or the reverse, and the organ is cut from the thorax, spread and inspected between glass plates. It shows a diffuse blue staining with superimposed bars of much deeper blue, each having a transverse collecting venule as axis. More of the tissue is involved in the bars of Chicago blue than in ordinary skeletal muscle—about one half all told. Near the arterioles the staining is neither greater nor less than elsewhere outside the barred regions. When the animal has been let live for a minute after injection the bands appear narrower because their margins are lost in a deep, general staining. Equalization of the staining is very rapid after death.

In some of our instances the abdomen was opened just prior to the injection, and the liver was seized and pulled down on one side at intervals to find whether the diaphragm contracted during the period of staining, as was regularly the case. In uninjected animals the organ showed no trace of barring, although the vascular pattern could be plainly seen when it was looked at between glass plates; and no bars appeared in animals receiving a half and half mixture of India ink and Ringer's solution in sufficient quantity to make the vessels stand out in black. Thus the possibility that the "mackerel sky" might be due in part to the vascular content was ruled out.

The question of whether the color pattern in the diaphragm is referable to a graded increase in permeability along the capillary, to increase in the amount of permeable surface, or to both combined, is

immaterial.   We have shown in a previous paper that only the possibility last mentioned explains the barring in ordinary skeletal muscle. The point to be stressed in the present connection is that the vascularization attests as definitely as in the case of pigeon muscle to special demands along the further capillary way.

DISCUSSION

An understanding of the conditions determining exchange between the blood and tissues is one of the ultimate goals of physiology; and it has attracted the attention that it deserves.   In proportion as conditions have been recognized which influence exchange, reasoning upon how and where this should take place has become precise and occasionally dogmatic.   There is reason to ask whether direct observation is not still a safer mode of approach to the problem presented by the process than *a priori* inference from such conditioning factors as have thus far come to light.   Having observed what happens to a given substance one is certain at least that it actually passes into or out of the blood at such and such regions and in such and such quantity.   In reasoning upon what should happen to it on the basis of known factors one is assuming that one knows them all, that there is none still hidden which plays a decisive rôle.   It is with this point in mind that our experiments have been carried out, of necessity with substances strange to the tissues.

The observations reported here make plain the fact that in frog muscle and the mammalian diaphragm the opportunity for dyes to pass from the blood to the tissues progressively increases along the capillary way.   In the frog this comes about mainly through a graded increase in permeability of the walls of these vessels, but in addition there occurs toward their further end some expansion of the surface through which exchange may take place.   In the diaphragm the expansion is abrupt and considerably greater.   While it will suffice to explain the especially abundant escape of substances near the diaphragmatic venules, that it plays the sole rôle in the graded increase seems unlikely, in view of the fact that permeability increases progressively along the capillaries of other mammalian muscles.   Chicken muscle exhibits a significantly different state of affairs.   It is supplied with what is to all intents and purposes a double circulation in closely

spaced, short capillaries, and the blood runs in opposite directions between muscle fibres lying almost side by side. No evidence of a gradient of permeability along the capillaries has been obtainable under the conditions; perhaps none is present. In a flight muscle of the pigeon (pectoralis major) there exists not only the same structural provision for abundant circulation as in the chicken but an elaborate collecting system of capillary vessels.

These diverse findings bespeak the need, emphasized in a previous paper, for an equalization of opportunity where shortcomings in local maintenance would reduce the efficiency of an entire fabric. As there pointed out, the parenchymal cells of the liver compete with one another for existence (12); and the lobular capillaries converge and progressively unite on the way to the venous center, the result being that the further a cell is from the blood source the greater is the quantity of blood passing it. Thus opportunity is equalized. In the urinary bladder the capillaries enlarge along their course and interlace in such manner that any further provision to equalize the conditions along them may be unnecessary, though it is far from being ruled out. Frog skin is supplied from a capillary meshwork practically devoid of such local differences in vascular calibre and mesh size as would suffice to put the connective tissue cells in the neighborhood of the venules on a parity with their fellows closer to the arterial blood. This end is gained by a sharply mounting gradient of capillary permeability (13).

The opportunity to get rid of wastes is as important for the tissues as that to procure materials; and hence the artifices just mentioned must be examined with both purposes in view. The provision in pigeon muscle of fan-shaped aggregates of capillaries converging upon the venules would seem manifestly appropriate to some special eliminative need, though what that need one can only conjecture. Structural indications of somewhat similar character are present about the collecting venules of the mammalian diaphragm. The increase in the wall area of the capillaries of frog muscle as these near the venules may serve more for elimination than supply. So too may the remarkable permeability of the small venules of muscle generally, which is only slightly less than that of the capillaries, and in frog skin even greater (13). Krogh (2) has worked out for muscle the relation of capillary spacing

to oxygen distribution, and while commenting upon the fact that the number of open capillaries in the exercising tissue is larger than will suffice to ensure oxygen, has suggested that it points to some other need. The local elimination of carbon dioxide presents no problem in this connection because it diffuses through tissues with enormous rapidity. Determinations of the relative rate at which the metabolic products of muscle activity pass through living tissue may yield an answer to some of the questions raised by the singular capillarization of pigeon muscle. The need for research on the comparative anatomy of the muscle vessels in its relation to muscle function is an obvious one.

Despite the high blood pressure of the fowl,—averaging about 160 mm. Hg (14),—and the abundant vascularization of its muscle, very little dye gets out into this tissue as compared with the amount in mammals. The point should be emphasized in connection with this difference, though not as explaining it necessarily, that tests with dyes disclose only the further and furthest limits of vascular permeability. Schulemann has proved that the rate of vital staining corresponds nearly with the diffusibility of the dye employed (4). The most highly diffusible of our materials, phenol red, spreads through water at about the same rate as dextrose and through gelatin more than three times as slowly (1). It may pass through the capillary wall more readily than some amino acids, but without question would be found to lag far behind many substances used by the tissues; for the rate at which dextrose itself gets into and out of the vessels is almost startlingly slow as compared with that of the salts of the blood (15). Unfortunately all highly colored substances of simple constitution that we have sought to utilize have yielded an equivocal vital staining or have been toxic.

In an accompanying paper the gradient of vascular permeability will be discussed in some of its general relations. Here the fact may properly be dwelt upon that recent investigation shows the differentiation of the vascular tube to transcend far, both structurally and functionally, the conceptions of it generally held. Until a few years ago the tripartite division,—into arteries, capillaries, and veins,—carried with it a belief that the capillaries are inert organs, mere passive instruments in exchange. The demonstration of their active contractility has invested them with new meanings. The structural modifications along some of them (as e.g. those of nail-fold, bladder, and

connective tissue (16)) and the gradient of permeability along others (muscle, skin (13)) constitute evidence that the capillaries, as well as the arteries and veins, undergo differentiation along their course. The final arterioles and least venules are now known (15, 1) to share in the functions of exchange allotted in the past solely to capillaries. One may expect that as knowledge grows in the future the classification of the vessels into three categories will be supplemented by a conception of the vascular tube as a single entity which undergoes modifications that are especially various and significant along that portion of it known as the capillary.

SUMMARY

A mounting gradient of permeability exists along the capillaries of frog muscle. In chicken muscle on the other hand none has been demonstrated; but the close-knit vascularization is arranged in duplicate in such manner that the blood runs in opposite directions through the capillaries of nearly adjacent fibres. In a flight muscle of the pigeon there exists in addition to this artifice what appears to be a special collecting system of venous capillaries. In the mammalian diaphragm indications of such a system are also to be found, and a gradient of capillary permeability like that in the other skeletal muscles is probably present.

These vascular conditions are briefly considered in terms of function.

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. Edge of the sartorius of *Rana pipiens*, cleared after injection of an ink-gelatin mass. The muscle has shortened somewhat with result that the vessels appear slightly contorted. Two transverse venules are seen with an arteriole between, paralleling them at a distance of about 1.3 mm. Many of the capillaries fork on the way to the venule, and cross connections become more frequent as the latter is approached. The result is an undoubted increase in vascular surface, marked in some regions, negligible in others. ×29.

FIG. 2. Distribution of trypan red to the sartorius of *Rana pipiens*. The 50 gm. animal was given 0.3 cc. of the dye solution in half a minute, while unanesthetized, and decapitated 3½ minutes later, when the muscle was dissected out and photographed between slides. Each of the transverse venules is the axis of a broad bar of stain which fades off in the direction of the arterioles. Only one of the latter is visible (the arrow points to it); but the microscope showed that each lay in the midst of unstained tissue. The general vascular arrangement is far less regular than in mammals, and so too, of course, is the distribution of the dye. The distance from venule to venule is in some cases more than 3 mm. ×5.

FIG. 3. Drawing of the vascularization of an injected, teased, and cleared muscle bundle from the diaphragm. A transverse arteriole is shown with venules to either side. Few capillaries traverse the arterial tree. Those given off from it increase on the way to the venule by forking, and on nearing it some quit their course, enlarge, and run slantingly to unite with the main stem. ×58.

PLATE 9

FIG. 4. Thin portion of an injected and cleared external oblique muscle of the fowl. The arteries appear paler than the veins because they contain red mass, while the veins hold black. They ramify side by side across the muscle, two veins to an artery, and break up into exceedingly fine and numerous capillaries which parallel the muscle fibres. Only the larger distributing and collecting vessels can here be made out. ×20.

FIG. 5. Arrangement of the smaller vessels of chicken muscle, as shown in a teased fragment of the pectoralis major. The preparation had been treated like that of Fig. 3. The final arterioles and venules lie nearly side by side but the fact can be made out that they are in different muscle planes. They connect, not with each other but with the nearest vessel of opposite kind which lies in the same plane. The length of the capillaries is only about 1/3 mm. The arrows point to artefacts which might be taken for vessels. ×66.
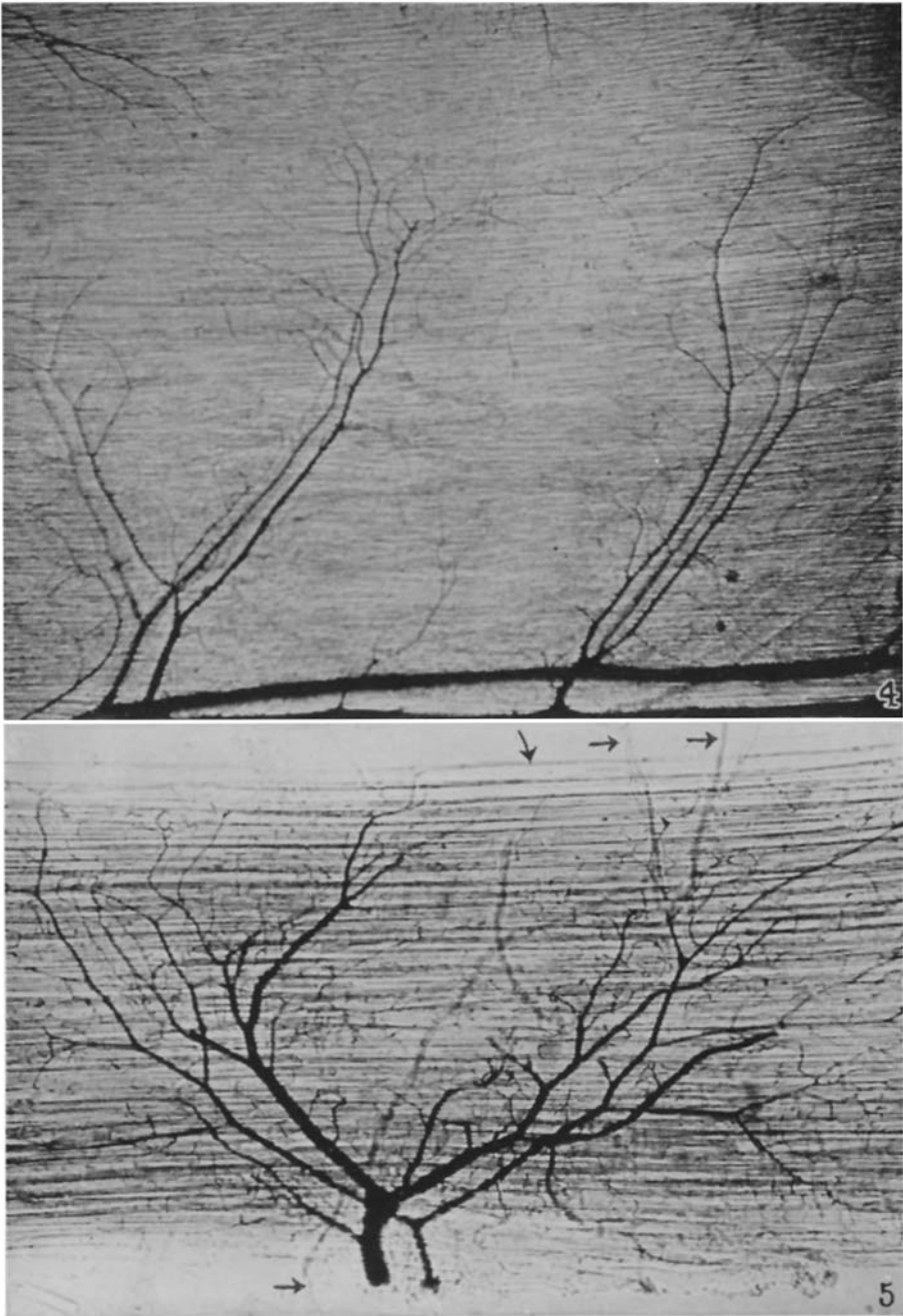
PLATE 10

FIG. 6. Half of the diaphragm of a 2000 gm. rabbit killed by cutting the carotids 9 seconds after the injection of 6.1 cc. of 8 per cent Chicago blue 6B in 2 minutes and 11 seconds. Photographed between glass plates. The "mackerel sky" barring with color in the muscular portion of the organ closely resembles that which is indicative in other mammalian muscles of a mounting gradient of permeability along the capillaries. The preparation thins into tendon at one end. Here the vessels are very sharply outlined, no stain having escaped. Natural size.

FIG. 7. The vessels in a single plane of the pectoralis major of the pigeon—from a specimen rubbed thin after injection and fixation. The transverse arterioles lie in tissue traversed by very few capillaries, whereas each venule constitutes a stem to which many such vessels converge fan-wise, after abruptly quitting their course beside the muscle fibre. The greatest distance between arteriole and venule is only about 1/5 mm. but the length of the capillaries is sometimes more than 2/5 mm. owing to the change in their direction. At the lower edge of the photograph the vessels appear closely crowded because two layers of them are superimposed. × 90.
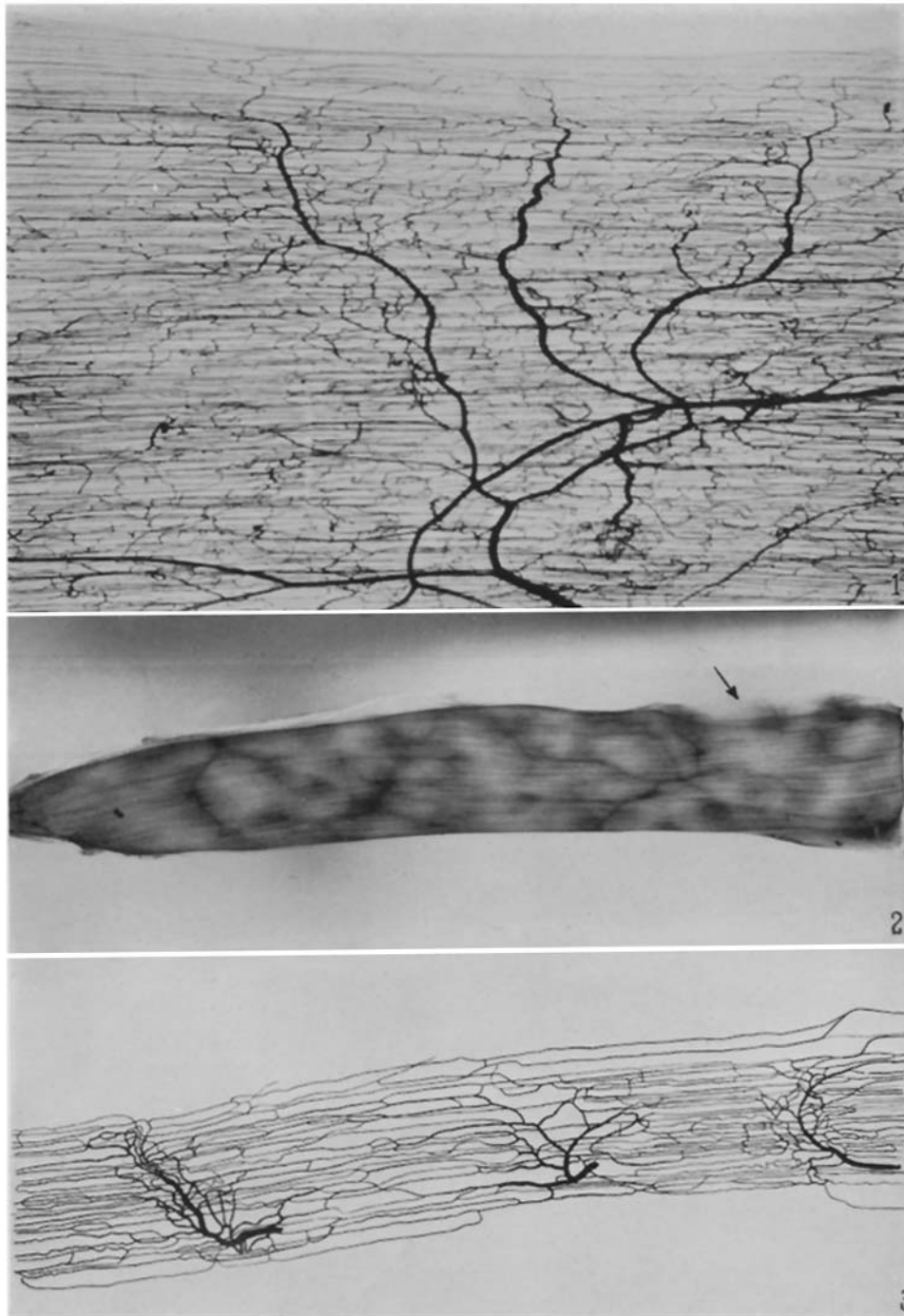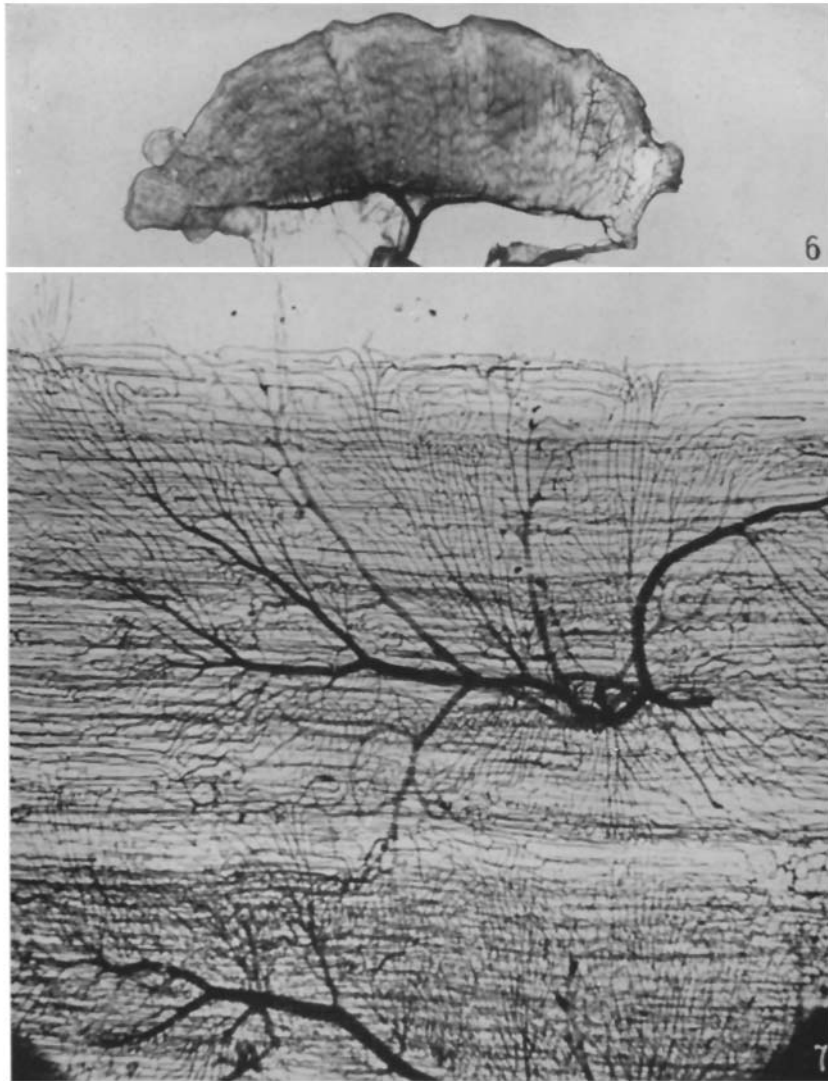
(Smith and Rous: Gradient of vascular permeability. II)