1909. Anat. Record 3

OBSERVATIONS ON LIVING GROWING LYMPHATICS IN THE TAIL OF THE FROG LARVA.

BY

ELIOT R. CLARK.

From the Anatomical Laboratory of the Johns Hopkins University.

Recent studies have led to a distinct advance in our knowledge of the lymphatic system. Yet there are many points in which this knowledge is still incomplete. The observations here recorded seem to have a bearing upon some of these points, and hence seem worthy of presentation to the Association.

The studies were made on living frog larvæ; the species used were Rana sylvatica, R. palustris and R. catesbiana. Two devices were employed, both of which were essential to the success of the observations, an upright chamber and chloretone anesthesia. The former permits observations to be carried on with the larva in its normal upright position. Chloretone serves to keep the larva motionless while under observation. The use of chloretone introduces slightly abnormal conditions. Yet by alternating the periods of anesthesia with return to fresh water, the same larva may be kept under observation for several hours daily, for three or four weeks. During this time growth continues, though somewhat retarded.

In order to preserve accurate records of the various stages, drawings were made both with and without the aid of the camera lucida. The micrometer eye-piece was employed in making measurements. With the assistance of careful records, little difficulty was experienced in finding the same structures—blood-vessel, lymphatic, even connective-tissue cell—in successive observations.

The fin expansion of the tail of the frog larva, in early stages, is rather opaque, owing to the presence of pigment and yolk granules. During this period it is possible to distinguish the course of the blood-vessels only by the moving blood-corpuscles. Gradually this opacity diminishes, until eventually, at lengths which vary for different species, the tail becomes beautifully transparent. Now the walls of blood-vessels, individual connective-tissue cells, nerves, wandering cells and lymphatics may be readily distinguished, furnishing a picture of rare simplicity and beauty. Little wonder that it has been selected so often as a field for the study of elementary problems in anatomy! From this period an accurate record may be kept of the changes which take place, until the picture is again somewhat clouded by the development of pigment cells and by the increase in thickness of the tissues.

The blood-vessels first demand a short description. When the tail becomes transparent, they form anastomosing loops in both dorsal and ventral fins, extending outward from the edge of the axial mass. The limit of the vascular area is roughly parallel to the free edge of the fin and leaves a wide non-vascular area. Sprouts are sent out forming secondary plexuses which gradually approach the edge of the fin, so as to give a festooned appearance. Hand in hand with this new growth there is a general expansion of the tissue with an increase in the distance between neighboring capillaries. Accompanying these processes, adaptive changes take place in the older capillary mesh-work. Here some of the capillaries increase in size to form arterioles and venules, while others disappear. These disappearing vessels may be readily observed in all stages. The sequence of events in a single degenerating vessel is usually a stasis, so that a cell in the lumen remains in equilibrium—a narrowing so that no other cells enter—the appearance of a solid portion, which extends until the whole vessel becomes a solid cord—the breaking of this cord and gradual shortening of the two ends until the only vestige may be a slight swelling on the wall of the blood-vessel.

In no instance during these observations has there been seen a lumen-containing portion of a blood-vessel isolated from the actively functioning vessels. The relation between blood-vessel and lymphatic will be mentioned later.

The lymphatics in the tail of the frog larva have often been studied, by Kölliker[1] in 1846, then independently by Remak[2] in

[1]Kölliker, Annal. d. Sc. Natur., 1846.
[2]Remak: Müller's Arch. f. Anat., Phys., u. wissensch. Med., 1850.

1850 and subsequently by numerous investigators, in the live animal, in fixed and in injected specimens. In our work, lymphatics were noticed during observations on the blood-vessels, as narrow, irregular, double-contoured structures with free ending toward the margin of the fin and traceable to the edge of the axial mass. They

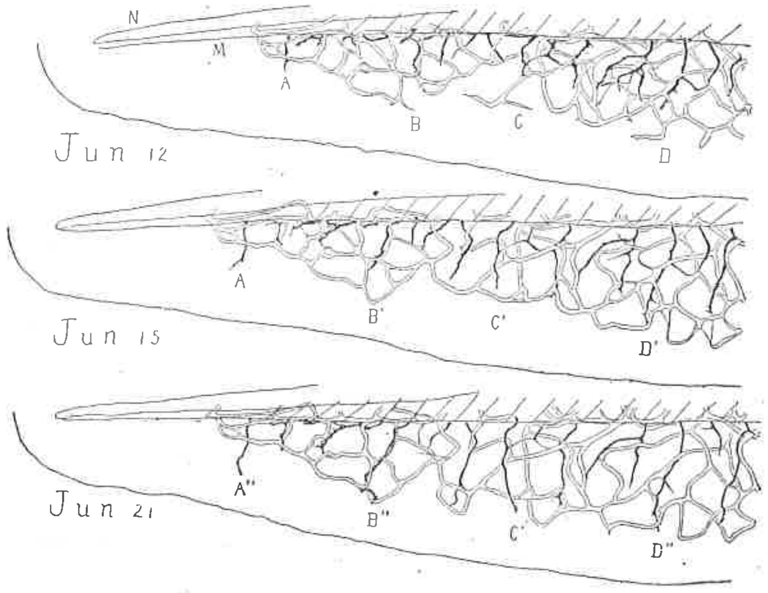

FIG. 1.—Three successive stages of growing lymphatics and blood-vessels in dorsal fin of Rana catesbiana larva. × 30.

Lymphatics in solid black, blood-vessels in lines. Vessels near tip are omitted. A,A', A", etc., indicate same vessels in different stages. M., muscle edge; N., notochord.

appeared independent of blood-vessels. In order to be quite certain, injection was resorted to. With the aid of Dr. Knower's[3] delicate injecting apparatus, a capillary glass tube with glass bulb to furnish pressure by air expansion, it was found possible to fill with India ink the vessels in question, quite independently of the blood-vessels.

[3]Knower: Anat. Rec., Vol. II, No. 5, Aug., 1908.

When the fin first becomes transparent, the only lymphatics to be seen are sprouts, some branched and some unbranched, often anastomosing with one another, extending out from under the cover of the axial musculature. They are of varying lengths, and in number correspond somewhat irregularly to muscle segments. Their tips stop considerably short of the limit of the blood-vascular area, so that we have three areas: a peripheral non-blood-vascular, non-lymphatic area, an intermediate blood-vascular non-lymphatic area, and a proximal area containing both blood-vessels and lymphatics, next the axial mass. As growth proceeds the lymphatics rapidly encroach upon the blood-vascular, non-lymphatic area, while both together grow into the non-blood-vascular area, until eventually the two systems are practically coextensive, reaching in older larvæ nearly to the fin border. During growth the primary anastomoses noted above disappear in part. Later there are gradually formed secondary anastomoses between neighboring capillaries. (Fig. 1.)

In a minute study of the growing capillary several questions at once present themselves. Does the lymphatic capillary grow out independently? What is its relation to blood-vessels, connective-tissue cells, wandering cells? Can we gain any clue as to the factors underlying the periphereal extension of lmyphatics—what is the stimulus?

If a single lymphatic capillary is selected for minute observation, it is found to present a characteristic appearance well described by Kölliker and Remak and well figured in Kölliker's Gewebelehre.[4] The wall is of irregular thickness; most of it is extremely delicate, while at intervals are nuclear thickenings. In the earliest observable stage there are small globules in this nuclear area (yolk?), which soon disappear leaving a granular appearance. From the walls extend numerous fine pointed projections, at various intervals, and of varying lengths. The diameter of the lumen of the lymphatic is considerably less than that of the blood-capillary. The lumen always extends beyond the last nuclear thickening. The tip ends in one or more fine pointed processes, usually somewhat longer than

[4]Von Ebner, A. Kölliker's Gewebelehre, III, 1899.

the processes at the sides. Into the bases of the larger of these processes, the lumen of the lymphatic may be followed for a short distance. The tip is never bulbous but rather pointed or angular.

When such a living tip is carefully studied, an extraordinary phenomenon is noted, for it is found that the appearance of the capillary is perpetually changing. So complex are these changes that they almost elude description. Most noticeable are the changes in the

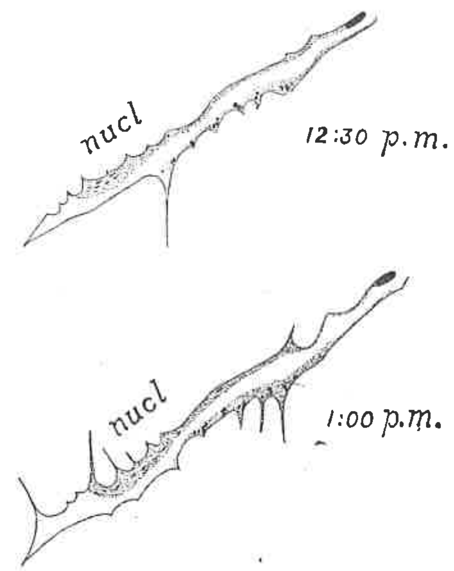

Fig. 2.—Successive drawings of same lymphatic tip in tail of Rana catesbiana larva, which had been stained with neutral red intra vitam. × 570. Nucl., nuclear thickening.

contour of the lymphatic. The fine pointed processes already noted are not constant, they are continually appearing and disappearing. They vary much in length, some form mere short blunt projections, while others reach a length of many micra with all intermediate gradations. They may appear at any portion of the wall, including the nuclear area. The longer processes are usually seen at and near the tip of the lymphatic, but not invariably—for occasionally long processes appear at the sides with those at the tip quite short. Just as they vary in length, so also they vary in the time of persistence. If careful drawings are made at ten or fifteen minute

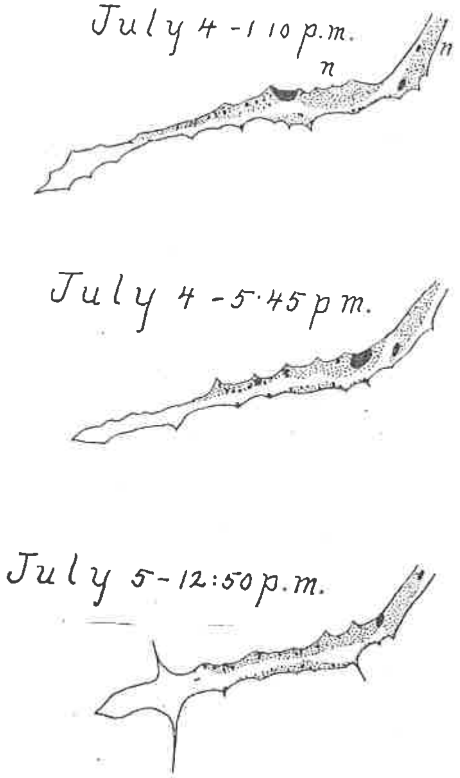

FIG. 3.—Same larva as in Fig. 2. Note shiftings of protoplasm of (n) nuclear thickenings as indicated by pigment granules.

intervals, in no two tracings will the pattern be identical. In fact, one gains the impression that were it possible to photograph the lymphatic at one minute intervals, careful study would reveal definite changes in successive pictures. Moreover, it is to be noted that these

observations were made on larvæ whose body processes were slowed by the use of chloretone. (Fig. 2.)

In addition to the unceasing change in the contour there are to be seen changes and shiftings in the wall. The nuclear thickenings are perpetually changing both shape and position. Now they appear crescent-shaped with the convexity encroaching on the lumen, again they become much elongated. Often the nuclear area is accompanied by a large black pigment granule. The nuclear thickening may shift its position relative to this pigment granule—being now at its proximal, now at its distal side. If a larva is stained with a weak solution of neutral red, there is a red granular coloration in the area of nuclear thickening. Later, instead of the red, there are to be seen here numerous small black granules. If a nuclear area containing these granules is observed closely, there will be noted a continuous shifting of the granules; sometimes several become grouped together, and again they separate. Two nuclear thickenings may appear in place of one,—and again two may appear to be moulded into one. (Fig. 3.)

While these changes are taking place, there may be, on the part of the capillary as a whole, a definite increase, or, more rarely, a decrease in length. The successive changes concerned in the increase are as follows: From a main lymphatic may be sent out numerous fine pointed processes. One of these processes persists and grows longer. As it increases in length, the lumen follows farther and farther into its base, until there is formed a short delicate-walled tube with one or two pointed tips, without a nuclear thickening. As this increases, gradually, from the wall of the main lymphatic, there passes into the branch a nuclear thickening. This whole tube now becomes longer and longer, the nuclear area passing further and further from the main lymphatic, but always remains at a distance from the tip. The lumen always extends beyond this nuclear thickening. (Fig. 4.)

After a time there appears in the wall a second nuclear thickening, and again a third and a fourth. These new thickenings seem to arise by division of the pre-existing ones, though definite proof of this has not yet been adduced by staining selected stages. Branches form in a

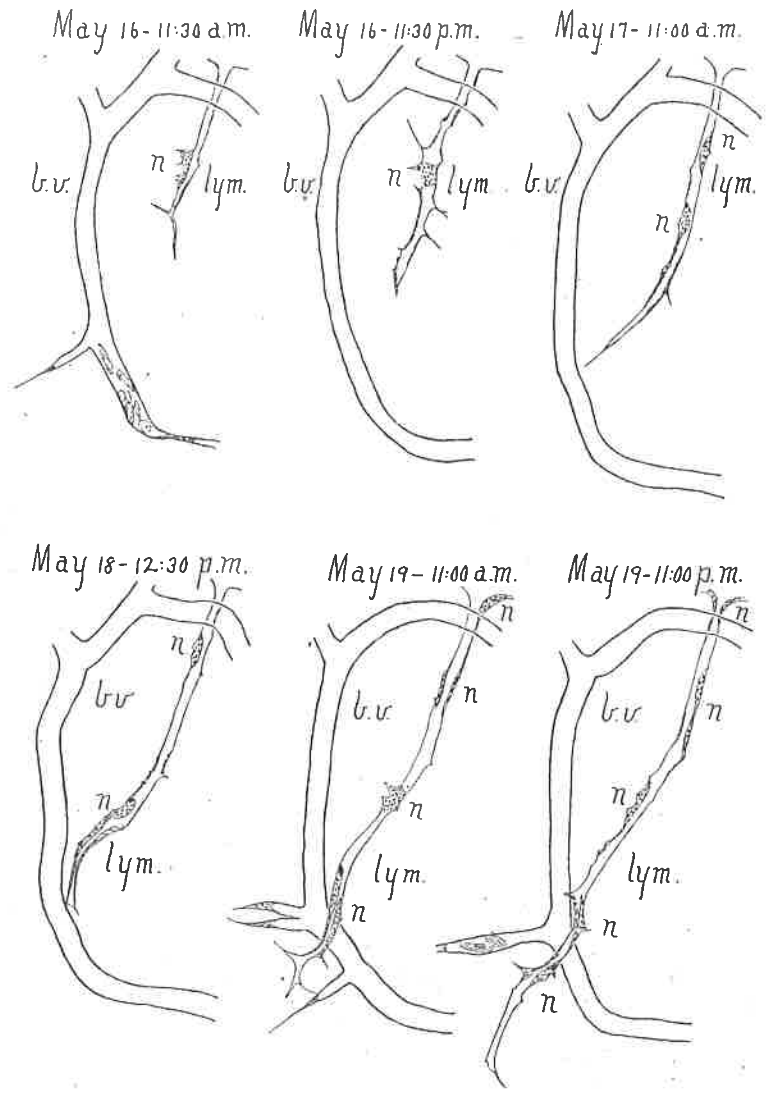

FIG. 4.—Successive stages in growth of lymphatic capillary in tail of Rana palustris larva. × 207. b.-v., blood-vessel; lym., lymphatic; n., nuclear thickening.

similar way. Anastomoses arise by the growing together of tips from neighboring capillaries or their branches, and appearance of a lumen in the solid connection thus formed. (Fig. 5.)

The shortening of a lymphatic capillary may be quite pronounced, for a branch which has attained the length of .2 mm. may be entirely withdrawn. The process of withdrawal has been studied less minutely than that of increase. It has been noted, however, that it takes

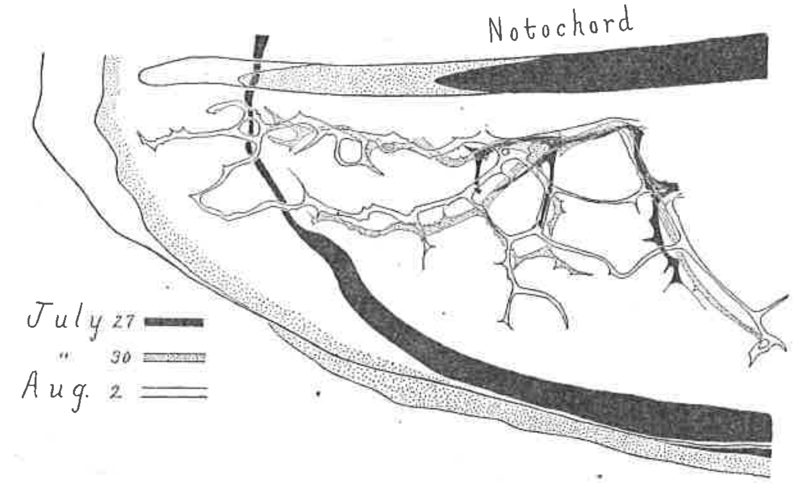

FIG. 5.—Three successive stages superimposed of lymphatics in tip of regenerating tail of Rana catesbiana larva. Camera lucida drawing. × 82. On July 21, about 4 mm. of tail of larva 12 mm. long was cut away. On August 2, regenerated part measured 3 mm.

place more slowly than does the increase; that two nuclear thickenings may approach one another; that the fine processes are shorter and less numerous; and that the lymphatic undergoing regression often ends in a long solid thread. In one instance such a long process contained a narrow lumen separated from the main lumen by a solid portion.

[a]Growth processes, especially branch and anastomosis formations, are well seen in a regenerating tail, for here the changes are more rapid than in the normal tail.

If we examine the growing lymphatic over a wide area, we find a continuous balance between neighboring sprouts. Of two or more sprouts which start in a new area, often only one increases while another may remain stationary or may be withdrawn.

Between neighboring sprouts which grow out to the fin margin the distance is fairly constant for any stage. As the larva grows, the tail expands in thickness and in length. The distance between neighboring lymphatic sprouts increases, and coincidently there arise branches and anastomoses. For a time these all lie in the same sagittal plane. Later, in the thicker portions next the axial mass, branches may be seen extending toward either surface of the tail. As the lymphatic grows longer, the lumen of the more central portions increases in size, and thus the capillary becomes converted into a larger duct.

The relation between the connective-tissue cells and the growing lymphatic was carefully studied. These cells, with their numerous branched processes form a richly anastomosing supporting network. Unfortunately their finest processes are invisible in the living animal, so that the relation of these finest fibrillæ cannot here be determined. Of the visible processes, the smallest seem sometimes to extend to the lymphatic, yet whether they actually join the lymphatic cannot be decided beyond dispute. The larger of the visible processes appear quite certainly to be independent of the lymphatic capillaries. The main bodies of the cells are distinctly separate from the lymphatic. In a study of the growing lymphatic nothing may be seen which would even remotely suggest the bodily addition of one of these cells to the growing capillary.

Wandering cells are always present in the fin, usually well scattered. No connection was noted between them and the growing lymphatic.

The relation between lymphatic and blood-vessel was studied with great interest, for around this point many controversies have arisen. As noted above, when the tail first becomes transparent, the blood-vascular area extends beyond the lymphatic area. Later the two become practically coextensive. The growth on the part of each is an invasive one—as is readily seen by noting their relations to selected fixed connective-tissue cells. During this invasion, however, the two remain totally separate.

Occasionally a lymphatic grows into an area in which no blood-capillary is present and in which none has been present. When the two are invading the same area, no regular relationship is maintained, for the lymphatic now runs parallel to and now crosses at right or oblique angle the blood-capillary. The lymphatic pays no attention to the new blood-capillary sprout, sometimes passing near by, and sometimes at a distance. Nor is the lymphatic influenced by the presence of the degenerating blood-vessels previously described. The entire process of degeneration may take place with no lymphatic near. Even when a lymphatic is near a degenerating capillary, no transfer of tissue may be detected. As no portion of a blood-vessel cut off from the main blood-vascular system has been observed, evidently there has been no opportunity for the growth of the lymphatic by appropriation of such a portion. Never have we observed an anastomosis between lymphatic and blood-capillary; or the direct passage of blood-cells from one to the other. In brief, all our evidence favors the absolute independence of the two systems in their peripheral extension. We thus differ from S. Mayer,[6] who finds the lymphatics formed in part from the blood-vessels. Mayer quite certainly confused the true lymphatics with the constricted blood-vessels—a confusion easily possible when the larva is placed on its side beneath a cover-slip, and the electric current used to assist the anesthesia. For it has often been shown that both electrical and mechanical stimuli cause constriction of blood-capillaries in the tail of the frog larva.

In quite late stages Dr. Knower has noted that the lymphatics near the axial mass are very close to the veins. Our observations in the living lymphatic have not yet been carried to this stage, but we have seen the same in the relation between the ventral caudal lymph trunk and the caudal vein, in cross-sections of the tail. So close are these two that they seem to share in part the same wall.

[6] S. Mayer: Sitzungsber, d. kais. Akad. d. Wissensch., Wien, Abt. 3, Bd. 91, 92, 1885.

It is not surprising that such pictures have given rise to difficulties in the interpretation of cross-sections.

Thus far the changes in the lymphatics which seem concerned in growth have been considered, but there is another process which is going on simultaneously, perhaps inseparably connected with growth, perhaps the phenomenon at the basis of the growth, namely, functional activity.

By accident a few red blood cells were extruded from a new forming blood-capillary sprout into the extra-vascular tissue. To our astonishment two days later a lymphatic capillary had grown down to this group of cells and was seen taking them in one after another, without the agency of leucocytes. We had subsequently many opportunities to observe a repetition of this process, which deserves a careful description. The changes undergone by capillary and cell are extremely characteristic. From the lymphatic is sent out a fine pointed process, indistinguishable from the processes previously mentioned. This gradually extends to the blood cell. After coming in contact with the cell, the delicate tip is lost to view against the deeper colored blood cell and for a time the mode of procedure may only be observed by noting the changes in the shape of the blood cell. After the fine tip has been lost to view, there appears opposite the point of contact, a slight blunt projection from the blood cell. This projection gradually becomes longer until the cell is pear-shaped. The narrow portion is always paler than the remainder of the cell. Gradually this nose-like projection becomes longer, until the cell assumes an elongated oval shape as if in a narrow passage. Soon there appears beyond the cell, the tip of the lymphatic, slightly dilated, ending in a point. Slowly the cell moves centralwards and as it advances it is usually preceded by a short constricted area of lymphatic, while the portion between the cell and the tip is dilated. Gradually the cell passes along the lymphatic to the main caudal trunk, along which it advances with a steady uninterrupted motion, as if borne on by a definite stream. In one instance in which the cell entered the lymphatic quite rapidly, the tip could be seen for about a half minute distinctly open. (Figs. 6 and 7.)

If we arrange in order of intensity the reactions on the part of the

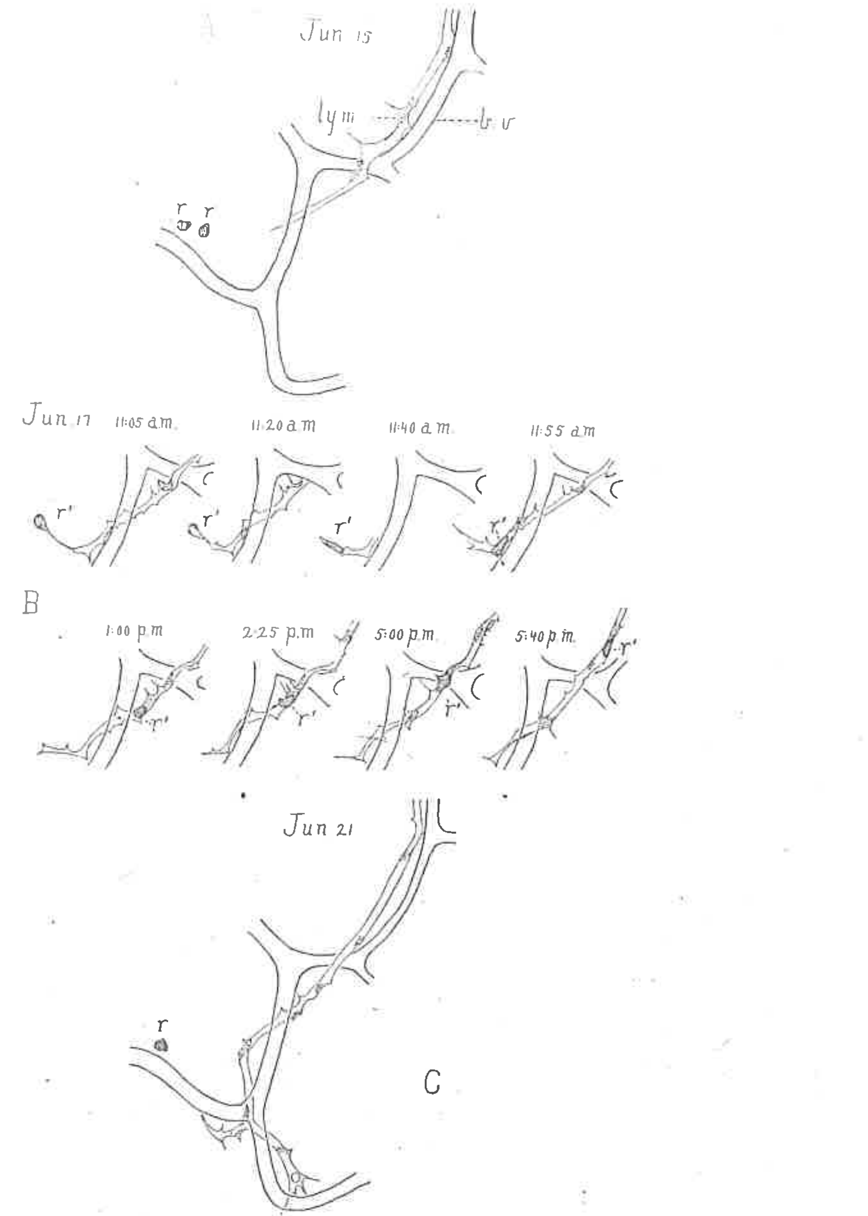

FIG. 6.—Successive stages in taking up of red blood cell by lymphatic capillary in tail of larva of Rana catesbiana. × 163. A before, B during, C after the process of taking up. r, r', two red blood cells, one of which (r') is removed, the other (r) left. lym., lymphatic; b.-v., blood-vessel.

lymphatic capillary as a whole to the stimulus caused by the presence of red blood cells in the tissue, we find the following series: If a single red blood cell is extruded, the lymphatic near by sends out a fine process, which takes in the cell and then disappears. If several

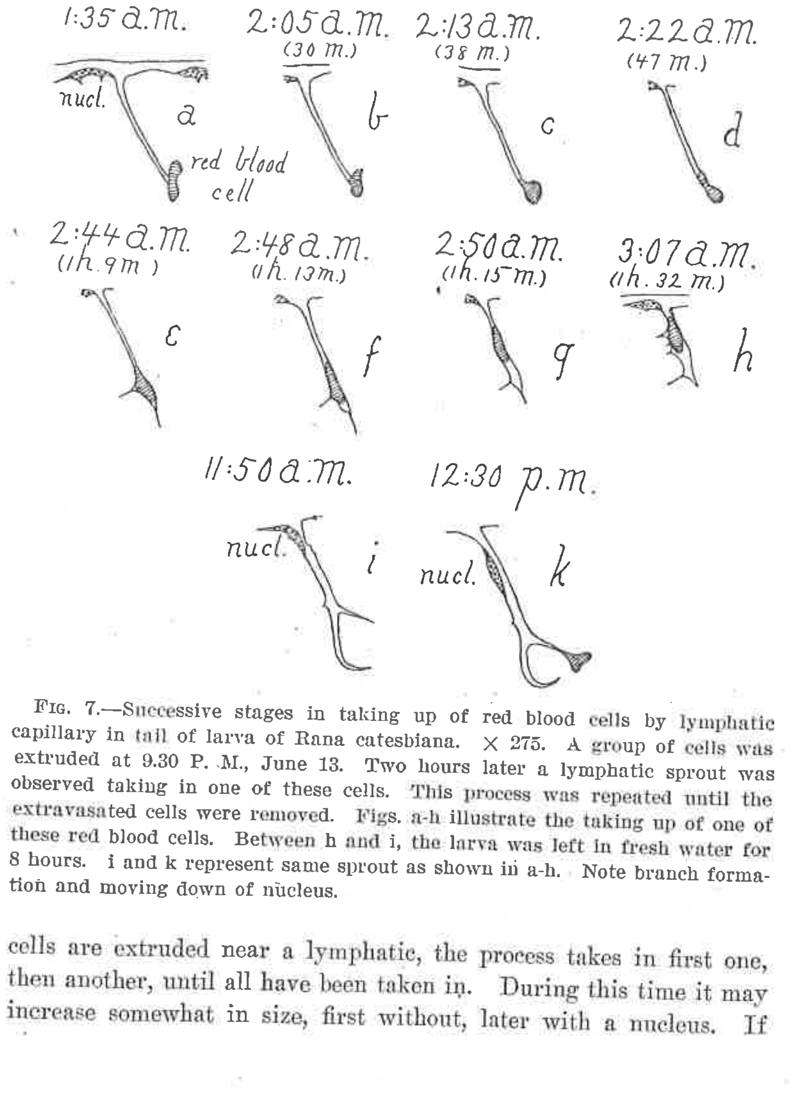

FIG. 7.—Successive stages in taking up of red blood cells by lymphatic capillary in tail of larva of Rana catesbiana. × 275. A group of cells was extruded at 9.30 P. M., June 13. Two hours later a lymphatic sprout was observed taking in one of these cells. This process was repeated until the extravasated cells were removed. Figs. a-h illustrate the taking up of one of these red blood cells. Between h and i, the larva was left in fresh water for 8 hours. i and k represent same sprout as shown in a-h. Note branch formation and moving down of nucleus.

cells are extruded near a lymphatic, the process takes in first one, then another, until all have been taken in. During this time it may increase somewhat in size, first without, later with a nucleus. If several cells are extruded at some distance from a lymphatic, the sprout sent out may reach a considerable size, with a nucleus, before the cells are taken in. If a large number of cells are extruded near a lymphatic, the sprout sent out may branch, the cells being taken in through two or more processes. After all the extruded cells are removed, the lymphatic formed may remain or may gradually be withdrawn.

The function changes in the lymphatic capillary revealed by this series of accidental yet beautifully clear-cut experiments give us a suggestion as to the meaning of the growth changes described above. If we compare the two, we find a striking parallel. In fact, if, in the description of the taking up of the cells, we should substitute for "red blood cell" a substance microscopically invisible, and make the necessary changes, the two descriptions would be practically identical. Thus the changes concerned with peripheral growth and with function seem inseparably connected. We cannot avoid the suspicion that the fine processes continuously sent out represent a reaction of the lymphatic to ultra-microscopic substances, perhaps products of cell metabolism, that the greater the accumulation of these substances the longer and more persistent the processes, and that it is the varying formation of such substances which regulates the peripheral growth of the lymphatic capillary.

In connection with recent studies of the lymphatic system the tail of the frog larva furnishes an excellent field for testing methods. The two devices principally used have been injections and serial sections. Some confusion has arisen in the results obtained by these two methods. It has been suggested that this confusion has arisen from limitations of methods, yet they have not been subjected to rigid tests. In the fin expansion of the tail of the frog larva, where every lymphatic may be seen, a record may be made of the living non-injected lymphatics to serve as a control. The lymphatics may now be injected to see whether the entire system will be filled with the injection mass, or the tail may be cut into serial sections to see whether a reconstruction may be made which shall correspond with the control drawing.

A bull-frog larva (Rana catesbiana) 16 mm. in length has been used for the latter test. A drawing was made of the lymphatics and blood-vessels during life. The tail was then cut into serial sections, ten micra thick and stained in hematoxylin and congo red. In attempting to reconstruct, it was found that, while blood-capillaries could often be fairly well reconstructed, it was impossible to reconstruct the lymphatics beyond the muscle margin. Further tests, however, are needed, with different stains, before definite statements are justifiable.

The injection method has been tested frequently and it is found that, while it carries us much farther than do reconstructions, the mass injected does not always fill the entire lymphatic system.

Let us now take a hurried glance over our results in their relation to present knowledge of the lymphatic system. Recent studies indicate that the first lymphatics arise from veins at various points. Of this primary origin we have made no test. As to the mode of extension of the lymphatic system into the different organs of the body, present views are divergent. Some observations lead to the conclusion that from several primary centers there is a centrifugal extension into the rest of the body, others that there is a transformation in situ of veins or mesenchymal tissue into lymphatics. The results here recorded are all in favor of the view that the peripheral lymphatics are formed by a process of centrifugal extension; that this extension, so far as relates to the endothelium, is strictly invasive, with no addition from connective-tissue cell, wandering cell or blood-vessel. Our observations indicate, however, that outside factors may exert a modifying influence on the growing lymphatic, that there is a close relationship between peripheral growth and functional activity. The question as to whether the lymphatic is open or closed is not definitely determined for all organs and tissues. The evidence here adduced favors a closed system of tubes, without direct openings into tissue-space or blood-vessel capable, however, of taking through its wall solid bodies of the size of red blood cells. But, perhaps most important, it has been demonstrated that the tail of the living frog larva, studied by special methods, furnishes an excellent field for the testing of problems relating to the growing and functioning lymphatic.

It is a pleasure to have this opportunity of expressing gratitude to Dr. Mall for his generous interest and numerous suggestions.

EXPERIMENTAL OBSERVATIONS ON THE DEVELOPMENT OF THE AMPHIBIAN EAR VESICLE.

BY

GEORGE L. STREETER,
University of Michigan.

The accompanying figure represents a reconstruction of the brain, eye and two ear vesicles of a tadpole about one month old, in which the experiment was made of transplanting the left ear vesicle to the right side in the space between the normal right ear vesicle and the eye. This experiment was made as a supplement to a series of

similar experiments showing the effect of change in environment upon the posture and development of the labyrinth, and which have been previously reported (Jour. Exper. Zool., Vol. IV, 1907).

In the present experiment the effort was made to determine the influence of two adjacent ear vesicles upon each other; to see if on transplanting a very young ear vesicle, while still a simple primitive epithelial cup, and placing it against another similar ear vesicle, whether the two would fuse and develop into a single large labyrinth,as has been supposed to occur in cyclopia, or whether the transplanted vesicle would retain its individuality and continue to develop as a separate structure.

The experiment was carried out on Rana pipiens larvæ during the premotile stage, at a time when the ear vesicle consists of an invaginated epithelial cup just in the process of being pinched off from the deeper layer of the skin. The procedure adopted was similar to that used in the experiments previously mentioned; in this case the left vesicle being loosened from its natural bed and transplanted in a pocket in the loose tissue closely against the front