STRUCTURE OF LIMULUS HEART MUSCLE.

WALTER J. MEEK.¹

Since the physiology of the *Limulus* heart has been so carefully investigated and that organ has been used so successfully in attacking many physiological problems, it has seemed desirable that something should be known concerning its histology. The chief objection to applying any of the results obtained on this form to the vertebrate heart has been that *Limulus* is a representative of a group unique in the animal kingdom and well removed even from its nearest relatives. This fact makes it all the more desirable to investigate the heart musculature in this form and to find what similarity, if any, it bears to the vertebrate type.

MATERIAL AND TECHNIQUE.

Heart tissue from adult *Limuli* was used. The most of the material was prepared at Wood's Hole where fresh tissue was also examined. The hearts from some animals that had been shipped to Chicago were also used. These animals were in good condition and nothing atypical was found in the tissues.

A number of fixatives were used, including Carnoy's solution, Perenyi's fluid, acetic-sublimate, and Zenker's solution. Of these Zenker's proved by far the most serviceable and satisfactory. The hearts were split open, washed in sea water to free them from blood

¹I take pleasure in thanking the Marine Biological Laboratory for material and also Dr. Bardeen and Dr. Erlanger, of the University of Wisconsin, for technical assistance. My thanks are also due Dr. Carlson, of the University of Chicago, for advice concerning the work.

and leucocytes, and then small pieces immersed in the fixative. The usual methods of embedding in paraffin and sectioning were employed. Sections were made from 3 to 6 micra in thickness.

Several different stains were employed, but iron hæmatoxylin followed by eosin was found to be the best for general study. The combination has been used largely by investigators and a comparison with their work therefore becomes easy. Orange G and fuchsin were frequently used as counter stains. In studying connective tissue distribution iron hæmatoxylin followed by Van Gieson's picro-fuchsin gave good results. A stain equally good and used with success was Mallory's connective tissue stain. Vanadium hæmatoxylin was also used in an attempt to demonstrate a sarcolemma. Fresh material was also treated with silver nitrate.

Fresh material was teased and studied in sea water or salt solution. Fresh tissue was also macerated in 20 per cent nitric acid, 40 per cent potassium hydroxide, 30 per cent alcohol, and saturated ammonium carbonate. Nitric acid is especially good as a macerating agent, since the preparation may be washed, passed into glycerine, and preserved. Material fixed in Zenker's solution and preserved in 70 per cent alcohol was also found to tease easily. These preparations could be stained with hæmatoxylin and eosin and then showed nearly all the details clearly.

GENERAL MORPHOLOGY.

The heart of *Limulus* is a hollow sack 15-20 centimeters long and 1.5-3 centimeters wide. It is swung in the pericardial sinus by eight pairs of connective tissue ligaments, the alary muscles, which form a lateral support for the heart and extend to the pericardium with which they fuse. On its dorsal surface the heart has eight pairs of ostia. The ostia are narrow slits connecting the pericardial cavity with the lumen of the heart, and they are supplied with inward projecting connective tissue lips which make efficient valves. Leaving the organ are eleven arteries; four pairs of lateral arteries from the four anterior segments, two aortic arches, and one median aorta. The aortic end of the heart is provided with a strong connective tissue valve around which the heart musculature curves concavely

on the dorsal side. The posterior end is attached to the carapace by a broad sheet of connective tissue. The outer surface of the heart looks as if it were longitudinally striated, an appearance which is due to a layer of elastic tissue strands.

MICROSCOPIC HISTOLOGY.

In cross section the heart is somewhat triangular in outline and may be seen to consist of three layers. (See Fig. 1.) The median layer (Fig. 1, b) is a fine, dense, connective tissue support, termed the basement membrane (Patten and Redebaugh, 1899). Outside of this is the longitudinal layer of elastic tissue fibers mentioned above. Inside the basement membrane is the muscular layer which consists of branching, anastomosing strands, arising from the basement membrane and running mostly in a circular direction. The muscular layer is thickest at the angles of the heart and thins out over the aortic valve. It is with this layer that we are most concerned.

By reference to Fig. 1 the general characteristics of the muscular heart wall can be understood. Trabeculæ of striated muscular tissue arise from the basement membrane, branching and anastomosing until a spongy heart wall is formed. No membrane limiting the lumen corresponding to the endocardium of the mammalian heart is present. In only one region is the lumen lined and that is at the aortic valve where for a short space the muscle layer lies between two connective tissue layers, the basement membrane above and the tissue of the valve beneath.

The blood circulates freely around the strands of the heart, passing into all the interstices and crevices, thus bathing the entire musculature. This arrangement for securing nutriment seems to be quite sufficient. A small amount of connective tissue appears between the trabeculæ, especially in the thicker parts of the heart wall. This can easily be traced back in origin to the basement membrane. It would seem that capillaries might enter along with this tissue, but as far as can be determined they do not do so, unless it be in the deeper parts immediately adjacent to the basement membrane.

A small fragment of the heart wall macerated in 20 per cent nitric

acid and teased with needles gives such pictures as represented by Fig. 2. The trabeculæ are at once apparent. They are of all sizes and run for the most part circularly around the lumen of the heart. These strands branch freely in all directions and join neighboring strands. Preparations teased in this way show even more plainly than the cross section described above, that the heart is a great network of muscle, whose strands form a spongy wall or a thick solid one according as they are mingled together. In the heavier heart walls the strands branch with rather acute angles, and in life the meshes between are no doubt mostly potential spaces. In the parts near the lumen the meshes are large. Often many trabeculæ fuse and form large sheets of muscle, which are really only large trabeculæ, since they again divide and the parts reunite with neighboring strands. (See Fig. 3.)

On teasing these trabeculæ with fine needles it is seen that they tend to split into smaller strands along lines fairly well determined at least for short distances. These smaller strands which may be regarded as the real heart fibers branch and anastomose with each other within the primary trabeculæ. This arrangement is particularly visible in tissue macerated in Zenker's solution, stained, and examined in glycerine under high power. Fig. 4 shows this arrangement semi-diagrammatically.

So far as can be determined neither the trabeculæ nor the secondary anastomosing strands composing the larger trabeculæ, end freely. Marceau (1904) has described such a condition in the lower vertebrates. He finds a large number of heart fibers ending freely among the trabeculæ in the Fishes, Batrachians, Saurians, and Ophidians. In the Chelonians and Crocodilians the free endings are quite rare, and in the higher vertebrates they do not occur at all. In teased specimens of *Limulus* heart muscle the endings found are always such as those shown in Fig. 2. The smaller branches here represent the heart fibers and it is very apparent that the blunt ends are due to transverse rupture by the needles. Conical or filiform terminations, which would be the characteristic shape or natural endings, have not been found. If they do occur they must be somewhat rare. In this particular the heart of *Limulus* is like the higher vertebrates.

The structure thus far described has been made out by teasing in macerating fluids. For more definite work stained preparations are necessary. The chief interest centers in cross and longi sections of the trabeculæ. Such sections determine the accuracy of the ideas already obtained and also give the structure of the fibers making up the trabeculæ.

When a specimen is sectioned parallel with the long axis of the heart most of the trabeculæ are seen in cross section. The field is filled with oval, circular, triangular, or short ribbon-like areas. These are the trabeculæ, and it is apparent that they vary greatly in size and shape. The smallest are about 12 micra, and the largest observed were about 90 micra in diameter. Near the basement membrane the areas are closely appressed, while toward the lumen there is considerable space between. The arrangement is irregular and the whole appearance is what one might expect from cutting across strands that form a spongy heart wall.

Figs. 5, 6, and 7 show four of these trabeculæ in cross section. The greater portion of each area is mostly filled with the cross sections of the contractile fibrils which appear as black dots. These have no very definite arrangement. In some cases they seem to have a reticulated appearance, as in Fig. 7. In others they are in more solid masses, as in Figs. 5 and 6. Only in the smallest is the strand ever completely filled with fibrils. Between the fibrils sarcoplasm is of course present. Clear areas are nearly always present in the cross sections. Some of these may be due to vacuolization produced by the fixative, but a certain number probably represent the poorly staining protoplasmic columns of the muscle fibers. In these columns are found the nuclei of the muscle fibers. Surrounding the fibrillar area is a thin cylinder of protoplasm which is bounded externally by a fine but perfectly definite line. Within this boundary and lying in the protoplasmic cylinder are found nuclei which in cross section resemble those found in the protoplasmic cylinder. (See Fig. 7 for illustration of these details.)

The number of nuclei in the clear areas of the fibrillar portions is variable; there may be of course none, and there may be as many as five or six in the larger trabeculæ. When more than one nucleus

appears at a given level each one probably belongs to a separate so-called fiber. The territory of each fiber is, however, impossible to determine, a fact that is due to the irregular arrangement of the fibrils. Fig. 6 illustrates this point. Here two nuclei appear and one would judge that at least two fibers composed the trabecula, but it would be impossible to divide the fibrillar area into two very definite regions. This fact is due to the free branching and anastomosing of the fibers within the primary trabeculae. Since these secondary strands have no well defined boundaries, yet branch and fuse with each other, the heart muscle must be considered a syncytium. In a syncytic structure one can scarcely speak of definite cells or fibers and this is plainly borne out by the confused fibrillar areas in the cross sections of the trabeculae. The contractile elements form strands which branch and fuse. These strands may be called fibers or cells for convenience, but they must not be confused in structure with such fibers as we have in skeletal muscle.

In longi sections, cross and longi striation is well brought out by the iron hæmatoxylin method. As in the heart muscle of all other forms it is evident that each contractile fibril is continuous throughout the entire musculature, ending only where the muscle fiber itself takes its origin. The clear areas of the cross sections now appear as longitudinal clefts mostly filled with protoplasm. In these lie the muscle nuclei. Fig. 8 shows a trabecula illustrating these points. These muscle nuclei are long and narrow in diameter, with edges which curve out toward the membrane of Krause. There is a scant chromatin network and at least one nucleolus is usually visible. The fibrils are grouped into strands, which illustrates the inner syncytic structure already discussed. Outside of these strands is again seen the cylinder of protoplasm and outside of this the fine linear boundary (*c* and *f* in Fig. 8). The peripheral nuclei are now seen to be oval in shape. They equal the muscle nuclei in diameter, but otherwise do not resemble them closely. The boundary membrane appearing as a fine line (*f* in Fig. 8) is attached to the Z line of the muscle fiber, the membrane of Krause, and for this reason it appears in a series of festoons rather than a straight line. It has proved quite a task to decide what relation this outer protoplasmic

cylinder with its limiting membrane and peripheral nuclei bears to the contractile portions within. The boundary line at first sight is strikingly similar to the membrane Heidenhain figures as a sarcolemma (1901) in human heart muscle. There is the same protoplasmic area just outside the outermost fibril and the membrane arches in festoons to meet the Z line. This gives the same effect that McCallum describes in mammals (1897), where he says the rounded edge of the sarcoplasmic discs makes up the edge of the fiber. The presence of nuclei just beneath this membrane seems opposed to the view that a sarcolemma is present unless it is assumed that there are two kinds of nuclei present in the fiber, one being at the periphery to attend to the growth of the cell. The facts are without question, it being merely a matter of interpretation. Of course if the trabeculæ were considered as having a sarcolemma the strands within could no longer be considered as the real heart fibers. The structure would still be a syncytium, but the significance of the secondary syncytium within the trabeculæ would be changed.

Most observers, however, agree that the lower forms do not have a sarcolemma (Renaut et Mollard, 1904), the heart fibers being bare. The simpler explanation would be that the trabeculæ are provided with a connective tissue sheath. This was tested by using standard connective tissue stains, and it was found that the limiting membrane stained blue with Mallory's and pink with Van Gieson's. Van Gieson's was not entirely decisive, however, since the Z line also took the same color. By keeping sections twenty minutes in the acid fuschin solution of Mallory's stain and then reducing the time in the aniline blue solution to about three minutes, it was found that the limiting membrane always stained blue, while Krause's membrane often took a bright purple color. This differentiation in color would indicate that the two structures were different. Heidenhain (1901) found that vanadium hæmatoxylin stained the sarcolemma and the Z line both blue, connective tissue taking a much lighter bluish color. In *Limulus* sections vanadium hæmatoxylin stained the basement membrane a faint blue and the muscle substance an orange, which seemed to indicate that the stain was ripe and working properly. The membrane in question, however, seemed little if at all

affected by the stain, and this is what one would expect if the structure were connective tissue. Being thin it would stain so faintly that it would make but little impression.

These results enable us to formulate our conception of the heart musculature. The heart wall is made up of branching, anastomosing trabeculæ which are individualized by connective tissue sheaths. Within these trabeculæ are the naked strands of muscle which also branch and fuse and thus form a true heart syncytium. Possibly the connective tissue sheath takes the place of a sarcolemma by functioning as a dialyzing membrane for the exchange of the nutriment. If it is related in any way to the development of the sarcolemma in higher forms it must be noted that the relation here is to the trabeculæ and not directly to the fibers within.

In *Limulus* then we have the same general conditions that are found in the lower vertebrate heart. There are no free endings of muscle fibers, but these even disappear with the higher reptiles. The connective tissue sheath seems more closely appressed than in the case of most forms figured by Marceau. With these two differences, neither of which can be fundamental, the heart muscle can scarcely be told from that of the lower vertebrates. To convince oneself of this it is only necessary to compare Marceau's figures of the lower vertebrates, particularly the fishes, frog, and turtle, with those figured for *Limulus*.

Physiologically the important fact brought out by a study of the *Limulus* heart musculature is that it is a syncytium practically indistinguishable from that of the vertebrate heart. Carlson (1904) has shown conclusively that the normal myocardium of *Limulus* is incapable of physiological conduction. When the nerve ganglion is removed the muscle responds to a local stimulus by a local contraction and there is no conduction whatever of the contraction to a more distant part of the organ. We have here then a heart, syncytic in structure, with a protoplasmic continuity throughout, which nevertheless does not conduct under normal conditions. This fact, that muscular continuity may be associated with the absence of muscular conduction, renders invalid the argument drawn from the syncytic structure in favor of the myogenic theory of conduction in the vertebrate heart.

A few more points may be of interest. The trabeculæ take their origin from the basement membrane. Here and in the connective tissue guarding the ostia are the only places where fibers have been found to end. The ending is not often conical, as noticed in skeletal muscle. The fibers rather fray out and the connective tissue inserts itself between the fibrils. The connection is thus one of fibrils to connective tissue rather than that of an entire strand.

The trabeculæ are often separated by narrow clefts into which the connective tissue sheath inserts itself, as may be seen in Fig. 9. This makes the trabeculæ on the average rather narrow in diameter. The function of the sheath in Fig. 9 may be a support, since the cross striation shows that the fibrils are not in synchrony.

Bands of Eberth are of course not found in the *Limulus* heart. These structures do not appear in man until after birth and are not found lower in the animal kingdom than in the birds. Their absence in *Limulus* is therefore of no significance.

SUMMARY.

The heart musculature of *Limulus* is a double syncytium. It consists of branching, anastomosing trabeculæ individualized by connective tissue sheaths, within which heart fibers branch and anastomose, thus forming a continuous network of contractile tissue.

The heart tissue studied agrees with all heart tissue in these fundamental facts.

1. It has the regular cross striation.
2. Contractile fibrils are continuous throughout the musculature, ending only where the fibers take their origin.
3. The trabeculæ are provided with a peripheral covering which may serve as a dialyzing membrane.
4. The heart muscle is a syncytium.

The fact that the heart musculature is syncytic would show that a continuity of musculature is not decisive evidence for the myogenic theory of conduction.

BIBLIOGRAPHY.

- CARLSON, A. J., 1904. The Nervous Origin of the Heart-beat in *Limulus* and the Nervous Nature of Coördination or Conduction in the Heart. *Am. Jour. Phys.*, Vol. XII, p. 67.
- HEIDENHAIN, 1901. Ueber die Structur des menschlichen Herzmuskels. *An. Anz.*, Vol. XX, p. 33.
- MACALLUM, 1897. On the Histology and the Histogenesis of the Heart Muscle Cell. *An. Anz.*, Vol. XII, p. 609.
- MARCEAU, 1904. Recherches sur la structure et le developpement compare des fibres cardiaque. *Ann. des Sc. Nat. Zool.*, Vol. XIX, p. 245.
- PATTEN AND REDEBAUGH, 1899. Studies on *Limulus*. *Jour. Morph.*, Vol. XVI, p. 129.
- RENAUT ET MOLLARD, 1904. Le Myocard. *Rev. Gen. d'Hist.*, Vol. I, p. 213.

EXPLANATION OF PLATE I.

FIG. 1. Semi-diagrammatic portion of entire heart wall. a—branching muscular layer. b—Basement membrane. c—Elastic tissue.

FIG. 2. Trabeculæ teased in 20 per cent nitric acid. Drawn with camera lucida. Leitz Ob. 6, Oc. 4. a—Blunt end of small strand.

FIG. 3. Large flat trabecula. Teased from 20 per cent nitric acid and drawn under camera lucida as above.

FIG. 4. Semi-diagrammatic. Constructed from thick paraffin section. Anastomosing trabeculæ are shown with anastomosing strands of muscle within. a—Sheath around trabeculæ. B—Strand of contractile tissue. d—Nucleus of contractile strand. c—Nucleus of sheath.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

EXPLANATION OF PLATE II.

FIG. 5. Cross-section of two small trabeculæ. Stained in iron hæmatoxylin and followed by Van Gieson's. Leitz Ob. 12, Oc. 3. Camera lucida. $\times 1,000$. Lettering the same as Fig. 7.

FIG. 6. Cross-section of a trabecula. Stained in iron hæmatoxylin and followed by Van Gieson's. Leitz Ob. 12, Oc. 3. Camera lucida. $\times 1,000$. Lettering the same as Fig. 7. Shows two nuclei at same level, each in its axis of protoplasm.

FIG. 7. Cross-section of a large trabecula. Stained in iron hæmatoxylin followed by eosin. Leitz Ob. 12, Oc. 3. Camera lucida. $\times 1,100$. a—Nuclei of connective-tissue sheath. b—Connective-tissue sheath around trabecula. c—Protoplasm within sheath. d—Nucleus of muscle fiber. e—Axial protoplasmic region in which nuclei are embedded.

FIG. 8. Longi section of a trabecula. Iron hæmatoxylin followed by Van Gieson's. Leitz Ob. 12, Oc. 3. Camera lucida. $\times 1,100$. f—Connective-tissue membrane. g—Strand of contractile fibrils. Other letters the same as in Fig. 7.

FIG. 9. Longi section stained with Mallory's connective-tissue stain. Color shown as in preceding figures. Leitz Ob. 12, Oc. 3. $\times 1,100$. a—Cleft dividing the trabecula into which the connective tissue sheath has entered. The appearance is similar to what Heidenhain figures as the "daughter sarcolemma."

FIG. 10. Longi section. Iron hæmatoxylin followed by eosin. $\times 1,100$. a—connective-tissue sheath which persists between the fusing trabeculæ until the cross striation becomes synchronous.

FIG. 5.

FIG. 9.

FIG. 7.

FIG. 6.

FIG. 10.

FIG. 8.