THE ORIGIN AND STRUCTURE OF A FIBROUS TISSUE WHICH APPEARS IN LIVING CULTURES OF ADULT FROG TISSUES.*

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Plates 31 to 36.

INTRODUCTION.

Within the past few years a considerable amount of investigation has been done upon the cultivation of living tissues outside the body, and various phases of the subject have been studied. Thus far, however, it appears that there has been very little consideration of the reactions that occur in the plasma clot of the tissue cultures. Harrison,[1] among others, has shown that a supporting framework of some sort is necessary in the medium in which the living tissue is imbedded in order to obtain a prolific growth and movement of the tissue cells. The fibrin net which is present in plasma or lymph cultures forms this framework. If the plasma or lymph be defibrinated and the resulting serum used as a medium in which to cultivate the tissues there is, in general, comparatively little growth or movement of the tissue cells. In this paper[2] the results of experiments with living cultures of adult frog tissues are presented, which show that a definite reaction frequently occurs in the plasma clot. As a result the fibrin net present in the clot gradually loses its typical structure and becomes transformed into a new fibrous tissue closely resembling in its appearance, structure, and function various types of white fibrous connective tissues.

The thanks of the author are due to Professor Ross G. Harrison

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[1] Harrison, R. G., The Reaction of Embryonic Cells to Solid Structures, *Jour. Exper. Zoöl.*, 1914, xvii, 521.

[2] A report of this work was given at the meeting of the American Society of Zoölogists held at Philadelphia, December 31, 1914.

for his suggestion of the problem and for his invaluable assistance and advice from time to time as the work progressed.

MATERIAL AND METHODS.

In the work reported in this paper, the tissue cultures have all been made by the hanging drop method, and the usual precautions to keep the cultures sterile have been observed. This method of tissue culture has become quite well known within the last few years, and a full description of the technique will not be given in this paper.[3] The plasma used has been secured in the following manner: An anesthetized frog is opened ventrally and the heart exposed. A hypodermic syringe (a five cubic centimeter glass Luer with a short fine needle is very satisfactory) is then inserted in the ventricle. If the frog has not been anesthetized too heavily, a considerable amount of blood (one to two cubic centimeters) can then be drawn out of the ventricle. When all the blood that can readily be secured —it may take some minutes—is obtained, the syringe is withdrawn and the blood centrifuged. In the present experiments it has been the rule to use only fresh plasma. In most cases both the plasma and the tissue for imbedding in it were obtained from the same animal. The tissue taken from the animal was placed in Ringer's solution and cut into small pieces.

During the course of the experiments it was found necessary to preserve and section a great many of the preparations, and in order to do this it was necessary to get the plasma clot with the imbedded tissue free from the cover-glass without injury. To accomplish this it was found convenient to coat the cover-glasses before being used with a thin coat of celloidin. This was done by immersing the cover-glasses in a weak solution and then letting them dry. The celloidin solution is sterile but if, for any reason, there is need of

[3] The reader is referred for further details regarding technique and for a general survey of the field to papers by the following authors: Harrison, R. G., The Outgrowth of the Nerve Fiber as a Mode of Protoplasmic Growth, *Jour. Exper. Zoöl.*, 1910, ix, 787. Carrel, A., and Burrows, M. T.,Cultivation of Tissues *in Vitro* and Its Technique, *Jour. Exper. Med.*, 1911, xiii, 387. Oppel, A., Causal-morphologische Zellenstudien. V. Mitteilung. Die aktive Epithelbewegung, ein Faktor beim Gestaltungs- und Erhaltungsgeschehen, *Arch. f. Entwcklngsmechn. d. Organ.*, 1912, xxxv, 371.

sterilizing the cover-glasses it can be done in a dry heat. The celloidin will stand a temperature of 100° C. without apparent change. Using these celloidin-coated cover-glasses it becomes a comparatively simple matter to get the plasma clots with the imbedded tissue free from the cover-glass. In general the clot will remain attached to the cover-glass in the killing fluid and through the washing and dehydrating up to the absolute alcohol, at which point the celloidin will be dissolved and the preparation will, in most cases, free itself from the cover-glass with no injury whatsoever. The preparation can then be imbedded and sectioned in the usual manner. Sections of the preserved preparations were cut at eight microns. Various methods of staining the preserved cultures have been tried and a description of these is given in a later section of this paper.

STUDY OF THE LIVING CULTURES.

As shown in the accompanying table (table I) a total of 996 cultures have been made from various tissues of the adult frog during the course of these experiments, and of this number 846 have been under observation and form the basis for the results reported in this paper. It is possible by a study of the living preparations to follow closely the reactions that occur in the plasma clot, and photographs

TABLE I.

Summary of the Results of the Experiments.

Material imbedded.	Total No. of cultures made.	No. of cultures rejected.	No. of cultures studied.	No. of cultures in which fibers appeared.
Liver	476	81	395	86
Spleen	170	50	120	55
Muscle	115	19	96	37
Dead tissues and corpuscles	135	None	135	0
Living blood corpuscles	30	None	30	3
Starch grains	20	None	20	0
Blank clots	50	None	50	0
Total	996	150	846	181

of the various stages can be made while the cultures are alive. The reactions that occur in the clot appear to be the same with any of the tissues tried, and the description given will suffice for any of the preparations. The plasma in these tissue cultures, as is well

known, clots within a few minutes after the piece of living tissue has been added, and the imbedded tissue is firmly held by the fibrin net which forms in it. This fibrin net is of the same character as would be formed whenever plasma clots, and when observed under the microscope shows a typical fibrin net structure. This condition of the fibrin net continues in a high percentage of the preparations during the entire history of the culture (table I). Growth may occur in the imbedded tissue and the cells move freely out into the clot and yet no transformation of the fibrin net take place. The tissues of the mature frog are very inert as compared with the embryonic tissues of various animals, and in many cultures very little or no growth or movement of tissue cells occur. The heart muscle tissue of the frog forms, as a rule, quite a definite exception to the above statement, and in most cultures of this tissue a considerable cellular growth and movement will occur. However, whether there has been any growth of the imbedded tissue or not, some of the cultures in the course of two or three days will show that a change is taking place in the plasma clot surrounding the tissue. In the region lying nearest to the imbedded tissue, small fiber-like structures appear. At first these are quite small and fairly indistinct. They are of the same color as the fibrin net but have a different index of refraction, and they are large enough to be easily seen with the low power of the microscope. These fiber-like structures increase in size and number and ramify in all directions through the clot. They generally reach a high state of development by about the fifth to seventh day after the making of the preparation. Their development, however, will continue after this until in many cases at the end of two weeks almost the entire plasma clot has been changed into a new tissue which has a very different appearance and structure from that of the typical fibrin net. So far as has been observed, and a number of the cultures have been kept alive for periods exceeding three weeks before they were preserved, these fibers which form in the plasma clot are permanent. The fibers are consolidated to the greatest extent near the imbedded piece of tissue and radiate from that region in all directions to the periphery of the clot. To one studying the fiber formation in the living cultures it appears that they are direct outgrowths from the imbedded tissue. Experiments

which are given later in this paper show that such is not the case, however, and that the fibers are formed from the elements of the fibrin net. The clots in which the fibers have formed become very firm and solid and very unlike in this respect an unchanged plasma clot in which the typical fibrin net is present. This fact is particularly evident when one tries to remove a clot from the cover-glass for permanent preservation. The clots in which the fibers have formed can be handled roughly without injuring them at all. The regular plasma clots are very tender and have to be handled with the greatest care when they are removed from the cover-glasses in order to prevent their serious injury.

Microphotographs have been made of a number of the living cultures to show different stages of the fiber formation. Figure 1 is a microphotograph of a culture of liver tissue at a magnification of 33 diameters taken when the culture was three days old. A few of the small, newly formed fibers are to be noted in the plasma immediately surrounding the upper edge of the tissue. All the fibers present in this culture are not seen, inasmuch as it was impossible to bring them all into focus. This preparation, in the size and number of the fibers present, is typical of the average condition found in a two to three day culture in which the reaction has taken place. Numerous examples, however, of still greater development have been observed. It has been found that the development of the fibers is more rapid and prolific with spleen tissue than it is with any other of the tissues that have so far been used. Figures 2 and 3 are both microphotographs of spleen cultures taken when they were four days old. The magnification is 27 diameters in both instances and the photographs show clearly how striking and prominent the fibers are in the living preparations. Numerous blood corpuscles can be seen scattered through the plasma. Figure 4 is a microphotograph of a nine day culture which has been magnified 40 diameters and gives one a clearer view of the network of fibers which has formed in the plasma clot. It can also be noted that around the edge of the imbedded tissue a specialized part of the plasma has developed and the fibers appear to arise from this region. Such a region is not present in the clot at first and the early fibers appear to arise from the edge of the imbedded tissue as shown in

figure 1. This region, in the older cultures, in which there is a transformation of the plasma, always occurs first in that region of the clot which is in direct contact with the imbedded tissue, and the changes which take place would therefore appear to be due to some action of the living tissue. The cultures from which figures 2 and 4 were taken were preserved, and a description of them is given later in this paper. In the cultures which are shown in figures 1 to 4 very little or no growth of the tissue cells took place, and, as has been noted, such is generally the case with most of the adult frog tissues. There are exceptions, however, and cultures have been under observation in which cell growth and movement as well as fiber formation could be noted. Such a condition is shown in figure 5, which is a photograph of a ten day liver culture. In this preparation a considerable number of fibers are to be noted in the plasma and others were present which are out of focus. To the left and below, it can be seen that a considerable outgrowth of cells from the tissue has occurred, among which are numerous large spindle cells. No relation is to be observed between these cells and the fibers. In cultures of heart muscle tissue a different condition is sometimes to be observed. An example of this is shown in figure 6, which is a photograph taken from a ten day culture of heart muscle tissue. In this culture quite a heavy fiber formation took place in the plasma surrounding the imbedded tissue during the first three or four days. Later spindle cells moved out from the tissue and followed the course of these fibers. This is the condition shown in the figure and several instances can be noted of the stereotropism of these cells. A similar condition has been observed in other cultures of this same tissue.[4]

STUDY OF THE PRESERVED CULTURES.

In order to make a histological examination of the fibers and their relations both to the imbedded tissue and the plasma clot, it was necessary to make and study mounted sections of the preserved cultures. A description of the methods used in preparing this material for microscopic study has already been given in a previous section of this paper.

In figure 7, which is a microphotograph of a spleen culture pre-

[4] Harrison, *Jour. Exper. Zoöl.*, 1914, *loc. cit.*

served at the end of the second day; an early stage in the fiber formation is to be seen. The fibrin net is for the most part unchanged, but a number of distinct fibers have been formed. These have arisen from the region of the clot lying nearest the tissue. Here the typical fibrin net structure has been partly lost, and it appears that there has been a consolidation or fusion of the fibrin elements to form a fibrous tissue. The evidence from the histological study of all the cultures apparently shows that the fibers have arisen through a transformation of the fibrin elements. However, the connection between the imbedded tissue and the fibers is so close in a great many instances that it is impossible to say from these observations alone that the fibers are not outgrowths of the imbedded tissue. Experiments, which are given in the following section of this paper, confirm the histological study of the cultures and make it certain that the new fibrous tissue arises from a transformation of the fibrin net. Figure 8 shows a microphotograph of a six day culture of spleen tissue at a magnification of 67 diameters. Figure 2, above, shows this same culture as it appeared while living at the time when it was four days old. In the two days intervening before it was preserved a considerable development of new fibers occurred, so that in figure 8 many more fibers are to be seen. This preparation is considered typical of a six day culture, and is worthy of careful consideration. The fiber formation shows a great advance over that pictured in figure 7. It will be noted that the fibrin net in the immediate vicinity of the imbedded tissue has been changed from the typical fibrin net structure into a dense, heavily staining tissue composed of wavy fibrous bundles in which, with sufficient magnification, the individual fibrils can be seen. From this dense fibrous area of the transformed fibrin net, bundles of fibers extend in all directions through the plasma clot. These bundles are heavier nearer the tissue and gradually grow smaller as they proceed out to the periphery and are finally lost in the undifferentiated fibrin net. They unite with other similar bundles of fibers at various places throughout their length. This condition can be seen in figure 9, which is a photograph of a portion of this culture magnified 667 diameters. In the upper part of this figure can be seen the dense fibrous tissue which lies next to the imbedded tissue, and proceeding from this area the wavy fibrous

bundles can be seen running to the less differentiated areas of the clot. These bundles can be seen to anastomose and interweave with each other to a considerable degree. In figure 10, which is also a photograph of a portion of this same culture magnified 1,000 diameters, the individual wavy fibrils which form the bundles can be seen. It is quite a difficult matter to show clearly the detailed structure of the fiber formation in the clots by means of microphotographs, especially with the higher magnifications. Accordingly in figure 11 a drawing of the same portion of this culture as is depicted in figure 10 is shown, which makes the structure of the transformed fibrin net still clearer. In the lower part of the figure a part of the imbedded tissue can be seen. In close contact with it is shown the heavy fibrous tissue with the bundles of fibers. These run to the periphery of the clot, gradually decreasing in size as they leave the region of the imbedded tissue, and are finally lost in the untransformed fibrin net. At the ending of the bundles, as well as in various other places shown in the drawing, an atypical arrangement of the fibrin elements can be noted. Apparently there is a fusion of these elements to form the fibrils and a grouping together of the fibrils to form the fibrous bundles. With the magnification used in this drawing ($\times$ 667) it is possible in many cases to trace the fibrils from their point of origin in the fibrin net into the fibrous bundles. The fibrin net in the region of the imbedded tissue has become almost completely transformed from its typical structure, and only small areas of the clot remain which are unchanged. In the outlying regions of the clot large areas of the untransformed fibrin net can be seen. The complete absence of all isolated tissue cells is to be noted in this region of the culture.

Another preparation of interest is shown in figure 12. This microphotograph is from a nine day culture of spleen tissue. In figure 4 is shown a photograph of this culture made while living and just before its preservation. The fiber formation in this culture did not begin as early as it usually does and the development of the fibers is not as far advanced as found in some of the younger cultures as, for example, in figure 8, which is a six day culture. In figure 12 attention should be called to the part of the plasma clot which is lying in contact with the imbedded tissue. In this area the

fibrin net has become so changed from its typical structure and takes a stain so much like the tissue, that in the photograph it is difficult to make out the exact boundary line between the clot and the imbedded tissue. This transformed area is similar to that shown in figure 8, but the transformation is more complete. From the central part of this area great bundles of fibers are given off which run to the periphery of the clot. The greater portion of the clot in this culture still retains its typical net structure.

If conditions remain favorable the fiber formation continues in the plasma clot until practically the whole area has become transformed into a new fibrous tissue. The fibrin net disappears and the clot in some of the older cultures appears as a reticular tissue. The preparation shown in figure 13, which is a photograph at 100 diameters, presents an example of such a case. This culture has two pieces of liver tissue imbedded in the clot and was fourteen days old when preserved. The appearance shown in this figure is not exceptional, and a number of other preparations have been made which show as advanced a condition of fiber formation. It can be noted that practically the entire clot has become changed into what appears to be a reticular tissue. Figure 14, which is a microphotograph of a portion of this culture at 667 diameters, pictures one of the least differentiated areas, and from this it can be seen that the clot has been almost completely changed in its structure. In figure 17 a drawing of a portion of this preparation may be seen which shows the structure of the transformed fibrin net more clearly than is possible with photographs. At both the upper and lower edges of the drawing are shown a few of the cells of the two pieces of imbedded tissue. The clot lying between these two pieces of tissue has almost completely lost its characteristic fibrin net structure and instead we find a new fibrous tissue containing a great many bundles of wavy fibers. To all appearances this new fibrous tissue is identical with true reticular tissue as pictured and described by Mall.[5] It is held by many authorities that the fibrils of reticular tissue are identical with the fibrils of areolar tissue.[6] In some cases it can even be demonstrated

[5] Mall, F. P., Reticulated Tissue, and Its Relation to the Connective Tissue Fibrils, *Johns Hopkins Hosp. Rep.*, 1893, i, 171, plates 17 and 18.

[6] Schäfer, E. A., Text Book of Microscopic Anatomy, in Quain, J., Anatomy, 11th edition, London and New York, 1912, ii, pt. 1, 123, 124, figure 203.

that the fibrils of reticular tissue are in direct continuity with the white fibrils of the adjacent areolar tissue. In other words, the fibrils composing a reticulum may join together to form the bundles of fibers in the nearby areolar tissue. Such a relation between the fibrils and the fibrous bundles is also found in the tissue cultures where, as shown for example in figures 11 and 17, the fibrous bundles are composed of a great many fibrils, and in many instances these fibrils can be traced to where they leave the bundle and join the fibrin net. The bundles of fibers are typical of those found in areolar tissue. They grow smaller as they get away from the imbedded tissue, and in the outlying regions we find them breaking up and the fibrils of which they are composed continuing to form a typical reticular tissue.

THE ORIGIN OF THE FIBERS.

Although the study of the prepared cultures gives evidence that the new fibrous tissue arises through a transformation of the fibrin elements of the clot, nevertheless the close connection existing in the cultures between the imbedded tissue and the bundles of fibers makes it impossible to say that they have not arisen as direct outgrowths of the imbedded tissue. In order to settle the matter definitely it was found necessary to make several series of experiments with special types of cultures.

In the first series a large number of cultures were made in which the living tissues were imbedded in defibrinated serum instead of plasma. The method used in this series was to take the plasma from the frog by the usual technique and to divide it into two parts. One part was used in making the regular type of plasma cultures with living tissues imbedded. The other part of the plasma was defibrinated and used in making other similar cultures. The two sets of cultures were identical in every way except for the fact that some had the plasma while the others had the defibrinated serum, and these latter owing to the absence of the fibrin could not form any clot around the imbedded tissue. The results obtained from these experiments have always been the same, namely, the serum preparations have always failed to show any fiber formation, while in many of the parallel cultures in which the plasma was used the transformation of the

fibrin net occurred. Such a result indicates that the fibers are formed by a transformation of the fibrin net and not as outgrowths of the imbedded tissue.

In another series of experiments preparations were made as usual with plasma, but no tissue was imbedded. The plasma will clot of itself shortly after having been exposed to the air, and it was thought worth while to determine if fibers would form in these blank clots in which no tissue was present. Blank cultures were also made in which the plasma was coagulated by the addition of an extract obtained from fresh muscle tissue. This extract was obtained by mashing muscle tissue of the frog in a small amount of Ringer's solution and then centrifuging it to get rid of the blood corpuscles and small pieces of the tissue. By adding a drop of this extract to the plasma a very firm clot can be had in a few minutes. All the cultures of this series gave negative results and no transformation of the fibrin net occurred.

In a third series of experiments plasma cultures were made as usual, except that either living blood corpuscles, blood corpuscles which had been killed by heating, or starch grains were imbedded in place of living tissues. In the cultures in which the dead corpuscles and the starch grains were imbedded negative results were obtained and no change of the fibrin net occurred. On the other hand, in three of the thirty cultures in which the living blood corpuscles were imbedded a transformation of the fibrin net occurred. In figure 15 is shown, at a magnification of 100 diameters, a microphotograph of a culture in which living blood corpuscles were imbedded without any tissue. It can be noted that in the region in which the corpuscles are present quite a complete transformation of the fibrin net has occurred and a new fibrous tissue with bundles of long wavy fibers has been formed. A study of the fibers reveals no apparent difference in structure from those formed when living tissue is imbedded in the plasma. The outlying regions of the clot still maintain the typical fibrin net structure. The fact then that the transformation of the fibrin net can occur and the fibers arise in cultures in which only living blood corpuscles have been imbedded shows definitely that the fibers are not formed as outgrowths of the imbedded tissue, but they have been formed through a transformation of the fibrin net.

This result confirms that obtained from the histological study of the prepared cultures, which is given above.

FACTORS INVOLVED IN FIBER FORMATION.

The experiments just mentioned in the previous section showed that the new fibrous tissue arose by a transformation of the fibrin net. They also indicated that this transformation was caused by the presence of living tissues or cells in the plasma, inasmuch as cultures in which starch grains or dead blood corpuscles were imbedded, as well as the blank clots, all failed entirely to show any transformation of the clot. It was deemed wise to experiment further in an endeavor to find out if the transformation of the clot was dependent upon the presence of living tissues. To do this several series of parallel cultures were made use of. In order to make the method of experimentation clear it may be well to give the complete data of one such series of cultures. On February 14, 1914, the following cultures were made in the same manner from the same plasma and under identical conditions:

10 cultures of living muscle tissue.
10 cultures of living liver tissue.
13 cultures of living spleen tissue.
10 cultures of dead spleen tissue (the tissue had been heated to 60° C. in order to kill it).

Two days later fibers began to appear in the plasma clots of a number of the muscle and liver cultures, and fibers were also present in eleven out of the thirteen living spleen cultures. In the preparations in which the spleen tissue had been killed by heating no fibers ever appeared, and the plasma clots of these cultures never showed any change whatever from the fibrin net structure. Six parallel series of cultures, which were essentially the same as the series just described, have been made from time to time during the course of the work and the various dead tissues which were imbedded have been killed both by heating and poisoning. The results have always been the same, namely, no transformation of the fibrin net has ever occurred in any of the cultures in which the dead tissues were imbedded, while the reaction has always been present in some of the parallel living tissue cultures. In some of the preparations in which

the dead tissues were imbedded the extract from fresh muscle tissue was used to coagulate the plasma. The addition of the tissue extract did not in any apparent manner change the result previously obtained and no fiber formation has occurred in the clot of any culture except those in which either living tissue or blood corpuscles were imbedded. Attention should be called to another line of evidence which is in harmony with the above, and that is that in the living cultures in which the fiber formation takes place, the fibers appear first in the part of the clot which is in contact with the living imbedded tissue, and this part of the clot always shows the most complete transformation. In some cases the outlying regions of the clot, which are considerably removed from the influence of the tissue, retain the fibrin net structure while a complete transformation of the clot may take place near the imbedded tissue (figure 12). The results from all the experiments strongly suggest that the reaction which causes the fiber formation is due to some property of the living tissues which have been imbedded. The author, however, does not regard the tests so far made as exhaustive enough to say with absolute certainty that the transformation of the clot can only take place under the influence of living tissues or cells. There is the possibility that the reaction is one which will occur under the influence of other conditions which as yet are not known. Whether or not such is found to be the case the essential thing to be noted is that the imbedded living tissues are by themselves able to bring about a radical transformation of the fibrin elements of the clot and the consequent formation of a new fibrous tissue which, in appearance at least, is a typical connective tissue.

It has been found possible by mechanical means, such as pulling and twisting the clots with dissecting needles, to cause the fibers to form very rapidly in the clots in which the living tissues have been imbedded.[7] In such cases the piece of living tissue is placed in the

[7] An account of some early work by Leo Loeb along this line that is suggestive is given by Adami, J. G., The Principles of Pathology, 2d edition, Philadelphia and New York, 1910, i, 40. To quote: "When a drop of uncoagulated lymph is placed between two glass slides, the mere act of pulling one slide over the other leads to the appearance of fibrils, which grow in length and bulk; which, like those of connective tissue, are not only intracellular, but actually traverse cell bodies situated in their path; which show themselves first in imme-

drop of plasma as usual. After the clot has formed around the imbedded tissue the formation of the fibers can be aided by manipulating the clot with needles and thereby exerting tension in various directions. Such a preparation is shown in figure 16 in which a piece of spleen tissue was imbedded and the clot manipulated with needles. The culture was then sealed as usual, left over night, and preserved the next morning. It will be noted that the fibers are very irregular and formed in the various regions following the path of the needles through the clot. Such a condition is different from that shown in the previous figures in which the fibers were allowed to develop slowly in an undisturbed clot. In either case the structure of the fibers appears to be the same and to have resulted from a consolidation or fusion of the elements of the fibrin net. In the disturbed clot these processes are evidently hastened by the introduction of the mechanical factor. Many cultures have been made during the course of the work in which the clots were disturbed. Also the effect has been noted of imbedding killed tissues and then disturbing the clots. So far such cultures have always failed to show the fiber formation except that, if the plasma is caused to clot by the addition of a little of the fresh tissue extract and then the clot is disturbed, there occasionally appears to be an attempt at fiber formation. Possibly it might be expected that such a result would be obtained, since it is probable that the fresh tissue extract contains the constituents of the living tissue which are responsible for the transformation of the clot.

Two points may be noted as a result of these experiments: first, the evidence at hand strongly indicates that the presence of living tissue or of living cells is necessary to cause a transformation of the plasma clot; and, second, that when either of these is present the introduction of a mechanical factor may aid in a more rapid formation of the fibers.

The fact that mechanical means could be used to assist in the formation of the fibers suggested to the author the possibility that

diate connection with these cells, the cells, as we now hold, liberating an enzyme that determines the modification of the more soluble protein into a precipitated or coagulated modification. But the lines of the precipitation are evidently along the lines of strain."

the fibers in the regular cultures were due to the action of living cells which had moved out from the imbedded tissue or that were present in the plasma; in other words, that perhaps the fibers marked the paths of the various isolated cells which had become separated from the tissue or of cells which were present in the plasma, such as blood corpuscles, lymphocytes, etc. In most of the cultures it can readily be observed that numerous blood cells are present in the plasma clots. In actively growing cultures also a great many cells move from the imbedded tissue to various regions of the clot. The cultures of heart muscle tissue of the adult frog show this to the greatest advantage, and in such cultures many instances can be noted in which the fibers are in close relation to the isolated spindle cells. Such a culture is shown in figure 18, which is a microphotograph at 100 diameters of a four day culture. Numerous spindle cells can be seen in the fibrin net to the right of the imbedded tissue. The path of these cells in their movement from the tissue to their present position can be traced by the appearance of the fibrin net. In such a culture it appears probable that the fibers which have been formed have arisen partly at least as a result of the action of the cells in forcing their way through the fibrin net. In the cultures of heart muscle tissue which have been under observation the formation of fibers is generally associated with the spindle cells which have moved through the clot from the imbedded tissue, whereas in cultures of spleen and liver tissue in which no growth or movement of tissue cells has taken place the fibers arise directly from that region of the plasma clot which is in contact with the imbedded tissue. A comparison of figure 8 with figure 18 will make this statement clear. In the first figure with spleen tissue, the fibers have arisen in close connection with the imbedded tissue, while in figure 18, with the heart muscle tissue, the fibers have, in general, arisen in close contact with the individual cells that have wandered out from the tissue, and there are very few fibers which are in contact with the imbedded tissue. In figure 19, which is a microphotograph at 667 diameters of a portion of figure 18, a better idea can be obtained of the relations existing between the spindle cells and the fibrous bundles in these cultures of heart muscle. The figure shows one of the spindle cells moving through the plasma clot. Below its path can be traced by

the appearance of the fibrin net which has been transformed into a fibrous tissue. Long fibrous bundles also radiate out laterally from the region of the cell. Above the fibrin net still retains its typical structure. In figure 20, which is a microphotograph of an eight day heart muscle culture at a magnification of 667 diameters, a condition is shown which is typical of that found in the older cultures of this tissue. The part of the clot lying next to the imbedded tissue and through which the cells have wandered has become almost entirely transformed into the bundles of fibers and with the cells lying between these a tissue is formed which is entirely similar, in its appearance at least, to the regular connective tissue of the frog. It is generally supposed that the fibroblasts which wander into a fibrin clot from the injured tissues during wound healing digest the fibrin and then form the new connective tissue fibers intracellularly. Such, however, is not the case with these fibers which have formed from the elements of the fibrin net. They are not digested by the tissue cells which have wandered into the clot, but, on the other hand, they are acting as permanent fibers and have combined with the tissue cells to form a connective tissue.

In the cultures of heart muscle tissue, then, the results indicate that the tension exerted by the isolated spindle cells in their movements through the plasma clot plays a part in the fiber formation. However, it can be shown that the movements of isolated tissue cells are not responsible for the formation of fibers in all the cultures, for the same reaction will take place in cultures in which there are no isolated cells present in the clot, and, therefore, the transformation which occurs must be due to the action of the living tissue as a whole without the aid of any apparent mechanical factor. In cultures of adult frog spleen and liver there is, in general, very little, and in some cases no growth or movement of tissue cells, and yet it is in cultures of these tissues that the most prolific fiber formation takes place. With care it has been found possible to make a culture in which the plasma is also free from blood corpuscles. Such a culture, providing there is no outgrowth of cells from the imbedded tissue and that a transformation of the fibrin clot has occurred by which the new fibrous tissue has been formed, demonstrates that the imbedded tissue can bring about the reaction without the aid of iso-

lated cells. A number of such instances have been noted during the course of the work and a microphotograph of such a culture is shown in figure 13, in which preparation the clot showed an almost complete absence of isolated cells and no movement of tissue cells occurred. Notwithstanding the absence of isolated cells in the clot an almost complete transformation of the fibrin net took place. Such a result makes it clear that the imbedded living tissue is, without the aid of isolated cells or of any other apparent mechanical factor, able to bring about the formation of the fibrous tissue from the fibrin elements of the clot.

THE NATURE OF THE NEW FIBROUS TISSUE.

In an endeavor to determine the nature of the new fibrous tissue, which arises in the cultures through a transformation of the fibrin elements of the clot, experiments with various specific stains have been made. Also the new tissue has been subjected to digestion and acid tests. It was hoped that these experiments would show definitely whether or not the fibers composing the new tissue were fibrin in character, although apparently identical in their appearance, structure, and function with the regular collagenous fibers of connective tissue.

Staining Reactions of the New Fibrous Tissue.—Two stains have been made use of, both of which are supposed to be specific to differentiate between fibrin and connective tissue; namely, Mallory's connective tissue stain modified according to Mall,[8] and Van Gieson's picrofuchsin stain. Early in the work it was found that with the Mallory stain the fibers formed in the fibrin net were stained a blue color which very closely resembled the color of the regular connective tissue fibrils when stained in the same manner. With this stain fibrin is supposed to stain red in contrast to the blue of the connective tissue. However, the fibrin of frog plasma stains a purplish blue instead of a red with this stain. The differentiation, though between the purplish blue of the undifferentiated fibrin net and the almost ultramarine blue of the new fibers, is very clear. All the microphotographs that are shown in this paper were made from

[8] Mall, F. P., On the Development of the Connective Tissues from the Connective-Tissue Syncytium, *Am. Jour. Anat.*, 1901–02, i, 338.

preparations which were stained with the Mallory stain, and from these it can be seen that the differentiation is very sharp between the fibrin net and the fibers which form in it. In comparison with the regular connective tissue fibers which can be studied in the imbedded tissues of the cultures it can be said that with the Mallory stain there is in most instances very little difference in color between them and the fibers which have been formed by the transformation of the plasma clot. The regular connective tissue fibers stain a perfect ultramarine blue, while the fibers which form in the clot in the same culture are generally a little darker in color and a little less clear. In short it may be said that when this stain is used the regular connective tissue fibers and the fibers formed in the clot stain so nearly the same that there is no real difference in their appearance. This result, however, is not obtained when the cultures are stained with Van Gieson's stain. This stain colors connective tissue fibers red and other tissues yellow. Using this stain on the preparations it has, thus far, been found impossible to obtain any differentiation between the undifferentiated plasma clot and the fibers formed in it, the entire clot with the fibers taking a yellow stain. The fibers can be clearly seen in the clot of a culture thus stained, but they have the same yellow color as the parts of the clot which have not been transformed, in distinction to the red color of the regular connective tissue fibers. With this stain, therefore, the evidence is that the fibers formed in the clot retain their fibrin character. A combination of the two stains has been used in a number of instances. In this case the culture is stained first with the Mallory stain, then partially decolorized with acid alcohol, and restained with Van Gieson. Preparations treated in this manner show a clear differentiation between the fibers in the clot which stain a light green, as do also the regular connective tissue fibers in the imbedded tissue, and the remainder of the clot which takes a pinkish stain.

Digestion and Acid Tests with the New Fibrous Tissue.—The fact that fresh fibrin is easily dissolved by digestion with pancreatin or by the use of a weak acid, whereas connective tissue fibers are not so affected, was used as a basis for determining the nature of the fibers formed in the clot. The method of experimentation was as follows: In a culture in which the fibers were present in the clot, the cover-

glass, to which were attached the clot and the imbedded tissue, was removed and immersed in the pancreatin solution at 37° C. or in a dilute solution of acetic acid. Cultures ranging in age from five to fourteen days have been experimented with in this manner. The result, with either of the tests, has always been that within a few hours the entire clot containing the fibers completely disappears leaving the imbedded tissue free. Such a result apparently indicates that the fibers formed in the clot remain fibrin in character. The tests, however, are not conclusive, because of the intimate relations existing between the fibrin net and the fibers which form in it. The destruction of the fibrin net either by digestion or with a weak acid naturally causes a breaking down of the fibrous bundles which are formed in it and a scattering of the individual fibrils, inasmuch as they are not attached to the imbedded tissue and therefore their only support is the fibrin clot in which they are formed. Thus far it has proved to be a difficult matter to get a test that can be used in these tissue culture preparations that will definitely distinguish between fibrin and connective tissue fibers. Mall[9] mentions the great difficulty he found in using the digestion and acid tests in the study of the early differentiation of the connective tissue fibers. He states that the results from various tests contradicted each other, so that he hesitated to draw any conclusions from them. Difficulties of the same kind have been encountered in this work, and the author does not lay great weight upon the results of the tests so far made as showing definitely whether or not the fibers formed are really connective tissue fibers.

In brief, the structure and function of the new fibrous tissue and the appearance with Mallory's stain all indicate that it is a true connective tissue. On the other hand, the Van Gieson stain and the acid and digestion tests give evidence that the new fibrous tissue retains the fibrin character of the fibrin net from which it formed. Work along various lines is in progress and it is hoped that as a result the true character of the new fibrous tissue can be definitely settled.

[9] Mall, *Am. Jour. Anat., loc. cit.*, p. 339.

DISCUSSION.

With the results of these experiments at hand it may be well to discuss briefly the significance of the changes which take place in the cultures of adult frog tissues. It is evident that in the reaction which occurs in the plasma clot, involving as it does a transformation of the fibrin net into a new fibrous tissue, we have a process which must be fundamental in wound healing. A wound under the most favorable conditions for healing becomes quickly filled with the fluid plasma and lymph which pour into it from various sources. This quickly coagulates and a fibrin net is formed.[10] Under the influence of the living injured tissues, and aided to some extent by the tension exerted on it through the movements of the tissues, this mass of fibrin soon loses its typical structure. As shown in this paper, the fibrin elements fuse or consolidate to form fibrils, and these unite to form long wavy fibrous bundles which freely intertwine and anastomose and ramify through the entire clot. In a short time there has arisen, in place of the fragile and easily destroyed plasma clot, a new fibrous tissue which is tough and resistant and which functions for the time, at least, as a regular connective tissue. The transformation of the clot also causes a shrinkage, and the wound space is thereby lessened, the edges of the wound are held firmly in place, and a support is formed for the outgrowth of the tissue cells.

From the work presented in this paper it also appears that in wound healing, at least, there is good reason for believing that this new fibrous tissue which arises by a direct transformation of the fibrin clot, without intracellular action, remains as a permanent connective tissue. Hertzler[11] has already put forth such a view which he bases on results obtained from wound healing. He shows

[10] For a summary of the part played by fibrin in wound healing, see Marchand, F., Der Process der Wundheilung, Stuttgart, 1901, 52–55. The fact that the fibrin elements in wound healing consolidate to form fibers is also shown; see Marchand, *loc. cit.*, pp. 245–255, figures 64–67.

[11] Hertzler, A. E., Pseudoperitoneum, Varicosity of the Peritoneum and Sclerosis of the Mesentery. With a Preliminary Note on the Development of Fibrous Tissue, *Jour. Am. Med. Assn.*, 1910, liv, 351; The Development of Fibrous Tissues in Peritoneal Adhesions, *Anat. Rec.*, 1915, ix, 83.

that the fibrous bundles are present in the clots in wound healing and also that they will take a specific connective tissue stain a short time after they are formed. Hertzler believes that the fibers arise through some chemical action. Although there is considerable support for the view that the connective tissue fibers, in general, arise through intercellular action,[12] the idea put forth by Hertzler that they arise by a transformation of the fibrin elements of the clot does not appear to have received any support. In the present paper it has been possible to show by the use of tissue culture methods that under the influence of living adult frog tissue a transformation of the fibrin elements of the clot may give rise to a new fibrous tissue which in appearance, when stained with Mallory's stain, structure, and function is identical with regular connective tissue. The experiments which have so far been made use of in an endeavor to determine the nature of the new fibrous tissue have failed to settle the question definitely, and until it is possible to get a test that will determine the question no definite statement regarding the nature of the tissue should be made. The work with the older cultures shows that the new fibrous tissue remains permanently (figure 17), and also that it is not digested by the tissue cells which wander into it, but, on the other hand, the two combine to form a typical areolar tissue (figure 20). There appears to be no reason for believing that the fibers of the new tissue would ever be replaced by other fibers formed intracellularly. Personally, from the study of the living and preserved cultures that has been made, the author feels that there is good ground for the belief that the fibers which have formed in the clot through a transformation of the fibrin elements are identical with those found in areolar and reticular tissues. Whether or not the work which is now being carried on, which embraces a study of the origin of the connective tissue fibers in wound healing and also in embryonic development, bears out this belief, as suggested above, a direct transformation of the clot into the new fibrous tissue such as has here been definitely shown must be a process which is of the highest value in wound healing.

[12] For an account of the various views held regarding the origin of the connective tissues, see Schäfer, *loc. cit.*, pp. 116–122. Adami, *loc. cit.*, p. 429.

SUMMARY.

In living cultures of various kinds of adult frog tissues, which have been made according to the hanging drop method, there occurs, in many cases, a transformation of the plasma clot by which it becomes entirely changed from a typical fibrin net both in appearance and structure.

The changes in the fibrin net generally begin when the culture is from two to three days old. During these changes it appears that the elements of the fibrin net fuse or consolidate, and as a result a great number of fine wavy fibrils are formed which unite to form wavy bundles of fibers, and these freely intertwine and anastomose as they ramify through the area of the plasma clot. The transformation of the fibrin net occurs first in the region of the clot which lies next to the imbedded tissue, gradually extends to the distal regions of the clot, and in time—as a rule in about two weeks—the entire plasma clot becomes changed from the fibrin net into a structure which to all appearances is identical with regular connective tissue. Photographs of both living and preserved cultures have been made to show the course of the transformation of the plasma clot and the development of the fibers.

Experiments have been made which show that the fibers which are formed are not outgrowths of the imbedded tissue. Also they are not formed by an intracellular action, but arise directly by a transformation of the fibrin elements of the plasma clot.

Experiments have been made which indicate that the transformation of the fibrin net will not occur unless it has come under the influence of living tissues or of living isolated cells. However, mechanical means, such as exerting tension on the clot with needles, may hasten the formation of the fibers. Also, in some cultures, movements of living isolated cells appear to aid in the formation of the fibers. The living tissues alone, however, are able to cause the fibers to form without the aid of any apparent mechanical factor. This is shown by cultures of various tissues in which no cell movement occurs and in which the plasma clot is undisturbed and yet a prolific formation of fibers may take place.

Experiments have been made in order to determine the true nature

of the transformed plasma clot and to see if the new fibrous tissue were still fibrin in character. The results that have so far been obtained from these tests have not been entirely conclusive and leave the question unsettled.

The transformation of the fibrin net results in a shrinkage of the clot. It also becomes very tough and resistant to injury and, therefore, entirely different from the fragile and easily destroyed fibrin net when in its original condition. It is believed that such a reaction must play an important part in wound healing. A study of the relation between connective tissue fibers formed in wound healing and in embryonic development to the fibers formed in the plasma clot is being made, and the results will appear in a later paper.

EXPLANATION OF PLATES.

All of the figures shown are microphotographs[13] with the exception of Nos. 11 and 17 which are drawings. Figures 1 to 6 inclusive were taken from the living cultures. The remainder of the figures were taken from the preserved cultures and were made with a Bausch and Lomb microphotographic apparatus equipped with a Zeiss microscope and apochromatic lenses.

PLATE 31.

FIG. 1. Three day culture of liver tissue. $\times 33$. From the upper edge of the imbedded tissue a number of small fibers can be seen projecting into the plasma clot.

FIG. 2. Four day culture of spleen tissue. $\times 27$. A prolific growth of fibers is shown. This culture was preserved. See figures 8 to 11.

FIG. 3. Four day culture of spleen tissue. $\times 27$. Two pieces of imbedded tissue are shown, from both of which a great many of the fibers are given off into the plasma. The pieces of tissue were not connected at first. Later they became connected by the fibrous formation in the clot and then cells wandered out on these fibers as shown in the figure. Numerous blood corpuscles are present in the clot. Also many small light areas are noticeable in the clot. These are the result of condensation on the glass slide.

FIG. 4. Nine day culture of spleen tissue. $\times 40$. Fibers are shown at a higher magnification. Note specialized area of the clot around the imbedded tissue from which the fibers arise. This culture was preserved. See figure 12.

FIG. 5. Ten day culture of liver tissue. $\times 33$. A considerable cellular movement from the imbedded tissue has occurred, as well as a prolific fiber formation.

FIG. 6. Ten day culture of heart muscle tissue. $\times 33$. A heavy cellular growth and fiber formation have occurred. At the lower edge of the tissue cells can be noted which are apparently travelling along the fibers.

[13] The author is glad of this opportunity to express his thanks to Professor Alexander Petrunkévitch for his advice and assistance in making some of the microphotographs.

PLATE 32.

FIG. 7. Two day culture of spleen tissue. × 100. An early stage in the fiber formation is shown in this figure. A small area of the clot which is in contact with the imbedded tissue is becoming transformed. The rest of the clot retains the typical fibrin net structure.

FIG. 8. Six day culture of spleen tissue. × 67. A heavy fiber formation is to be noted in the clot. A dense fibrous tissue has been formed in the clot almost entirely around the imbedded tissue, and from this area the fibers radiate to the outlying regions. See figure 2; also figure 11 which shows a drawing of a part of this region.

FIG. 9. A part of the culture shown in figure 8. × 667. A portion of some of the bundles of wavy fibers are shown and also some of the untransformed clot. Above can be seen a part of the dense fibrous tissue which surrounds the imbedded tissue.

FIG. 10. A part of the culture shown in figure 8. × 1,000. The fine fibrils which compose the bundles of fibers can be noted, as well as some of the untransformed fibrin elements of the clot.

PLATE 33.

FIG. 11. A drawing of a part of the culture shown in figure 8. × 667. Below a small portion of the imbedded tissue is shown. Lying in close contact with this is the dense fibrous tissue which has formed in the clot. Bundles of fibers, which compose this area, radiate to the outlying regions of the clot, gradually grow smaller, and finally end in the untransformed fibrin net. The elements of the net appear to fuse together to form the fibrils which in turn unite to form the fibrous bundles. No isolated cells are present in this region of the culture.

PLATE 34.

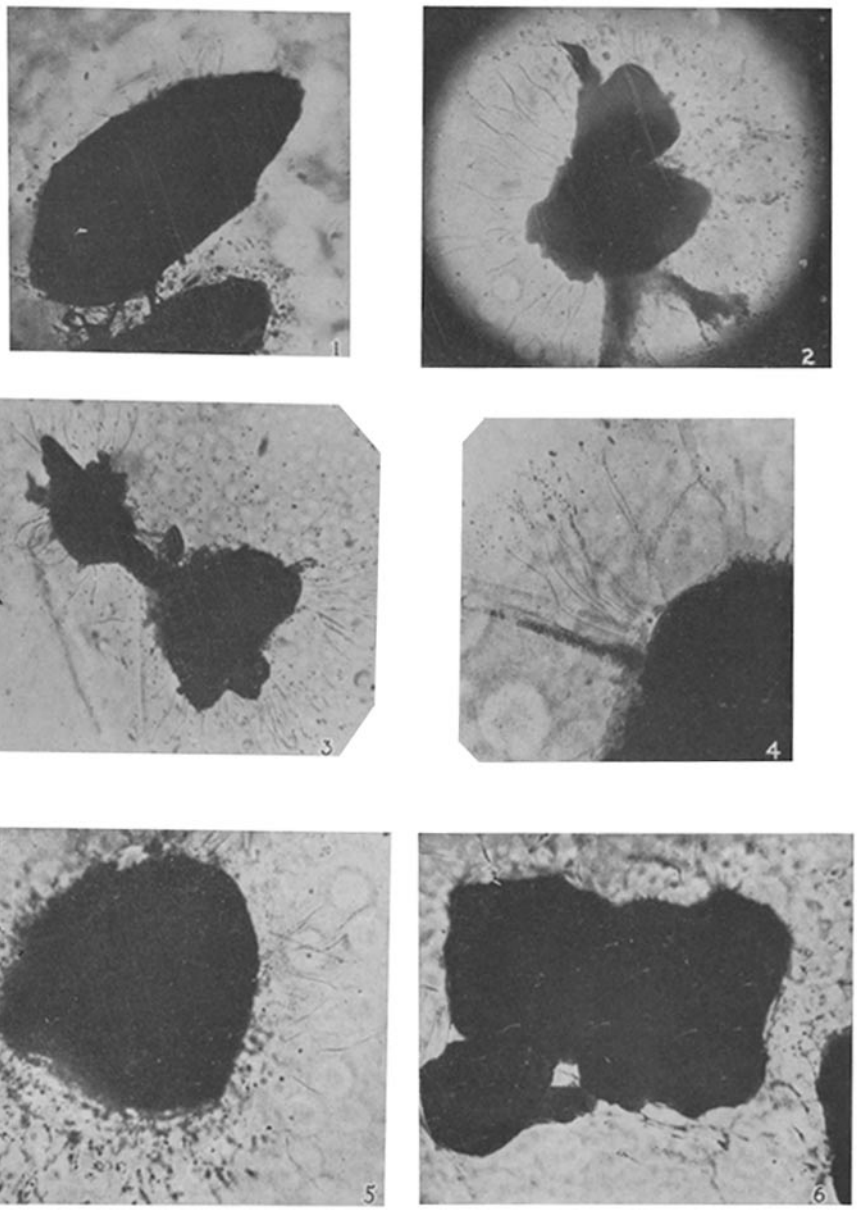
FIG. 12. Nine day culture of spleen tissue. × 100. The portion of the clot lying in contact with the imbedded tissue has become changed from the fibrin net to a dense fibrous tissue from which large bundles of fibers are given off. The transformation has been so complete in this region that it is difficult to make out the boundary between the clot and the imbedded tissue. See figure 4.

FIG. 13. Fourteen day culture of liver tissue. × 100. Two pieces of the imbedded tissue are shown. The portion of the clot lying between them has become almost completely transformed into what gives every appearance of being a reticular tissue. See figure 17, which shows a drawing of this same culture.

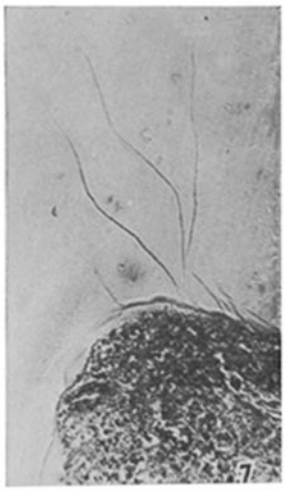
FIG. 14. A portion of the new fibrous tissue formed from the clot of the culture shown in figure 13. × 667.

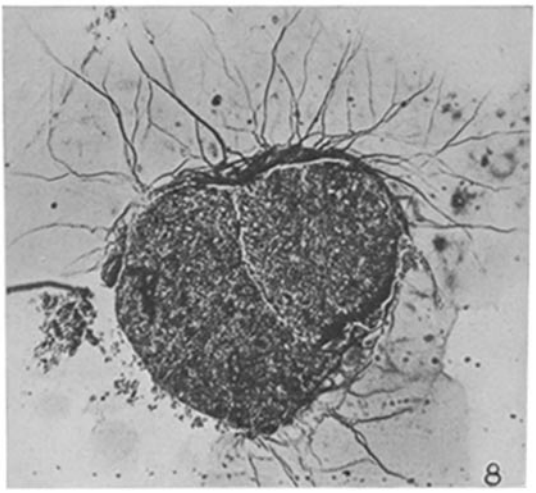
FIG. 15. A preparation in which living blood corpuscles were imbedded in place of a piece of tissue. × 100. The fibers have formed in the region in which the corpuscles were imbedded.

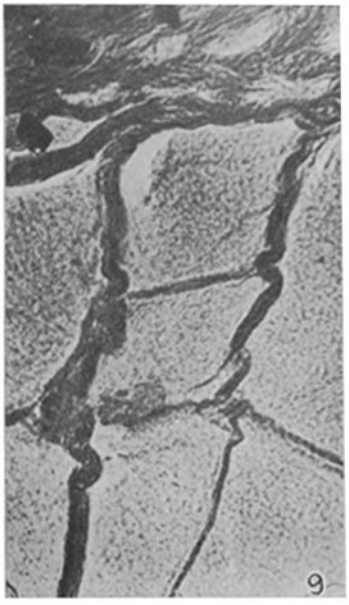
FIG. 16. A preparation in which the clot was disturbed by exerting tension with needles. × 100.

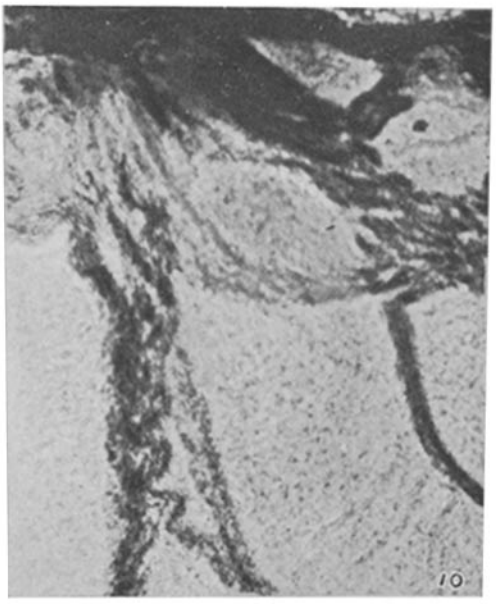


(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)









(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

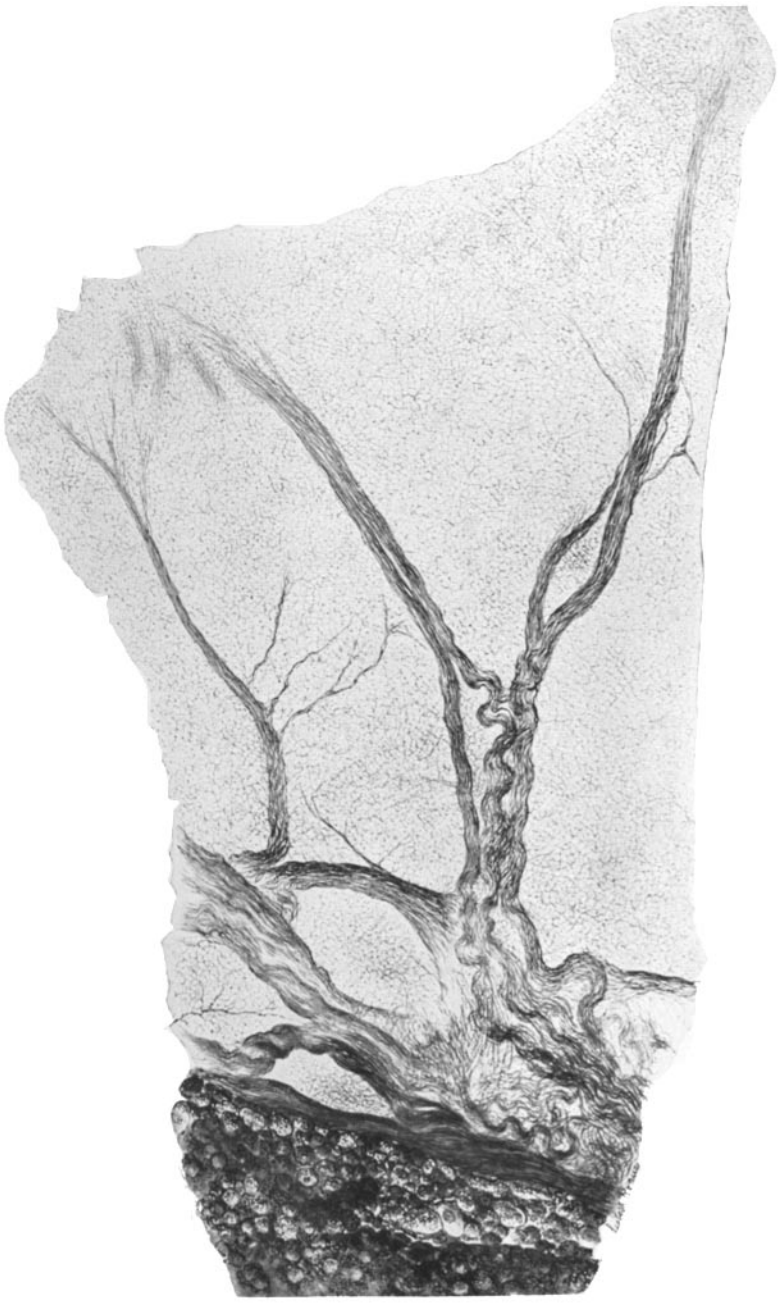
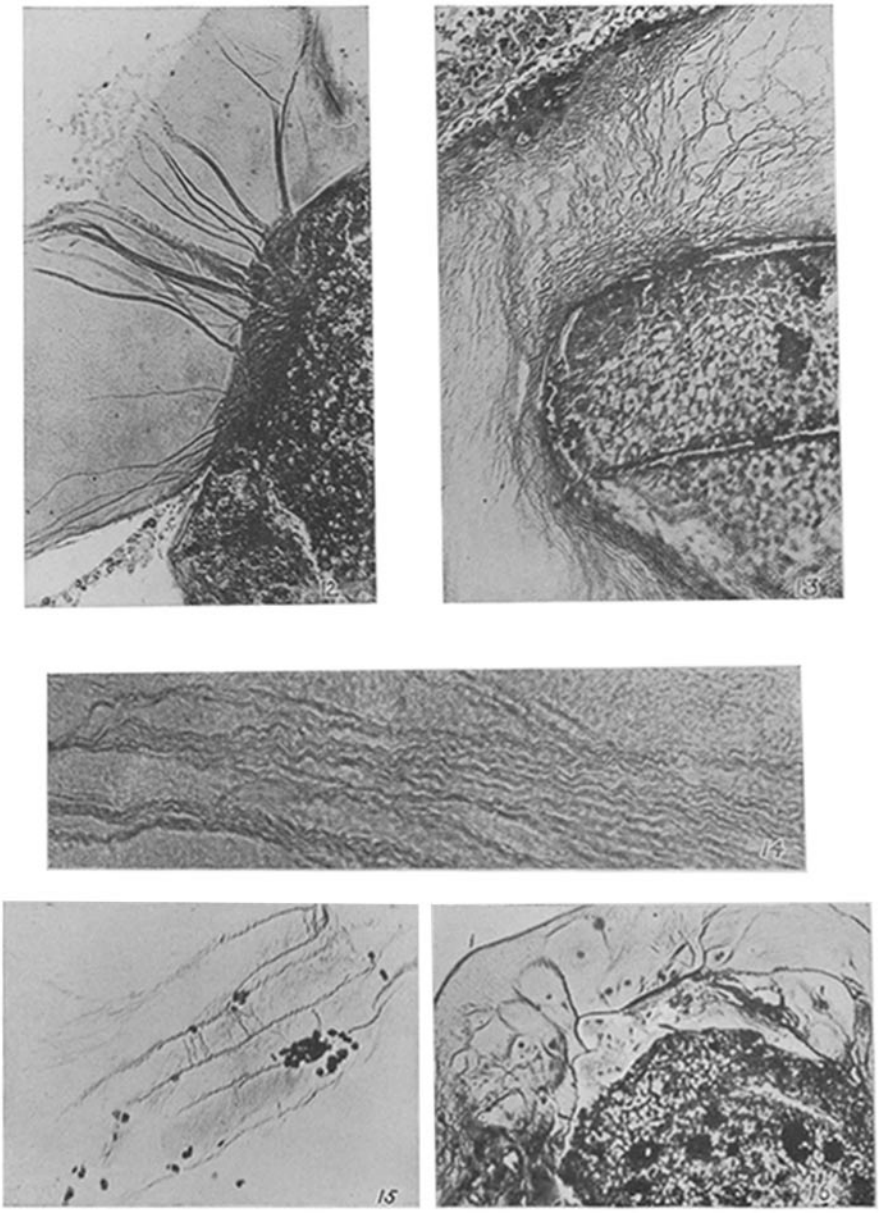


FIG. 11.

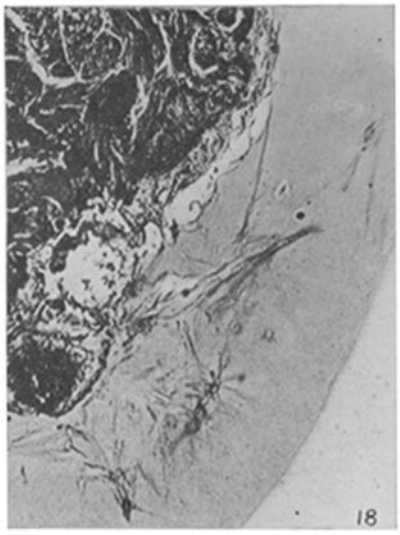
(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

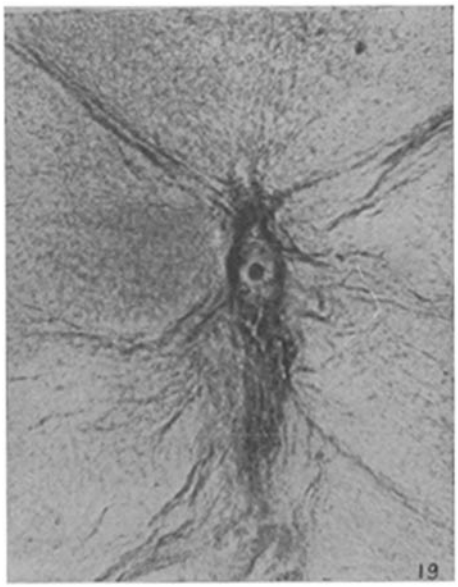


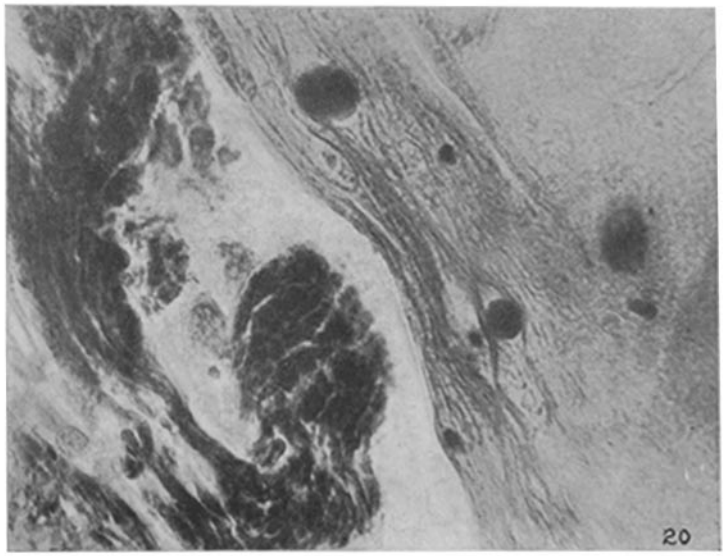
(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

FIG. 17.

(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)







(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

PLATE 35.

FIG. 17. A drawing of a part of the culture shown in figure 13. ×667. It can be seen that practically the entire clot has been transformed into what appears to be a reticular tissue. Only very small areas are present in which a fibrin net appears. The most complete transformation of the clot lies nearest to the imbedded tissue.

PLATE 36.

FIG. 18. Four day culture of heart muscle tissue. ×100. The fibers have formed in close connection with the cells which have wandered out from the tissue.

FIG. 19. A part of the culture shown in figure 18. ×667. One of the spindle cells is shown. It has moved out from the imbedded tissue and its path can be traced by the appearance of the fibrin net, which has, in the vicinity of the cell, been largely changed into a fibrous tissue.

FIG. 20. A portion of an eight day heart muscle tissue culture. ×667. The clot lying in contact with the imbedded tissue has become changed into the new fibrous tissue with its bundles of fibers, and these with the tissue cells which have wandered out from the imbedded tissue give a typical areolar tissue structure.