Resumen por el autor, George A. Baitsell.
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Estudio del desarrollo del tejido conectivo en los Anfibios.

El esbozo primitivo del tejido conectivo de los embriones de rana es un material amorfo y gelatinoso, el cual a causa de su escasa avidez hacia los colorantes durante las primeras fases del desarrollo, durante las cuales se tiñe ligeramente por los diversos métodos empleados, es muy dificil de poner en evidencia. Esta substancia fundamental puede observarse alrededor del notocordio a raiz de la formación de este último y un poco antes de aparecer la yema caudal del embrión. Un poco más tarde este material, que entrará a formar parte del tejido conectivo, rodea la médula espinal y una capa formada por él se extiende ventralmente a lo largo de la pared del cuerpo, y al cabo de cierto tiempo termina rodeando por completo la cavidad del cuerpo. La formación de esta substancia alrededor del notocordio tiene lugar antes de haber aparecido un sincicio de células mesenquimatosas en esta región.

Es evidente, por lo tanto, que esta substancia fundamental primitiva del tejido conectivo se ha formado por secreción intercelular de las células embrionarias y no a consecuencia de una fusión sincicial del protoplasma. Las fibras del tejido conectivo comienzan a aparecer en la substancia fundamental a raíz de formarse esta. Pueden constituir la substancia fundamental en regiones desprovistas de células, de modo que es evidente que no se han originado por una acción intracelular. En algunos casos puede comprobarse que las fibras se originan por una transformación gradual de la substancia fundamental, primero en una delicada estructura reticular y después en las largas fibras típicas del tejido conectivo. En sus rasgos morfológicos por lo menos, este proceso coincide con el observado previamente por el autor en la transformación del coágulo sanguíneo.

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A STUDY OF THE DEVELOPMENT OF CONNECTIVE TISSUE IN THE AMPHIBIA

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SIX FIGURES (FOUR PLATES)

INTRODUCTION

The studies in which the author has been engaged for several years have shown, in brief, that the plasma clot obtained from frog's blood is of such a nature that, when influenced by the proper factors, it is possible to radically transform its structure. The reactions of the clot were studied in tissue cultures ('15) and in wound healing ('16). In both these cases it was possible to demonstrate clearly that the clot could be transformed into a fibrous tissue which was apparently identical in structure, save for the absence of connective tissue cells, with regular connective tissue. In wounds made in frog skin, the experiments further showed that the fibrous tissue which developed from the fibrin clot functioned, at least temporarily, as a normal connective tissue and there was no evidence to indicate that it would ever be replaced. In the final paper of the series ('17)—in which a study of the normal clotting and the transformation of the clot under the influence of certain mechanical factors was studied with the aid of a microscope equipped for dark field illumination—it was possible to demonstrate that the transformation of the clot was brought about by a fusion and consolidation of the fine elements of which it is composed. This process resulted in the formation of long fibers which united to form wavy, fibrous bundles. These bundles of fibers anastomosed with other bundles, ramified in various directions throughout the clot and the result was the formation of a fibrous material which closely resembled regular connective tissue.

The above results led naturally to the question as to whether a similar process normally takes place in the histogenesis of the connective tissues in amphibian embryos and thus to the present study in which this problem is considered. It is worthy of note that, notwithstanding the great amount of work that has been done upon the development of connective tissue, no general agreement has been reached. At present there are two main theories held regarding connective tissue formation—the intracellular theory and the intercellular theory—and, in general, the results of the various investigators support one or the other of these. In order to get the matter clearly in mind it may be well to set forth a statement of the opposing views, which are as follows:

According to the intercellular theory there is early formed in development an amorphous, gelatinous, non-living ground substance. It is generally held that this is formed as a secretion of the mesenchyme cells. Later, fibers are formed in this ground substance by a deposit or secretion which is given off by the cells of the tissue. It is held that the cells have no morphological connection whatever with the fibers which form in the ground substance.

The intercellular theory substantially as stated above was the original theory held for connective tissue formation and was first stated by Henle. With various minor modifications, it has been supported by v. Kölliker, Merkel, Ranvier, v. Ebner, Renaut, Schäfer, Laguesse, and others. The material used in the researches of these investigators was obtained from many different sources and included both embryonic and adult tissue.

The intercellular theory has been, perhaps, most strongly supported by Merkel. In his last paper ('08) he gives a splendid review of the question of connective tissue development as well as the results of his own studies. His conclusions are that the primitive origin of all connective tissues lies in an amorphous gelatinous substance which is secreted by the syncytium of the mesenchyme cells. This secretion constitutes the ground substance and in some cases it is formed sparingly and in other cases very abundantly (e.g., Amphibia, umbilical cord). The fibers, according to Merkel, are really strands of the amorphous ground substance and they increase in size through an assimilation of the surrounding ground substance. The cells play no direct part in the formation of the fibers but serve only to produce the ground substance by secretion. Merkel also believes that the formation of the connective fibers is due in every case to the influence of mechanical factors acting on the ground substance; e.g., where there is a decided stretching, as in a tendon, parallel fibers are formed from the ground substance.

According to the intracellular theory, the ground substance which is early formed is regarded as living material, although it is intercellular in position. The fibers which form in this ground substance are considered by some investigators to be strands of cell cytoplasm which have been given off from the periphery of the connective tissue cells. Others believe that a transformation of the entire cell into fibers takes place. In either case, in the first stages of fiber development, all the fibers are regarded as being merely modified strands of cell cytoplasm. The fibers when once formed by the cells may, and often do, become separated from the cells but nevertheless it is held that they are able to grow both in thickness and in length by intussusception due to the assimilation of food material. The living intercellular material is also able to grow in the same manner and it increases greatly in amount in proportion to the cells present.

The intracellular view has been modified by the Hansen-Mall theory, which is widely held at present. In accordance with this theory, the ground substance arises as a result of a syncytium of the mesenchyme cells and is therefore regarded as being a common living exoplasm of the mesenchyme cells. In this exoplasm, each of the nuclei of the mesenchyme cells are surrounded by a minute mass of cytoplasm which constitutes the endoplasm of the cells. According to this theory the fibers arise in the common exoplasm either in the regions in immediate contact with the cells or in the regions of the exoplasm which may be far removed from cells.

The above view regarding connective tissue formation is clearly set forth by Mall ('02) who says:[1]

In very early embryos the mesenchyme is composed of individual cells which increase rapidly in protoplasm and then unite to form a dense syncytium. The protoplasm of the syncytium grows more rapidly than the nuclei divide, so that in a short time we have an extensive syncytium with a relatively small number of nuclei. In its form the syncytium appears as large bands of protoplasm with spaces between them filled at times with cells and at other times with fluid. The second condition we have in the umbilical cord of young human embryos. About this time the protoplasm of the syncytium differentiates into a fibrillar part, which forms the main portion of the syncytium—the exoplasm—and a granular part, which surrounds the nucleus—the endoplasm.

The view of Mall, as stated above, is in quite close agreement with that of Hansen ('99 and '05) which is based upon his work on the development of cartilage. He believes that,[2] in many cases, it is not possible to distinguish between protoplasm and ground substance and also that[3] cells of cartilage are to be considered as an endoplasm and the ground substance as a common ectoplasm which is more or less independent of the endoplasm.

In the concluding section of this paper will be found an analysis of these two theories and a discussion of the relations shown to them by the results obtained from the present work. It may, however, be stated at this time that the results reported in the present paper[4] strongly support the intercellular theory of connective tissue development, and furthermore they show that the process of connective tissue formation closely resembles, morphologically at least, the formation of fibrous tissue by transformation of a plasma clot.

[1] Mall, 1902, p. 331.

[2] Hansen, 1899, p. 434.

[3] Hansen, 1905, p. 747.

[4] A report of this work was given at the meeting of the National Academy of Sciences held in New Haven, Nov., 1919, and an abstract of the work is published in the Proceedings of the National Academy, 1920, vi, 77.

MATERIAL AND METHODS

In the present work, embryonic amphibian material has been used. It was obtained from a number of species, chiefly Rana sylvatica, R. palustris, R. catesbiana and Amblystoma punctatum. From each of these species, five series of nine or ten embryos, each ranging from the medullary plate stage by regular gradations to well developed free swimming larvae were obtained for preservation. In all about 200 embryos were selected for permanent preservation. In addition to this, through the kindness of Professor R. G. Harrison, access was had to several series of transverse, sagittal and frontal serial sections of R. palustris, cut 7.5μ and stained in haematoxylin and Congo red. Figures 1 and 2 are taken from this series.

The following preserving fluids were used: *a)* Zenker with 5 per cent glacial acetic acid; *b)* Zenker without acetic acid; *c)* saturated solution of mercuric chloride with 5 per cent glacial acetic acid; *d)* saturated solution of mercuric chloride without acetic acid; *e)* 80 per cent alcohol. Several series of embryos were preserved in these fixing fluids. They were later imbedded in paraffin, and transverse, sagittal and frontal serial sections were made ranging from 7 to 10 micra in thickness.

The following stains have been used: *a)* Delafield's haematoxylin; *b)* iron haematoxylin; *c)* picro-fuchsin connective tissue stain; *d)* Mallory's anilin blue connective tissue stain, and *e)* Mall's modification of the Mallory stain. With the large amount of material that was available, preserved by various methods, and with the various methods of staining which were used, it was possible to test a large number of combinations and to determine the combination which was best adapted for the present work. Considerable time was spent in making this comparative study of the preserving fluids and stains and their reactions with the tissues, and it is believed that the combination, consisting of Zenker with acetic for the killing fluid and Mallory's aniline blue connective tissue stain which was finally adopted and used for most of the material is the best possible one for the problem in hand.

With regard to this combination, it may be well to say in the first place that, of all the preserving fluids tried, Zenker with acetic gave the best results. The only objection to this fluid is that it has a tendency to harden the yolk material in the embryos somewhat more than some of the other fluids and this makes the sectioning of the earlier stages difficult. However, the difference here is not great and the superior preservation in the Zenker fluid warrants its use.

As to the staining, it was found that the Mallory connective tissue stain was superior to any of the other stains tried for the work in hand. A comparative study was made of the reactions of this stain as originally given and the same stain as modified by Mall ('01–'02). The results obtained show very clearly that the modified Mallory does not give such a clear differentiation between cytoplasm and ground substance as does the original Mallory stain. Mall says,[5] "My best specimens were obtained by staining the sections with Mallory's connective tissue stain which tinges the nuclei and surrounding endoplasm, if present, slightly red and the exoplasm of the syncytium intensely blue. We have modified this stain somewhat by omitting the water and intensifying the blue."

The results of the comparative study have convinced me that the intensification of the blue is a mistake in that thereby the differentiation between the cell cytoplasm and the ground substance of the developing connective tissue is largely lost. In material preserved in Zenker and correctly stained with Mallory's original stain there is a clear differentiation between the cells, which are of varying shades of red, and the ground substance which is invariably blue. It is possible therefore in such material to speak with certainty as to the limits of the cell boundaries and the relations the cells bear to the common intercellular ground substance. When the blue is intensified the entire field takes this color and the differentiation becomes obscured or even lost.

[5] Mall, 1902, p. 338.

STUDY OF PREPARED MATERIAL

If sections of a frog embryo, taken shortly after the notochord is formed and before the tail bud stage is reached, are studied, it will be found that the notochord of the embryo is surrounded by a mass of transparent gelatinous material (primitive ground substance). Such a stage is shown in figure 1 which was made from a portion of a frontal section of a 3.2 mm. embryo of Rana palustris. The magnification is 285 diameters. Above a portion of the central nervous system is shown. Extending posteriorly, in the median line, is the notochord imbedded in a gelatinous cell-free matrix or primitive ground substance which extends laterally between the muscle plates in either direction.

In figure 2 is shown a portion of a transverse section through a 4 mm. embryo of the same species but slightly older than that shown in figure 1. The magnification is 322 diameters. The notochord is shown in the center of the figure. Lying above it is a portion of the ventral wall of the medullary tube. The embryonic muscle cells can be seen lying to the right and left of the notochord. Filling the space between the notochord and the muscle cells is the gelatinous material mentioned above, which constitutes the primitive ground substance. This material also extends dorsally along the sides of the spinal cord and laterally into the muscle tissues. As is shown in the figure, the ground substance in some regions shows a fine fibrillation running through it. In other regions it appears homogeneous.

At the stage in embryonic development shown in figures 1 and 2, the mesenchyme cells, which later wander all through the cavities of the embryo, are not present in this region in which the primitive ground substance is first formed. The case is clear, therefore, that the ground substance which is here present surrounding the notochord has not arisen by syncytial fusion of cell cytoplasm.

The primitive ground substance having been formed as shown in figures 1 and 2, it is next found that, in an embryo of a little later stage, cells begin to detach themselves from the cell masses and wander through it. These cells, when they first appear, are for the most part more or less rounded in shape and contain a considerable amount of yolk. As they move through the ground substance they become amoeboid and also in some cases spindle-shaped; a form which is regarded as being typical of the so-called connective tissue fibroblasts. It is frequently possible to trace the movements of the cells by the altered appearance of the gelatinous ground substance.

In figure 3 is shown a portion of an unstained transverse section through a 9 mm. embryo at a magnification of 322 diameters, at the stage when the cells are starting to move into and through the ground substance, as described just above. In this figure a portion of the notochord is shown with embryonic muscle tissue lying on either side. Both the notochord and the muscle tissue are imbedded in a matrix of the ground substance which is especially heavy in the region just below the notochord. From this region, it spreads to the right and the left surrounding the bundles of muscle fibers and extending to the body wall on either side. At the stage shown numerous cells are to be seen in the portion of the ground substance which lies below the notochord. These cells vary considerably in shape; some are rounded, while others have assumed a spindle-shape.

In figure 4 is shown a portion of a transverse section through the tail region of an 11 mm. embryo which was preserved in Zenker and stained with Mallory's stain. The magnification is 760 diameters. The portion of the section shown in this figure lies just below the notochord. At this point there is a considerable area of the primitive ground substance. It is surrounded ventrally and laterally by developing muscle tissue and dorsally by the notochord. In this figure it is desired to lay particular emphasis upon the relations shown between the ground substance and the cells which are present in it. The cells which have wandered into this region have for the most part become amoeboid or spindle-shaped in type and they possess long cytoplasmic processes which stretch out in various directions through the ground substance. The figure shows that, in a preparation of this kind which has been properly stained with the Mallory connective tissue stain, the differential color reaction between ground substance and cell bodies is so clear that the cell processes can be differentiated from the ground substance and traced to their definite endings, even though they may be drawn out extremely fine. Attention should also be called to the color reactions of the muscle tissue which bounds the ground substance. It will be noted that the bundles or muscle fibers show the characteristic color reaction of the cytoplasm of the mesenchyme cells lying in the ground substance. These results demonstrate that the Mallory connective tissue stain differentiates definitely the living substance, present in the cells and in the muscle tissue, from the intercellular ground substance.

It is worth while to note in this connection that Mall says[6] "In specimens of this kind it is easy to view these cells with their endoplasm as the connective-tissue cells and the exoplasm of the syncytium as the intercellular substance were not the development of the syncytium taken into consideration." And also[7] "while my results are now decidedly in favor of Flemming's view, the reader will soon see that if other methods and interpretations are employed (which I now consider false), it will be quite as easy to see the fibers developing between the cells as within them." Inasmuch as in the present work the study of the various stages has shown, in general, that the formation of the ground substance takes place before there is any attempt to form a syncytium by the mesenchyme cells—in fact before they have left the cell groups, wandered into the ground substance and there assumed the irregular shapes characteristic of mesenchyme cells—it is evident that the formation of the ground substance cannot be due to a cytoplasmic syncytium. This fact leads definitely to the conclusion, as Mall suggests above, that the cells with their endoplasm are the connective tissue cells complete and the material in which they lie is not exoplasm but an intercellular substance or ground substance. The cells which move into the common, intercellular ground substance are separate entities; not a part of it but apart from it.

[6] Mall, 1902, p. 336.
[7] Mall, 1902, p. 330.

The presence of a ground substance formed prior to the appearance of the mesenchyme cells has been demonstrated by various investigators but notably by Szily ('07) who, working on chick and fish material, says[8] "Vor dem Auftreten der Mesenchymzellen sind die Lücken and Spalten der Embryonalanlage durch ein feines Fasersystem ausgefüllt." He holds, however, that this cell-free, fibrous, supporting tissue, instead of being a secretion from the cells, arises from fine fibrous protoplasmic processes of the epithelial and endothelial cells. The mesenchyme cells appear later and enter into a protoplasmic union with the fibers already present. The embryonic connective tissue is made up of the mesenchyme cells and the fibrillar intercellular material. The mesenchyme cells furthermore serve for the nourishment and growth of the fibers which, in the meantime, have become separated from the cells of which they previously formed a part. Szily therefore believes that, in the formation of the embryonic supporting tissues, all three of the primary germ layers take an active part, and the final product is a mixed tissue in which the product of any one germ layer cannot be identified.

The present work agrees with the work of Szily in demonstrating the presence of a primitive ground substance prior to the advent of the mesenchyme cells. No evidence has been found, however, of its formation by means of protoplasmic processes of the surrounding cells. The study of the embryonic cells in the amphibian material, at the early stage at which the primitive ground substance is formed, shows them to have a clear, sharply defined, regular membrane with no processes of any kind. There is no question but that with a differential stain, such as Mallory's which was used, protoplasmic processes could be demonstrated if they were present. It is easy enough to do so a little later with the mesenchyme cells when they wander into the primitive ground substance as shown in figures 4 and 6. The conclusion is, therefore, that the primitive ground substance in the amphibian embryo arises as a cellular secretion and not by the fusion of cytoplasmic processes.

[8] Szily, 1907, p. 741. Cf. also Isaacs, 1919.

In the discussion of the earlier stages, it has already been noted that not only is the ground substance formed prior to the advent of the mesenchyme cells but also, in some regions, it shows from the earliest stages a fine fibrillation. In the stage shown in figure 4, this fibrillation of the ground substance has markedly increased and it is found that practically all the ground substance is permeated with the fibers. In some regions the fine fibers have united to form fibrous bundles and in general there appears to be a tendency for this to take place. A careful study of this and similar preparations reveals the fact, as is depicted in the figure, that there is no morphological connection between the cells which are present in the ground substance and the fibers. The latter have formed directly from the ground substance by a modification of the elements of which it is composed.

The preparations give evidence that the movements of the cells through the ground substance may supply, in part at least, a mechanical factor which aids in the formation of the fibers. For example, it will be noted in figure 4 that the fiber formation is particularly heavy in the portion of the ground substance lying near the ventral opening in the muscle tissue through which, apparently, a number of the amoeboid cells have wandered into the ground substance. The preparations show many similar instances of regions in which the movements of the cells through the ground substance have supplied a mechanical stimulus and thereby brought about the fiber formation. There is also the possibility that the cells in their movements may have some secretory action which also modifies the ground substance. However, this point is clear from the study of the preparations and should be emphasized that in no case has any morphological connection been found between the cells in the ground substance and the fibers.

In this connection attention should be called to the results previously obtained by the author ('17) in certain of the studies on the plasma clot in which it was demonstrated that, under the influence of certain mechanical factors, it was possible to transform a homogeneous fibrin clot into a fibrous tissue which, in its morphology, very closely resembled various types of fibrous connective tissues. In such cases it was possible to observe all stages in the process from an apparently homogeneous ground substance or matrix to the heavy fibrous tissue. By the use of a microscope equipped for dark field illumination it was possible to analyze the transformation process and to demonstrate that the fibers arose as a result of a fusion and consolidation of the minute elements of the fibrin clot which under ordinary illumination were entirely invisible. In experiments of this type, inasmuch as cells were not present, it was possible to state with absolute certainty that the fibers had arisen through a modification of the ground substance of the clot.

In other types of experiments with plasma clot preparation in which cells were present, it was shown ('15) that a transformation of the clot, and consequently fiber formation, could be brought about by mechanical factors induced by the movements of the cells through the clot. A case of this is shown in figure 5[9] which is a drawing of a portion of a plasma clot preparation at a magnification of 950 diameters in which a piece of muscle tissue had been imbedded. A spindle-shaped cell has left the piece of imbedded tissue and is moving through the fibrin clot. In this preparation, the path of the cell can be traced from the imbedded tissue through the clot to its present position, by means of the fibrillar structure which has arisen in its wake in the ground substance of the clot. In this and similar cases it is clear that the fiber formation in the clot has taken place by the introduction of mechanical factors through the cell movements with a consequent fusion and consolidation of the minute elements of the fibrin clot.

In figure 6 is shown a portion of a transverse section through the tail region of a 75 mm. embryo of R. catesbiana at a magnification of 75 diameters. In this embryo the connective tissue is more mature in type with a practically complete transformation of the ground substance into a fibrous tissue. It will be noted in the figure that the densest formation of connective tissue is in the region immediately surrounding the notochord and also

[9] Baitsell, 1915. A photomicrograph of this preparation is published in this paper as fig. 19.

ventrally in the median line. From the median region of the embryo the connective tissue sheet extends laterally in either direction among the bands of muscle fibers—transverse sections of which are to be seen in the figure—until the body wall is reached. Between the body wall and the muscle tissue there is also a compact layer of the connective tissue. The fibrillation of the connective tissue is heaviest around the notochord. In the region of the muscle tissue the fibrillation is comparatively light and the ground substance closely resembles in its structure that found in young tadpoles as shown in previous figures. Under a higher magnification it will be seen that numerous spindle-shaped cells are scattered in various regions of the preparation lying in cavities on the connective tissue. In some cases they are stretched along the bundles of fibers. Here again the Mallory stain shows a clear differentiation between the cytoplasm of the cell bodies and the common ground substance in which they are imbedded and in no case has it been possible to demonstrate any morphological connection between the cells and the fibers.

DISCUSSION

The researches of Studnicka ('03), Hansen ('05), Mall ('02) and others, upon various phases of connective tissue development in different species of animals, demonstrate conclusively that a rigid adherence to the intracellular theory, in which it is held that the fibers develop directly through a transformation of cell cytoplasm, is no longer possible. The result has been that the intracellular theory as originally stated has been modified by the Hansen-Mall theory which holds, as stated earlier in this paper, that the fibers develop, in general, in a common, living intercellular material, termed the exoplasm, which has been formed as a result of a syncytium of the mesenchyme cells.

At the present time, the inadequacy of the unmodified intracellular theory has been recognized even by Flemming, whose work on the development of connective tissue fibers in the Salamander larvae ('91) is generally regarded as the corner stone of the intracellular theory. Flemming admits that the results of his researches, in which he showed that the fibers arose through a direct transformation of the cell cytoplasm, do not explain authenticated cases in which it is known that, in the later stages of development of certain connective tissues, an independent growth of the bundles of connective tissue fibers takes place. In such cases Flemming holds ('06) to the Hansen view of a living exoplasm which can nourish itself, grow by intussusception and differentiate new connective tissue fibers.[10]

A review and summary of the question is given by Heidenhain in his Plasma und Zelle ('07). He says[11] that the general opinion of most of the recent investigators is that the first fibrillar differentiation in connective tissue formation arises by a transformation of the cell protoplasm. It is also generally held that a secondary fiber differentiation arises from an organized living matrix independently of cells. Cases are known however (e.g., v. Ebner's work) where the cells apparently first produce a structureless intercellular substance which secondarily produces fibers. With regard to the activities of the organized living intercellular substance (exoplasm), Heidenhain says:[12]

[10] Flemming, 1906, p. 7. To quote: ". . . . ; wie sind aber die späteren Zustände zu denken, wo die schon gebildeten Fibrillen aus der Zelle herausgerückt und Intercellularsubstanz geworden sind? Ich antworte darauf mit Hansen . . . . : dann liegen eben die Fibrillen in dem Territorium von Intercellularsubstanz, das von der betreffenden Zelle geschaffen worden ist, das aus ihrem 'Ektoplasma' (Hansen) hervorgegangen ist; diese Territorien sind mitlebendig, wie ich mir die ganze Intercellularsubstanz so denke . . . . , es können in ihnen Vorgänge fortspielen, die zu einem Längenwachstum der Fibrillen durch Intussusception führen. Ein solches, intussusceptionelles Längenwachstum der Fibrillenbündel wären wir ja übrigens auch genötigt anzunehmen, wenn wir eine freie intercelluläre Fibrillenentstehung voraussetzen wollten. Denn wenn ich mir eine embryonale Sehne in ihren frühesten Zuständen denke und dann spätere dagegenhalte, wo die Bündel 10- und mehrmal so lang geworden sind als zu der Zeit wo sie eben entstanden waren, wie soll das ohne eigene Wachstumverlängerung der Bündel abgegangen sein?" And again (p. 9): "Es bestände danach die gesamte Intercellularsubstanz des Bindegewebes aus solchen vereinigten Ektoplasmen von Zellen, die fibrillär umgewandelt wurden und die, wie ich mit Hansen glaube, mitlebend fortbestehen unter dem vitalen Einflusz der produzierenden Zellen und zur Entwickelung neuer intercellulärer Formteile im stande bleiben."

[11] Heidenhain, 1907, p. 37.

[12] Heidenhain, 1907, p. 37.

> Innerhalb der einmal angelegten Intercellularsubstanz wachsen die Bindegewebsbündel späterhin selbständig weiter fort, in die Länge sowohl in die Dicke. Dasz das Längenwachstum ohne eine besondere Tätigkeit der Zellen vor sich geht, ist wohl immer angenommen worden; was das Dickenwachstum anlangt, so war früher die Anschauung vielfach verbreitet, dasz die Zellen neue Fibrillen auf die Oberfläche der erstmals angelegten Bündel abscheiden. Indessen ist dies nur eine Formel, welche zwar aus dem cellularen Prinzip sich ergibt, aber unmöglich überall zutreffen kann. Tatsache ist, dasz man im lockeren Bindegewebe die Zellen oft nur sehr sparsam eingestreut findet, und dasz die Bindegewebsbündel auf weite Strecken hin mit ihnen in keiner Berührung stehen, also nur durch eigene Assimilation wachsen können.

Heidenhain further holds[13] that the living intercellular material is of a more passive nature, in fact a sort of a living framework which lacks many of characteristics of true cell cytoplasm, but still retains at least for a time the power of growth through intussusception, and the ability to differentiate new fibrillar structures. To quote: "Die Metaplasmen sind in Verhältnis zu den eingeschlossenen Zellen eine lebende Substanz besonderer Art, welche in eine andere Bahn der Entwickelung übergegangen ist, ohne dasz eine Möglichkeit der Rückverwandlung in Protoplasma besteht."

The adoption of this view of a living exoplasm with the power to assimilate food material and the ability to differentiate new fibrillar structures independently of the typical cells at once raises a number of questions. In the first place this living exoplasm must be regarded, as stated by Heidenhain, as being of a lower grade of living matter than that found in the regular cell bodies. It possesses only certain of the vital activities and entirely lacks others. In other words, it is necessary to conclude that, in the connective tissues of an animal, there are different degrees of living matter, beginning with the nucleus of the cell as the highest type, then grading into the endoplasm of the cell bodies and finally into the common exoplasm.

In the second place, the postulation of a living exoplasm modifies the cell theory to a great extent, and delegates many of the powers supposedly possessed only by cells to masses of intercellular material, in considerable areas of which even nuclei

[13] Heidenhain, 1907, p. 48.

may be entirely lacking. This raises at once the question as to how such a material is governed. Heidenhain believes[14] that "den Intercellularsubstanzen jene eigenartige Automatie des Lebens zukommt, welche alle lebenden Teile besitzen." He also holds that there is an external regulation of some sort but "Woher diese Regulation stammt, ist uns einstweilen verborgen."

A consideration of the intracellular theory as modified by the studies of Hansen and Mall and the intercellular theory reveals a quite close agreement. This rests upon the recognition in both theories that a common intercellular ground substance is present in which fibers form independently of cells. The difference between the two theories lies in the question of the nature of the intercellular ground substance; the intercellular theory holding that it is a secretion product of the embryonic cells and the Hansen-Mall theory holding that it is a special type of living matter formed by a syncytium of the mesenchyme cells.

In this connection, the present researches give evidence that the ground substance is nothing more than a type of secretion given off by the embryonic cells. This conclusion is based, first, upon the fact that ground substance is formed originally before any syncytium of mesenchyme cells could have been formed and, second, that material which has been properly stained with Mallory's connective tissue stain shows, at all stages, a clear and consistent differentiation in the color reaction between cell cytoplasm and the ground substance.

The ground substance having been formed, the next step we have to consider is the formation of the connective tissue fibers. In this connection from the intracellular standpoint, Flemming and others have clearly demonstrated that the cells in developing connective tissue contain intracellular fibers which they regard as being connective tissue fibers. The presence at times of these intracellular fibrillar structures cannot be questioned but conclusive proof is lacking that they become the connective tissue fibers; there is the possibility that they are transient fibrillations due to various factors, examples of which can be found in many types of cells.

[14] Heidenhain, 1907, p. 48.

In connection with this point, Merkel[15] rightly calls attention to the fact that it is only the formation of the fibers in the first stages of connective tissue development concerning which there is a difference of opinion at present. For a long time there has been no question but that in the later stages of connective tissue development there is a complete separation of fibers and cells. Since this is the case, the logical conclusion appears to be that the fibrillar structures which are present in the connective tissue cells at certain times are not connective tissue fibers, for there is no apparent reason why the fibers in the early stages of connective tissue development should be formed in the cells and later the process change and the fibers be formed in the ground substance independently of cells.

Considerable stress has been laid at times upon the spindle shape of the connective tissue cells or fibroblasts. I regard the shape of the connective tissue cells as almost entirely dependent upon the external factors which the cells encounter. The study of the material shows that when the mesenchyme cells first enter the ground substance they are, in general, rounded, typically-shaped cells. Later, in the ground substance, they assume various irregular shapes, as shown in figure 4, among which will be found a number of spindle-shaped fibroblasts. The idea has been more or less prevalent that cells of the spindle type are in some way 'spinning fibers' and therefore the shape has been regarded as a constant factor for the fibroblasts. The studies of various types of cells in tissue cultures[16] show that the shape of the cell is not a constant factor. Certain cells when grouped together in the cell masses show a typical cell body. The same cells when separated assume various irregular shapes, such as stellate, amoeboid and spindle, depending upon the nature of the medium and whether the cell is exhibiting movement. If the cell begins to move in a certain direction along a fiber or other support it will elongate in a direction parallel to the movement and assume thereby a spindle shape.[17] It is believed,

[15] Merkel, 1908, p. 381.
[16] Harrison, 1914, pp. 535–8. Cf. Figs. 8–12. Matsumoto, 1918, pp. 555 et seq.
[17] Harrison, 1914. Cf. figs. 4–7.

therefore, that the spindle shape of a cell indicates a response to the environment and not a process of fiber formation.

With regard to fiber formation the present work demonstrates that the fibers may develop in regions of the ground substance which are free from cells. At later stages when cells are present the study of the material gives no evidence of any morphological connection between them and the fibers in the ground substance. The complete process of fiber formation from the ground substance can be observed and the transformation into a fibrous tissue appears as a gradual development from a fine fibrillation through various stages until the well developed fibrous condition is reached.

The previous studies of the author, with the plasma clot, show clearly how a morphological transformation of this character may take place, by means of a fusion and consolidation of the minute elements of which the clot is composed, in response to mechanical factors such as tension or pressure. It was suggested at that time[18] that "a reaction of this kind plays an important part in the ontogeny of the individual in the formation of the various connective and supporting structures. The well known fact that they, in general, are laid down in exact correspondence to the definite stresses of the organism leads to the conclusion that in their formation some arrangement must have been present which would respond to the various mechanical factors introduced during development, such as has been shown to be the case with the plasma clot. The generally accepted view of the intracellular origin of the connective tissues does not give any adequate explanation of this fact."

From the morphological standpoint, the results of the present study indicate that the formation of connective tissue in the amphibian embryo is similar to the process which takes place in transformation of the plasma clot. The intercellular ground substance of developing connective tissue may therefore be compared in its morphology to the plasma clot. This ground substance when first formed appears homogeneous or with a

[18] Baitsell, 1917, p. 130.

fine fibrillation. The process of transformation into a fibrous tissue is a progressive one. The fibrillation increases, bundles of fibers are formed and in time the entire ground substance, which at first showed such a high degree of homogeneity, becomes transformed into a fibrous tissue. It is indicated that this transformation occurs as the results of the introduction of mechanical factors in the embryo. These factors may be due to certain lines of tension in the embryo corresponding to the inherent polarity of the organism or, just as in the plasma clot, the movements of the cells through the ground substance may introduce mechanical factors which aid in the transformation of the ground substance into a fibrous tissue. The cells, however, are to be regarded primarily as assimilative and secretive agents, chiefly concerned in the formation of the undifferentiated ground substance.

SUMMARY

1. The primitive forerunner of connective tissue in amphibian embryos is a gelatinous material (primitive ground substance) which can be demonstrated around the notochord soon after it is formed and shortly before the embryo has reached the tail bud stage. A little later this material, which is to form in general the ground substance of the connective tissues, surrounds the medullary cord and a layer of it, following the body wall, extends ventrally on either side and in time completely encircles the body cavity. The formation of this matrix around the notochord takes place before there is any syncytium of the mesenchyme cells in this region. It is evident, therefore, that this primitive ground substance of connective tissue has arisen as an intercellular secretion of the embryonic cells and not by a cytoplasmic syncytium.

2. The ground substance having been formed, cells begin to move into it and wander through it. These are spherical at first, but as they move through the ground substance, they soon change into various shapes, becoming stellate, spindle-shaped, etc. The study of the sections shows that, in general, individual cells do not separate from the cell masses and move out into the

various cavities and open spaces of the embryo until after the formation of the secreted ground substance which is the forerunner of the connective tissues. It appears evident therefore that cells, whether in tissue cultures as shown by Harrison[19] or in the developing embryo, have need of a supporting framework of some kind in order for movement from the main masses to take place.

3. The connective tissue fibers begin to arise in the ground substance soon after it has formed. In some cases, they form in the ground substance in regions which are free from cells so that it is certain that they have not arisen by an intracellular action. The fibers are evidently formed by a gradual transformation of the ground substance, first, into a delicate net-like structure and then into the long fibers which are typical of connective tissue. In its morphological features, at least, this process gives evidence of being identical with the one previously observed in the transformation of the plasma clot.

4. In those regions of the ground substance into which the cells have wandered, the formation of the fibers can also be shown to be due to changes in the ground substance and not to a sloughing off of the cell protoplasm. A differential stain, such as Mallory's connective tissue stain, shows definitely the boundaries of the cell cytoplasm and of the ground substance. It is suggested that the movements of the cells through the ground substance introduce mechanical factors which aid in fiber formation just as has been shown to be the case in plasma clots. In any case, the fact that the fibers can form in a cell-free ground substance shows that no part of the cell cytoplasm is necessary for their differentiation from the ground substance; nor is it possible to demonstrate any morphological connection between the cells and the fibers in the ground substance.

[19] Harrison, 1914, pp. 540 and 543.

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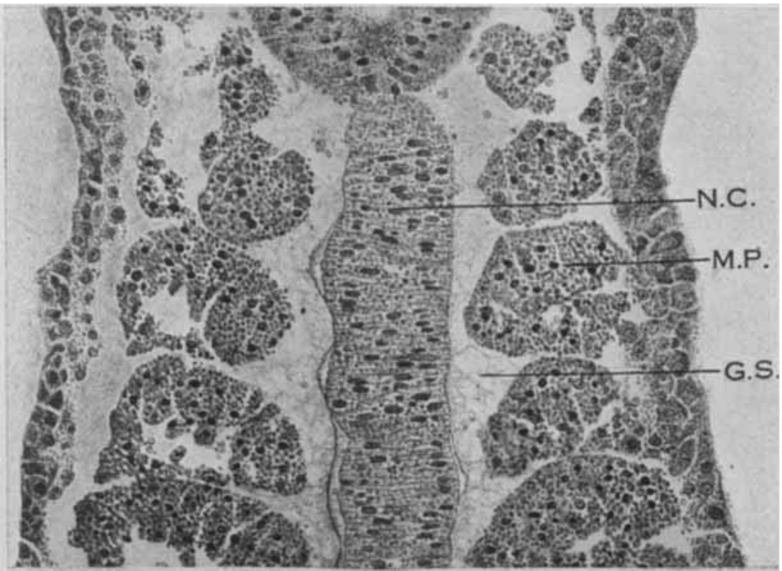
For complete bibliographies of this subject see references to Heidenhain, Flemming or Merkel above.

PLATE 1

EXPLANATION OF FIGURES

1 Portion of sagittal section of 3.2 mm. embryo of R. palustris. × 285. The notochord (N.C.) is shown imbedded in the cell-free connective tissue ground substance (G.S.). The latter extends laterally on either side into the muscle plates.

2 Portion of transverse section of 4 mm. embryo of R. palustris. × 322. A section of the notochord (N.C.) is shown imbedded in the connective tissue ground substance (G.S.).



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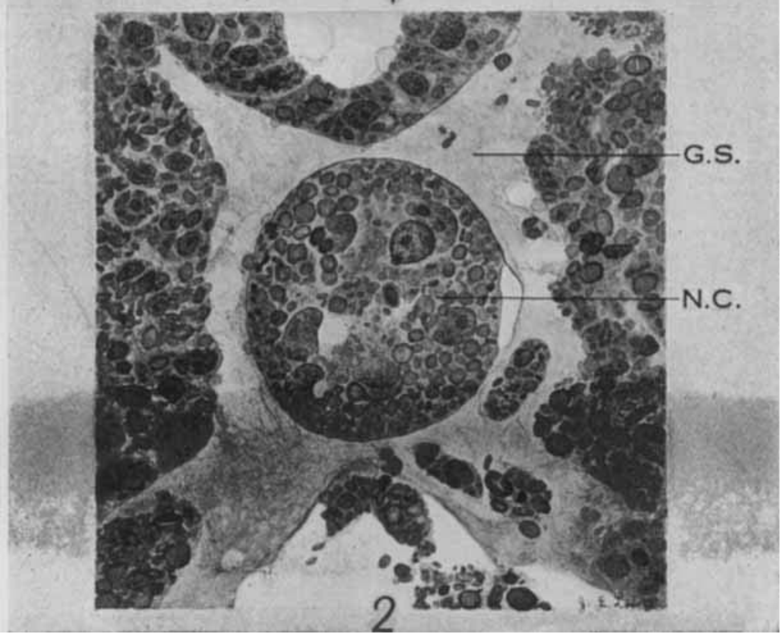

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PLATE 2

EXPLANATION OF FIGURES

3 Portion of 9 mm. embryo of R. sylvatica. × 322. The ground substance (G.S.) is shown lying below the notochord (N.C.) and extending laterally on either side between the muscle plates to the body wall. At this stage some of the mesenchyme cells are beginning to move into the ground substance.

6 Portion of a transverse section through the tail region of a 75 mm. embryo of R. catesbiana. × 75. The figure shows a portion of the notochord surrounded by the connective tissue ground substance (G.S.). In this late stage the ground substance, particularly around the notochord (N.C.), is largely composed of bundles of typical connective tissue fibers.

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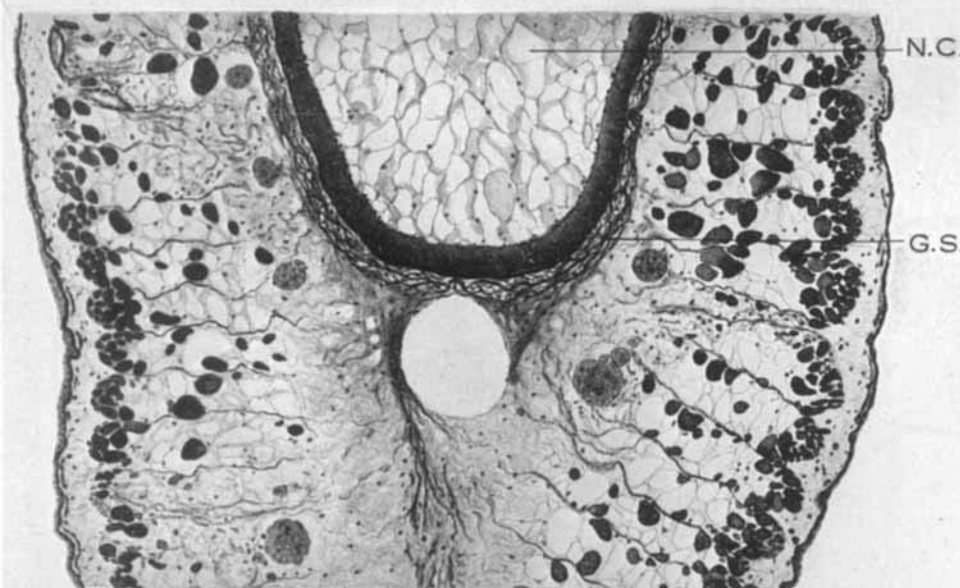

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PLATE 3

EXPLANATION OF FIGURES

4 Portion of transverse section of 11 mm. embryo of R. sylvatica. × 760. Mallory connective tissue stain. The region shown lies just below the notochord as in figure 3. The connective tissue ground substance (G.S.) contains a number of wandering mesenchyme cells which take the same stain as do the surrounding muscle fibers (M.F.)—both types of cells being clearly differentiated by the stain from the secreted connective tissue ground substance which is formed before any cells are present in it.

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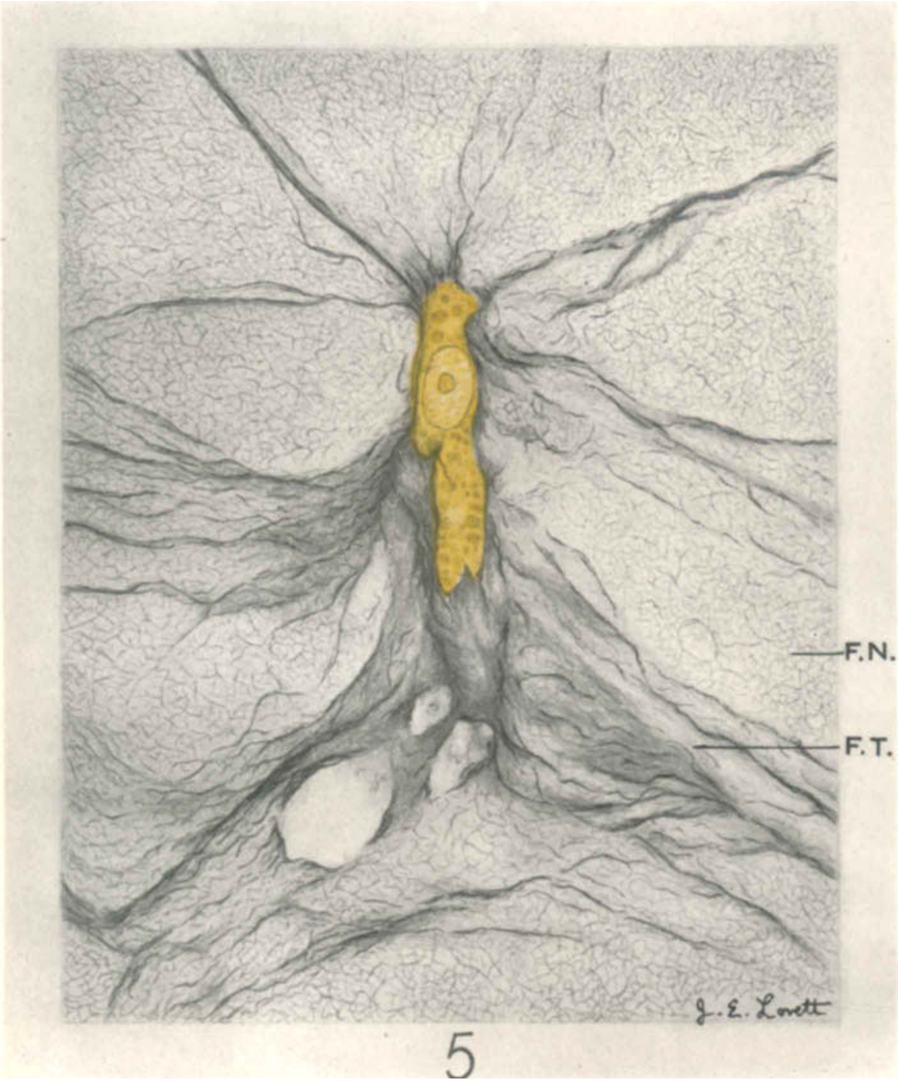
PLATE 4

EXPLANATION OF FIGURE

5 Section of plasma clot from a tissure culture. × 950. The figure shows the transformation of the fibrin net (F. N.) of a plasma clot into a fibrous tissue (F. T.) as a result of the mechanical factors induced by the movements of the cell through it. Cf. Baitsell, 1915, fig. 19.



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