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A STUDY OF THE DIFFERENTIATION OF TISSUES IN THE REGENERATING CRUSTACEAN LIMB.[1]

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WITH 8 PLATES.

CONTENTS.

[1]This paper is one of a series of experimental and anatomical studies of regeneration ('05, '06[1], '06[2], '06[3], '07[1], '07[2], '08).

Introduction.

The recent work by Reed ('04) on regeneration in the crayfish, Ost ('06) on Oniscus, and Steele ('08) on the regeneration of the compound crustacean eye, draws attention to certain fundamental questions. Their results are in marked contrast with the observations made on vertebrates where it appears that a regenerating tissue almost invariably arises from similar pre-existing tissue. The important relations of this subject to the problem of both the normal and pathological origin and cytomorphosis[2] of new tissue cells, render it desirable to investigate further the histogenesis of regenerating tissues. The present paper contains the results of a cytological study of the cells and tissues in the regenerating chela of the lobster, a crustacean which has been especially favorable for this study, both on account of its highly developed capacity for regeneration and because of the unsurpassed opportunity for obtaining suitable material at the lobster hatcheries.

The results to be described deal more especially with (1) the cytological changes observed in the regenerating ectodermal cells; (2) the genesis of striated muscle, nerve, and connective tissue; and (3) the morphology of certain structures at the breaking joint of the limb, which as far as the writer is aware, has not previously been considered.

I desire to express my thanks to Professor C. S. Minot, of the Harvard Medical School, for interest and encouragement during the

[2]This term is used "to designate comprehensively all the structural modifications which cells or successive generations of cells may undergo, from the earliest undifferentiated stage to their final destruction." See Minot ('01, p. 494.)

progress of the present work. I am also indebted to Professor A. D. Mead, of Brown University, for generously permitting me the use of materials and apparatus at the Experiment Station of the Rhode Island Commission of Inland Fisheries; and to Professor R. J. Terry, of Washington University, for valuable suggestions during the completion of the present paper.

II. Material and Methods.

A serious obstacle encountered in determining the origin and differentiation of a regenerating tissue is the difficulty of securing a complete series of successive developmental stages. If a number of animals are operated upon at the same time and material fixed at successive intervals, it is usually found necessary in subsequent study to make an extensive rearrangement of the material on account of variations in size and rate of growth. These technical difficulties were considerably lessened in the present work because of the excellent material obtained at the lobster hatchery of the State Experiment Station at Wickford, Rhode Island. From a hatchery pool containing several thousand lobster fry, it was possible to select a large number of animals which were practically equal in age and size, and which had all moulted to the same stage within less than twenty-four hours. This last point is of considerable importance, since it had been ascertained in previous experiments ('06) that the age of the animal and the time, in relation to moulting, at which the operation is made are important factors influencing the rate of regeneration. These young lobsters were also further favorable for study because the exoskeleton had not as yet attained such an amount of chitin and lime salts as to necessitate decalcification before sectioning.

The operation consisted in the autotomous removal of both chelæ. The lobsters, about 300 in number and all practically equal in age and size, were placed in floating cars and kept in as nearly normal condition as possible. Specimens were then preserved at two, four, and eight hour intervals throughout a period of fourteen days, in which time the chelæ had undergone complete regeneration. From this succession of stages about 54 were selected, sections of the chelæ were cut 5 micra in thickness and mounted in series. In order to

facilitate a comparative study, the specimens were all cut in a nearly constant plane. In addition to this series, sections of the regenerating limbs of older lobsters were also studied.

The material used was fixed in a saturated 35 per cent alcoholic solution of corrosive sublimate. Several other fixing agents were tried, but all solutions containing acids proved unsatisfactory on account of their injurious reaction with the chitin and salts of the exoskeleton. The nuclear and cytoplasmic stains employed were alum hæmotoxylin, Heidenhain's iron-alum-hæmatoxylin, Congo-red, eosin, and Mallory's connective tissue stain.

III. Anatomical Structure of the Chela.

1. *Segments, Muscles, and Joints.*—The following account is confined to those structural characteristics of the limb which are essential for the subsequent description of its regeneration.

The chela of the lobster is composed of six segments (Fig. 37). Proceeding in a disto-proximal direction these segments are known successively as a dactylopodite or dactyl (*d*), propodite (*p*), carpopodite (*c*), meropodite or meros (*m*), ischipododite (*i*), and basipodite (*bs*). A seventh basal segment not involved in the present study is the coxopodite. The index is the distal part of the propodite which opposes the dactyl and forms one jaw of the large claw. All of these seven segments are united to each other by flexible joints with the exception of the last two,—the ischiopodite and basipodite. These are fused together into an immovable joint or "breaking-joint," a structure especially adapted to the process of autotomy. Each joint is like a hinge, articulating at two points and permitting only a simple flexion and extension of the segment. The musculature of the chela consists of a pair of opposing muscles for each of the segments with the exception of ischiopodite (Fig. 37). The pair of muscles in any given segment is attached proximally to the chitin of the proximal region of the segment. Distally the converging muscle bundles are attached to a chitinous plate or ingrowth of the exoskeleton of the next segment distad. The origin and insertion of the extensor and flexor are on opposite sides of the joint. In each of the first three pairs of muscles, the flexor is larger than the opposing extensor.

The ischiopodite is an exception to the above description, for the muscles are not arranged to act in opposition. There are two muscles in this segment, but they both have their origin on the same side of the limb, *i. e.*, on the ventral side of the fourth joint (Fig. 37, e^4). One muscle is slightly larger than the other, but both are smaller than any other muscles of the chela. The larger muscle is inserted partly on the morphologically anterior side and partly on the dorsal surface of the exoskeleton in the proximal part of the ischiopodite. The smaller muscle is inserted directly on the morphologically anterior wall of the segment. Both muscles, therefore, appear to function as extensors. A similar arrangement of muscles has been described by Fredericq ('92) in the ischiopodite of the crab. That a similar condition exists in the crayfish is not so clear, however, for Reed ('04) states that the ischiopodite of the cheliped in the crayfish possesses "both an extensor and a flexor" (p. 310).

2. *The "Breaking Joint."*—a. *Exoskeleton.*—By the breaking joint (Fig. 37, *bk*) is meant the region in which a fusion has taken place between the ischiopodite and the basipodite. Since it is at this region that the chela is amputated in autotomy and the new limb begins to regenerate, a more detailed description of the breaking plane or joint is essential for our present purpose.

The external appearance of the breaking joint in the adult has been described by Herrick ('95) as a "fine hair line leading from the small spur next to the articular facet on the under side, round the anterior border to the upper side of the joint. It then bends forward and abruptly backward, crossing the small proximal end of the joint, to near its point of departure" (p. 10). This hair line represents the plane of union of the basipodite and ischiopodite into a solid compound segment. In the first three larval stages there is a movable articulation between these two segments and as described by Herrick, "there is no true fusion of the segments until after the fifth stage" (p. 102). It may be questioned, however, whether there is any longer a functional articulation of these segments even during the fourth and fifth stages of development. Although externally the joint may present the appearance of a free articulation as indicated in Herrick's figures, the examination of longitudinal sections

of the fourth stage limb shows a different condition. As may be seen in Figs. 1 and 2, there is strictly no free articulation evident, especially on the morphologically ventral side (see also Fig. 3, *bk*), where there is an almost perfect continuity of the exoskeleton from one segment to the other. On the dorsal side the union is less complete. A difference between the two sides of the breaking joint is evident even in the adult where the chitin is still much thicker on the ventral than on the dorsal surface. In section the region of the breaking joint can easily be identified by the characteristic coloration which the chitin takes with Mallory's connective tissue stain. With this reagent the exoskeleton, with the exception of the outer lamellæ of chitin, takes on a bright blue color, whereas the line of fusion between the two segments is sharply differentiated by becoming dark red.

The internal structure of the breaking plane of the limb may be described as follows. The epithelium (epidermis or "hypodermis") of the exoskeleton is continuous across the breaking plane, from one segment to the other. The connective tissue occupies the interior of the breaking joint and is complex in its arrangements. Certain structural relations of this tissue were discovered, which differ considerably from those previously recorded. They will presently be described.

It can be readily appreciated that the mechanism for preventing an excessive loss of blood is an important element in the perfection of a breaking joint adapted to the autotomous removal of the limb. Such a mechanism is supplied by the connective tissue structures. Fredericq ('92) was among the first to describe the separation of the blood cavities of the basipodite and ischiopodite by a "cloison membraneuse" or circular diaphragm, stretched across the distal part of the basipodite (p. 177). Similar descriptions have been written by Andrews ('90) for Libina, and by Herrick ('98) and Reed ('09) for the lobster and the crayfish. This transverse membrane was at once interpreted as a structure for preventing hemorrhage after autotomy. Andrews describes the membranous fold in the breaking joint of the spider crab as extending "from the epidermis to the central nerve and blood vessels," and states that after autotomy the membrane covers

the entire surface of the stump except at the centre, "where there is a rounded hole with a little torn tissue and blood exposed". Reed states that in the lobster and the hermit crab "the opening through the membrane for the nerve and the blood vessel is in about the centre of the exposed surface when the leg is thrown off" (p. 309). In an earlier description of the lobster, the writer ('05) also described this membrane as "extending almost entirely over the basipodite" and "perforated only at the centre by an artery, the blood sinus, and a large nerve" (p. 89). It was with considerable surprise, therefore, that it was found in the present study of serial sections of the chela that the above descriptions cannot, for the lobster at least, be regarded as strictly accurate, particularly in regard to the relation of the blood sinuses.

In order to obtain a more thorough conception of these structural relations, graphic reconstructions of the breaking joint have been made from serial sections of the right chela of a fourth stage lobster. The tissue structures of the breaking joint at this stage are perhaps somewhat simpler than in the chela of an older animal, but their essential relations are, I believe, practically the same. It should also be stated that these reconstructions are in one detail not complete, since the connective tissue network in which the arteries and nerves are partly embedded as they pass along the walls of the large venous blood sinuses, is not represented in its entirety. Figs. 1 and 2, respectively, show the outer and inner, or morphologically posterior and anterior, parts of the breaking joint and adjacent segments. Fig. 3 represents a median section between the two parts shown in Figs. 1 and 2. The latter figures do not show quite half of each side.

b. *Blood Vessels and Nerves.*—It may be observed in these figures that there are two nerves, two arteries, and a large venous sinus (divided into two separate channels) which pass through the region of the breaking joint. The two nerves n^1, n^2, lie on the inner or medial side of the joint, the larger nerve (n^1) giving off a small branch as it leaves the basipodite. The main artery (a^1) passes along the outer wall. In the basipodite it gives off a small branch (a^2) which takes an oblique course across toward the inner wall of the ischiopodite. The venous blood of the chela is carried toward

the body through an immense blood sinus (*vs*). As this sinus passes through the ischiopodite it is temporarily separated into two channels by a connective tissue partition or septum (*S*). One of these channels passes ventrally and the other dorsally, through the breaking joint. The two nerves and arteries lie within the septum and adjacent connective tissue separating the two venous channels.

In the course of these two blood channels just as they enter the basipodite after having passed through the breaking joint, there occur two very interesting valve-like folds of connective tissue. These two structures arise in the following manner from the median septum separating the blood channels. As the septum extends proximally into the basipodite it becomes divided into two plates or folds (v^1, v^2) much like the arms of an inverted "Y," one of the arms passing toward the dorsal wall and the other toward the ventral wall of the segment. The fold extending toward the dorsal wall is somewhat shorter and thicker than the one on the ventral side. These two folds do not completely unite with the walls of the basipodite. A part of the border of each fold retains a free edge, which, together with the opposing wall of the segment, completely encloses the lumen of each blood channel. Beyond (*i. e.*, proximally) these folds the two blood channels reunite to form again a common venous sinus (vs^2).

While endeavoring to understand the significance of these folds as they were first seen in the serial sections, it was suggested that they might possibly function as valves for the venous blood channels, the valves closing when the limb had been autotomously removed. Following up this suggestion, serial sections of specimens which had been fixed immediately after the autotomy of the chela, were then examined. It was at once evident that the folds in question had assumed in these specimens a position different from that observed in sections of a chela before the limb had been removed. Each fold had now become distended in a distal direction as if caught by an outward blood pressure, the result being the complete closure of the ruptured end of the two blood channels (Fig. 4, v^2). Desiring to make a still further test, a number of live lobsters were then obtained and the chelæ autotomously amputated. At first there was a short

jet of blood from the exposed stump, and then a slight bulging of the tissues over the surface of the stump with no further escape of blood. The location of the two venous channels could be distinguished quite readily by the transparency of the now turgid valves closing each lumen. The two valves were then gently pressed inwards with the tip of a pencil and in each case, a slight opening of the valve being effected, there at once followed a jet of blood. The valvular function of these folds seems, therefore, to be clearly demonstrated.

This description may be summarized with the statement that a septum of connective tissue extends across the cavity of the limb in such a way as to divide the venous sinus into two channels in its passage through the breaking joint. Through this septum and the adjacent connective tissue pass the two arteries and nerves. Proximally the septum forms two folds which function as valves for the venous channels.

It can readily be appreciated how in previous descriptions some of the relations of these structures may have been overlooked. For when the valves have closed after autotomy, the remaining stump presents every appearance of a continuous membrane extending over its exposed surface, "perforated only by a nerve and a blood vessel," so that in the following statement Fredericq ('92) even indicates a doubt regarding the passage of the vein, "Seul le nerf mixte de la patte, l'artère (et un sinus veineux?) franchissant cette limite et passent du basipodite dans l'ischiopodite, à travers un orifice étroit, creusé dans la membrane obturatrice" (p. 177).

c. *An Embryonic or Transitory Muscle.*—Both Fredericq ('92) and Morgan ('00) have recorded the fact that in the chela of the adult crayfish or lobster "no muscle passes from the ischiopodite to the basipodite". In other words, in the adult lobster no muscle extends across the breaking joint. It was with considerable surprise, therefore, that it was found that this is not true for the larval lobster. In the study of sections of the chela of larval animals, a well developed muscle was discovered extending from the ischiopodite to the basipodite, and consequently passing through the breaking joint. This muscle is easily seen in a longitudinal section of the limb (Fig. 5, *m*). It is attached to the inner side of the ischiopodite, passes

through the ventro-anterior region of the breaking joint, and is inserted upon the inner wall of the basipodite. It functions as a flexor. This muscle persists throughout the first four larval stages of the lobster's development. During the fifth stage, however, it begins to degenerate, and at the sixth stage the former muscle is represented by a mere bundle of connective tissue (Fig. 6, *ct.*), containing a few remnants of the degenerating muscle fibers (*md*).

It is interesting to note that the stage of the lobster's development at which the degeneration of this muscle occurs is not only the one just preceding the stage in which the asymmetrical differentiation of the chela first becomes evident, but, as the writer ('08) has previously shown, it is also the stage after which the asymmetrical differentiation of the chelæ can no longer be experimentally controlled.

The discovery of this muscle introduces a new fact to be taken into account in the question of the morphological significance of the breaking joint. Andrews ('90) maintains that the origin of the breaking joint may be "explained as a modification of a former free joint" (p. 142). On the other hand Reed ('04) concludes, from a comparative study of the crustacean limb, that it "seems, therefore, erroneous to state that the breaking joint corresponds to the lost joint" (p. 311). Without entering into a discussion of this question, it may be observed that the existence of a muscle in the chela of the larval lobster which crosses the breaking joint and is attached to its two adjacent segments, seems a strong point in favor of Andrew's conclusion.

d. *Autotomy.*—The process of autotomy or defensive mutilation among crustaceans has been fully described by Fredericq and Morgan. When the nerve within the chela is experimentally stimulated a vigorous contraction occurs in the muscles of the basal segment, followed by a sudden snapping off of the limb at the breaking joint, leaving the exposed surface of the stump as smooth as if cut with a keen-edged knife. Regarding autotomy in the larval lobsters, I can confirm Herrick's observation that the "casting of the claw" does not occur before the fourth larval stage. In experiments with several hundreds of first, second, and third stage lobsters in which one or both chelæ were removed, I have never observed the limb to

be thrown off autotomously, although under a slight tension, such as may be produced by a gentle pull with a small forceps, the chela will, however, usually part in the region between the basipodite and ischiopodite, *i. e.*, at the joint which is destined to develop later into the breaking joint of the adult animal.

IV. Early Stages of the Regenerating Limb.

1. *The Readjustment of Injured Tissues.*—Fig. 4 represents a longitudinal section of the stump of the right chela just after autotomy. The valve (v^2) has been carried outwards as the result of the amputation and now closes the venous channel *vc*. The exposed edge of the layer of epithelial cells of the epidermis (*e*) projects slightly above the level of the broken chitin (*ch*) of the stump. Shortly after autotomy the vascular cavities at the distal end of the stump become filled with a dense mass of nucleated blood corpuscles (Fig. 7, *bc*). The muscle (Figs. 2 and 5, *m*), which was torn by the operation, degenerates and is not restored again in the later regeneration of the limb, a result which might be expected, for it will be recalled that normally this muscle degenerates during the fifth stage and is absent in the adult lobster.

A protecting crust of coagulated blood plasma and blood corpuscles soon becomes evident over the exposed surface of the stump. This blood clot (Fig. 7, *bcl*) forms above, or in other words distal to, the connective tissue membrane extending across the limb. The relation of this blood clot to the connective tissue membrane differs somewhat from that described by Reed ('04) as occurring in the crayfish, where the "blood cells form a plug at the opening through the membrane, and collect in a thick layer just beneath the ectoderm cells, which form the proximal layer of the membrane of the breaking joint. . . . After a time these blood cells arrange themselves in horizontal lines crowded close under the ectoderm cells of the membrane." Reed further states that "in a few hours from the time that the leg is thrown off the membrane takes on the appearance of dead tissue. . . . The blood cells soon degenerate, and most of them are thrown away with the membrane, when this is later cast off" (p. 312). In contrast to this, as may be observed in Figs. 4

and 7, the corresponding membrane of the chela of the lobster is composed only of connective tissue and does not appear to degenerate to any appreciable degree. On the contrary, in the lobster the original connective tissue membrane and the valves for the venous sinuses persist throughout the regenerative activities, and retain their original function in the new limb. In Fig. 7 it may be seen that as the regenerating epidermal cells begin to migrate across the stump they pass between the outer blood clot (*bcl*), and the connective tissue membrane and the venous valves. The venous valves no longer exerting a resistance to the blood stream, now begin to assume their original and normal position (Figs. 7 and 8). There is little, if any, degeneration of the connective tissue. In the blood clot, however, the corpuscles soon deteriorate, the nuclei become flattened in the disto-proximal direction of the limb, and both the nuclei and the cytoplasm of the clot assume a deeper stain with hæmatoxylin and Congo red.

2. *Migration of Epidermal Cells.*—As the blood clot forms, the epithelium of the exoskeleton retracts slightly within the stump. There is, however, no other noticeable change in the position of the epidermal cells until after the fourteenth hour following the operation. The first regenerative activity which is then observed is a migration of ectodermal cells from all sides of the epidermal wall. This movement of the epidermal cells appears at first to be more pronounced in the region of the limb nerves on the inner side of the stump; in a section taken through the outer side of the stump twenty-four hours after autotomy (Fig. 7) the migrating epidermal cells (e^1, e^1) from the opposite sides are still quite widely separated, but in a section from the inner side of the stump the migrating cells are found much nearer each other. These cells continue advancing centripetally until they unite to form a complete layer of epidermal cells over the surface of the stump. During the migration, the congested mass of blood corpuscles within the distal end of the stump begins to disintegrate, but the old connective tissue remains intact as already described.

3. *Nuclear Changes in the Epidermal Cells.*—The epidermal epithelium consists of low columnar or cuboidal cells, apparently

fused into a true syncytium. Indeed, Huxley's description of the epidermal epithelium of the crayfish applies equally well to the lobster,—"It is found to consist of a protoplasmic substance in which close set nuclei are embedded . . . there can be no doubt that it is really an aggregate of nucleated cells, though the limits between the individual cells are rarely visible in the fresh state" (p. 178). It may be added that in the lobster at least, cell boundaries are no more clearly defined even after fixation and staining. In view of these facts it becomes evident that in making observations upon cell changes in such a tissue, attention is necessarily more largely directed toward the nuclei of the tissue cells.

The characteristic structure of normal epithelial nuclei as they appear immediately after the autotomy of the limb is shown in Fig. 11. They may be described as more or less elongated or oval in form with a clearly defined nuclear membrane. A structural characteristic is the segregation of the chromatin into relatively large angular masses or karyosomes. These karyosomes take on a heavy stain with hæmatoxylin, and are rather evenly distributed throughout the periphery of the nucleus. The ground substance stains a uniformly light color. The four nuclei in Fig. 11 were from the epidermal epithelium just below the breaking plane and from a region at the inner side of the basipodite near the limb nerves.

Fig. 12 represents epidermal nuclei fourteen hours after autotomy. To facilitate a more direct comparison of structural characteristics, these nuclei were taken from practically the same region as the nuclei shown in Fig. 11, *i. e.*, from the inner side of the basipodite,—a region of the epidermis where regenerative activities are first observed. Of these four nuclei, *a* and *b* are proximal of *c* and *d* and farthest from the region of the first regenerative changes. In contrasting *c* and *d* with *a* and *b*, and also comparing with Fig. 11, it may be noticed that the nuclei are becoming larger in size and the chromatin is undergoing certain changes. In *a* and *b* the karyosomes are still large and are similar to those in Fig. 11. But in *c* and *d* the chromatin is more finely subdivided and arranged in a reticulum of chromatin strands and knots. In nuclei *a* and *c* a round, centrally located body is seen, which takes a relatively lighter stain and has the

characteristics of a nucleolus. Whether the presence of nucleoli is typical of this stage is, however, questionable, for with the same stain they can also be found, although less frequently, in the normal or resting nuclei.

The nuclei shown in Fig. 13 are from a limb fixed twenty-four hours after autotomy. At this time the migrating epidermal cells have progressed considerably in their advance over the surface of the stump. The nuclei drawn are from the same specimen as are those of Fig. 12, but they have been selected from a region where the advancing epithelium has already partly closed over the inner side of the stump. As the epidermal nuclei migrate, their long axes rotate through an angle of about 90 degrees from their former position, and in such a way that at the center of the stump the long axis of the nucleus assumes a position practically parallel to the long axis of the limb (Fig. 8). In Fig. 13 the nuclei *c* and *d* are nearer the center of the stump. As the nuclei approach the central region, there is an increase in their shortest diameter, giving each nucleus a broader and less elongated form. They now also become more widely separated from one another. In the earlier stages of migration the lower or proximal ends of the nuclei tend to assume a tapering or pointed form.

A striking structural characteristic is the unequal distribution of the chromatic material (giving the nuclei a "loaded" appearance). The proximal end of each nucleus takes a relatively deep stain, while the upper or distal end of the nucleus is so pale that frequently little or no stain can be detected except in the few scattered granules of chromatin. In nuclei *a*, *b*, and *d*, one can see that the boundary between the light and dark areas, or zones, lies at such an angle across the nucleus that the darker zone occupies the lower and left region, and *vice versa* for the lighter zone. The nuclear contents have much the appearance of having been centrifuged. In nucleus *c* the plane separating the two zones is more nearly equatorial in position. It will also be recalled that these nuclei were taken from the left side of the section of the limb (*c* being nearest the center of the limb). In nuclei taken from the opposite or right side of the same section, it is interesting to find the localization of the two zones just reversed, *i. e.*, the

more lightly stained area is now at the upper and left side, whereas the darker zone at the proximal end is directed toward the right side of the nucleus. In studying the sections, a considerable enlargement or expansion of the nucleus is frequently found at the distal end, or region of the light zone, in some cases being even more marked than is shown in nucleus *d* (Fig. 13). At this stage of migration the former karyosomes have now almost disappeared. The chromatin has disintegrated into fine granules, which appear to be either peripherally distributed or axially segregated into a dense mass. It is also to be observed that the denser central mass is always located at the darker pole of the nucleus.

The significance of this polarization of the nuclear contents (if it may be thus designated) is not readily apparent. It can hardly be considered an artifact resulting from faulty fixation or staining. On the contrary, its regular occurrence, its reversal at opposite sides of the limb, the frequent expansion of the lighter pole, the association of the axial chromatin mass with the darker pole, together with the fact that similar conditions are even more conspicuous in later stages of the regenerating limb as will be presently described, is evidence which supports the conclusion that this polarization of nuclear contents is a characteristic structural feature of certain phases in the regenerative activity of the epidermal cells.[3]

Rand's ('04) discussion of the regenerating epidermis of the earthworm applies equally well to the migrating epidermal cells in the regenerating lobster's limb. He says: "The movement of the cells at the margin of the layer is not a passive one, resulting from external pressure. Nor is there the slightest ground for supposing the existence of a force acting from a position anterior to the cut epidermal edges and serving to pull the epidermal cells over the cicatrix. We are compelled, therefore, to look in the individual epidermal cell itself for the immediate source of the activity. This activity, by whatever mechanism effected, must be occasioned by an agency external to the cell, viz., by some factor of the conditions resulting from the injury." Beyond this we must admit that as yet we

[3]The resemblance of the "polarized" nuclei in appearance to certain stages of synapsis of the nuclei in spermatogonia may be noted, although it has suggested no further explanation of the "polarization."

have not succeeded even in determining whether the stimulus thus controlling the cell activity is some "chemical peculiarity" of the injured and exposed tissue, or whether it is some "inter-action" of the epidermal cells, questions which are merely preliminary to understanding the factors which may account for the apparent polarization of nuclear contents just described. These nuclear changes might possibly be classed among the phenomena designated as "cytotaxis" (Roux, '96), although in doing so it is not evident that we advance materially in a solution of the problem. At the end of the next fourteen hours, *i. e.*, thirty-eight hours after autotomy, the epidermal cells have spread completely over the stump and the first mitotic cell division appears. During this period the nuclei have assumed a more spherical form and appear somewhat larger. Both the nuclear sap and chromatin have become more equally distributed. The chromatin is now more coarsely granular, and as the nucleus approaches the prophase of mitosis, the chromatin collects in conspicuous rod and knot-like masses or chromosomes (Fig. 14, *a*). The nuclear membrane then disappears and the cell passes through the various mitotic phases, of which a later metaphase is shown in Fig. 14, *b*.

The volumetric increase of nuclear material during the initial regenerative activities presents another fact for consideration. In many respects the problem arising from the phenomena of cytomorphosis and regulation in the reproduction of a part of the organism are comparable with similar problems in the development of the original embryo. In both processes the differentiation of anatomical structure is preceded by the formation of a more or less undifferentiated mass of cells, characterized, in the case of embryonic development, by the segmentation of the ovum, and in the case of regeneration, by the proliferation of a mass of cells from the tissues in the region of injury. While the present data hardly justify an extended discussion, attention may, however, be called to the fact that in both regenerative and ontogenetic processes, the initial cytological changes are attended by a characteristic increase in the amount of nuclear material.[4]

[4]It should be observed that in the case of regeneration, at least in the earlier stages, the increase of nuclear material apparently is not associated with cell division.

In regard to the segmentation of the ovum, Professor Minot ('08) emphasizes this increase in nuclear material as a characteristic feature of "the process of rejuvenation," and as a result of his studies of the structural relations of cytoplasmic and nuclear material during the development of the organism, concludes "that as we define senescence as an increase and differentiation of the protoplasm, so we must define rejuvenation as an increase of the nuclear material," p. 167. Whether we shall be justified in attaching a similar significance to the corresponding nuclear changes in regeneration, or whether in the latter case the nuclear phenomena are fundamentally different in nature, are questions for further investigation.

4. *Cytoplasm.*—In describing the cytoplasm of the epidermal cells, it is necessary to consider the cells collectively, on account of the ill-defined or even non-existent cell boundaries. In the normal or resting cell the cytoplasm has a reticular structure of rather even appearance and is traversed by larger delicate supportive fibrils lying more or less parallel to the long axis of the cell. As the epidermal cells migrate over the surface of the stump, their cytoplasm ceases to be characterized by the presence of fibrillar structures, but appears to be composed of fine granules rather uniformly distributed (Figs. 8 and 9). A definite reticular structure is no longer evident. The inner surface of this sheet of migrating cells, instead of being smooth, presents an uneven contour due to numerous rounded inward projections of cytoplasm. These projecting masses of cytoplasm never show definite limiting membranes, but their boundaries can, however, be traced upward into the general layer of cytoplasm as far as the level of the epidermal nuclei, the relations of each mass being such as to indicate that it represents the unit of cytoplasm coming under the influence of each nucleus. The volume of cytoplasm appears to have increased, as is indicated by the wider separation of the nuclei and the greater depth of the epithelium after its migration has been completed.

One result of these observations on the epidermal cells is to emphasize the fact that in the migration of these cells as the first step in regeneration, there occurs a parallel series of definite structural

changes in cytoplasm and nucleus. It remains for further observation to answer the interesting question whether the parallel occurrence of these structural changes is due to the existence of a significant correlation between the nucleus and the cytoplasm in the regenerative activities of the cell. Such a correlation is supported, for example, by Eycleshymer's ('04) thorough work on the muscle cell of Necturus, in which that investigator finds strong evidence that the nuclear material plays a most important rôle in cytoplasmic synthesis. He suggests that "cellular degeneration and regeneration are accompanied by volumetric, structural and chemical changes in chromatin" (p. 307).

5. *Cell Division.*—Reference has already been made to the fact that mitotic cell division was not observed until the second day following the operation, and then only in the epidermal cells. Thirty-eight hours after the amputation, two mitotic figures were found in serial sections of the right chela and four figures in the left chela of the same specimen. Twelve hours later, eighteen mitotic figures were counted. Preceding mitosis the nucleus migrates toward the outer surface of the epidermis so that the mitotic figures are always found near the periphery of the epithelium. Regarding the question of amitosis in the early stages of regeneration, it may be said that there was no evidence found for direct or amitotic division of the epidermal cells during the thirty-eight hours preceding the first mitosis. Each migrating nucleus seems to maintain its original unity up to the time at which mitosis first occurs.

Without entering further into a discussion of this interesting question of amitosis, attention may be called to the contrast of the present results as compared with observations on the compound crustacean eye, where it appears that in "all cases of the regeneration of the eye the nuclei are increased by amitotic division" (Steele, '08, p. 183). Steele, referring to Reed's ('04) conclusion that mitotic figures do not occur in the early stages of the regenerating limb of the crayfish, suggests (p. 184) that amitotic division may possibly have taken place "during the preparatory stages at least." In regard to this question, the evidence, in the lobster at least, is of a negative character. It appears rather that an increase in both the quantity of cytoplasm and

the size of the nuclei, together with their wider separation during migration are sufficient to account for the formation of the first epithelium over the injured surface of the limb.

Similar preliminary regenerative conditions have also been observed in other animal forms. Among the vertebrates, Barfurth ('90), from a study of regeneration in the amphibian tail, concludes that the new cells which first cover the wound "stammen her vom persistierenden Epithel der Wundränder, sind nicht etwa durch Theilung aus diesen Epidermiszellen hervorgegangen, sondern haben sich aus dem Epithelverbande losgelöst, sind embryonal beweglich (amöboid) geworden und schieben sich langsam über die Wundfläche vor, bis sie mit den Zellen der anderen Seite Fühlung gewonnen haben". The process continues until "eine mehrfache Schicht die Wunde bedeckt" (p. 417). Among invertebrates, Rand ('04, p. 39), in his study of regeneration in the earthworm, states that "the wound surface becomes completely covered by an epidermal layer derived from the existing epidermis, without the occurrence of cell proliferation in that layer".

V. The Formation of Segments and Joints.

In this section will be described the earlier stages in the regeneration of the limb. The description of the further differentiation of the tissues and the sequence in which the segments develop is deferred to a later section.

At the time when the first mitotic cell division appears there is already present a thin lamella of chitinogenous cuticle formed on the outer surface of the epidermal cells (Fig. 9, ch^1). The fact that a cuticle could be detected as early as the twenty-fourth hour after operation indicates that cuticle differentiation may begin before cell division has occurred.

With the appearance of mitosis, there soon follows a rapid accumulation of cells in the central region of the epidermal disc of cells which has formed over the injured surface of the stump. This disc of ectodermal cells consequently becomes thicker, its cuticle increases in amount, and at the end of fifty hours the regenerating cells (Fig. 9) are seen pushing outward and breaking through the blood clot at the exterior, to form a papilla-like mass near the center of the stump.

Examined in section, the papilla is seen to consist of an evagination of regenerating epidermal cells. Toward the end of the third day after the operation, the regenerating bud has increased considerably in size (Fig. 10). A characteristic developmental phenomenon is that the lateral or outer wall of the bud grows faster than the inner or more mesial wall, so that, of the two sides, the outer wall becomes both thicker and larger in extent of surface, and contains a greater number of mitotic figures and nuclei. It is also on the outer or ventral sides that the first appearance of joint formation becomes apparent (Fig. 31). The result of this asymmetrical growth is that the regenerating bud bends upward or inward toward the body of the animal; a position which evidently reduces the chances for injury through friction with external objects.

It is near the end of the third day, too, that the first differentiation of limb segments becomes evident. A slight groove appears near the apex of the bud, and thus marks the first step in the development of the two jaws of the claw. It is characteristic that this groove is not located directly at the apex of the bud, but is somewhat ventral to the tip, with the result that during the earlier stages of development the dactyl is relatively larger than the opposing segment or index of the claw. The fact that this relation in the size of the dactyl and the index becomes reversed in the fully regenerated claw, is especially interesting since it is a process parallel with the series of changes which occur in the normal ontogeny of the claw (Emmel '06). During the next six days the outlines of the dactylopodite and propodite become more definite; the anlagen of all the segments appear, and eventually the four joints of the regenerating limb become clearly defined (Fig. 31-36).

As each point develops, an ectodermal invagination arises in relation with it. This invagination, which more or less completely surrounds the joint, soon develops, at two opposite sides of the joint, into two proximally directed processes, which are destined to form attachments for the flexor and extensor muscles. In the interior of each invagination, there is secreted a lamella of chitin which is readily demonstrated by its differential stain with Congo red.

During the development of the joints the epidermal cells present

characteristic structural changes. The mitotic figures occur at the periphery of the bud, the spindle being almost invariably parallel with the surface. A dividing nucleus is rarely found at a deeper level. The cytoplasm is granular in appearance. No cell walls were detected. In the vicinity of the mitotic figure, however, the mass of cytoplasm immediately surrounding the dividing nucleus is less granular and takes on a lighter stain.

Fig. 15 (five days and ten hours after operation) represents a group of epidermal cells in the region of the invagination for the flexor muscle of the fourth segment. The four nuclei at the right in this figure are migrating inward in the process of invagination. The three nuclei at the left are in the epidermal wall of the segment, while the remaining nucleus, in the prophase (?) of mitosis, occupies the typical superficial position. In these nuclei there may again be seen a polarization of the nuclear material similar to that described in the earlier stages of the regenerating cells. The dark staining chromatic elements are found at the inner or more mesial pole of the nucleus (with the exception of the nucleus undergoing mitosis). At the same time the opposite pole of the nucleus takes only a light stain and evidently contains a much smaller amount of chromatin. Here again the position of the plane of division between these light and dark zones varies with the position of the nucleus. (In Fig. 15 the left and right parts are respectively distal and proximal with reference to the limb.) The conditions generally found may be stated as follows: In nuclei lying approximately transverse to the long axis of the regenerating bud, the darker staining material is at the inner end or pole of the nucleus. In nuclei not lying in a transverse plane, as occurs in the region of joint formation, the chromatic material, although still found in the inner region of the nucleus, now becomes shifted either to the proximal or to the distal side of the nucleus according as the inner pole of the nucleus is directed in a distal or a proximal direction. The darker pole also frequently contains a darker body or nucleolus, while at the lighter pole of the nucleus there was usually found a reticular arrangement of fine granular elements. The inner or darker pole of the nucleus was generally found smaller and more tapering or pointed in form as

compared with the more expanded outer peripheral region. Here again the significance of this apparent polarization of the nuclear contents is not clear, but, as was mentioned in the case of migrating nuclei, the general occurrence of these structural characters in different specimens and with different stains, and the variation of the polarization of the elements in conformity with variations in the position of the nuclei, hardly permits of the interpretation of this phenomenon as a mere artifact.

As the invagination of the ectodermal cells at the joints advances, the involved nuclei undergo further typical changes. Fig. 17 (seven days, six hours) represents several nuclei taken at a later stage in the development of the same invagination shown in Fig. 15 (five days, ten hours). A marked difference is at once apparent in these two groups of nuclei. In Fig. 17 the chromatin is evenly distributed, and the nuclei have become elongated to such a degree that frequently they are more than two and one-half times longer than at an earlier stage. See Fig. 16 (six days, six hours) for intermediate stages. It will also be observed in this latter figure that at the center of the invaginating mass of cells, a thin lamella of chitin (*mp*) is now becoming evident, which later serves for muscle attachment.

VI. Origin of New Cells.

We have already learned that the regenerative process is initiated by epidermal cells, which migrate across the wound, form a layer over the injured surface, then multiply by mitotic division and evaginate to form the new bud. It is evident, therefore, that the outer layer of cells covering the regenerating limb bud is entirely of ectodermal origin.

The evagination of the ectodermal cells encloses a cavity at the center of the bud, which becomes filled with a core of cells. The origin of this core of cells becomes at once a vital question in the further study of the histogenesis. Since the interior of the fully developed limb consists of such tissues as striated muscle and connective tissue, it might be expected that this internal core of cells would be derived from similar tissues in the old stump, and consequently would be mesodermal in origin.

A careful study of the successive stages of the regenerating bud, however, does not warrant such a conclusion. Fig. 9 is typical of the conditions found at early stages of the regenerative process. The section is taken through the large limb nerve (n^2) which is here seen passing through a mass of connective tissue (ct). The regenerating layer of ectodermal cells (ec) is increasing in thickness. Between it and the connective tissue membrane enclosing the nerve trunk, a small space has arisen in which may be observed several nuclei lying in what seems to be a syncytial mass of cytoplasm. In their form, structure, and reaction to stains, these nuclei resemble the regenerating ectodermal nuclei. At certain points they are evidently migrating directly from the ectodermal layer of cells; and occasionally nuclei are seen which seem to be migrating inward from the edges of the old epidermis. On the other hand no evidence was obtained indicating any proliferation of the old connective tissue cells. Mitotic figures were not found, nor did there seem to be any migration of the connective tissue cells into the space beneath the evaginating ectodermal plate. Occasional blood cells, however, were observed within this space.

Fig. 10 (two days, twenty-two hours) is a section of a regenerating bud in which the invagination for the two jaws of the claw has begun. The central core of cells is now considerably larger. A few blood corpuscles are present in the proximal region of the core, but the nuclei of the central mass of cells still resemble the ectodermal nuclei. A slight migration of ectodermal cells may be observed from the sides of the limb bud, but it is in the region of the invagination for the first joint that the migration is most extensive. From this advancing invagination the ectodermal cells migrate in large numbers, and thus fill the central cavity of the regenerating bud. In this central mass of cells mitotic figures were very rarely observed. As other limb joints are formed, a similar migration of ectodermal cells was found to occur at the invagination for each limb segment. No evidence was found indicating the possible derivation of these internal cells from the underlying connective tissue of the old stump; if it does occur, it seems evident that it cannot be to any large degree. This conclusion is based upon (1) the

lack of evidence of cell division in the connective tissue, and (2) the fact that at no stage of development did there appear to be a migration of connective tissue cells into the regenerating bud.

In a word, the results of these observations lead to the unexpected conclusion that certainly the greater part, and probably the entire mass, of cells composing the interior as well as the wall of the regenerating limb bud, are ectodermal in origin.

VII. The Differentiation of Tissues.

1. *Striated Muscle*—a. *Histogenesis.*—The first differentiation of striated muscle, as indicated by the formation of myofibrillæ, became apparent as early as five days and twenty-two hours after amputation. On the sixth day the fibrillæ were readily distinguished. At the center of a longitudinal section through the second segment or propodite (Fig. 16, six days, six hours) may be seen the tongue of ectodermal cells invaginated for the flexor muscle of the dactyl. The space between the epidermal cells (*e*) of the regenerating exoskeleton, seen at the lower side of the figure, and the invagination, is filled by a mass of cells, the ectodermal origin of which has already been considered. It is within this central mass of cells that the myofibrillæ (*mf*) first appear. These fibrillæ, instead of extending as straight fibers between their origin in the invagination and their insertion in the epidermal wall, are considerably curved, being convex in a proximal direction. This curvature of the myofibrils does not entirely disappear until after the moult of the lobster has occurred and the regenerated limb has become functional. It may be justly questioned whether there is any correlation between the appearance of these fibrils and functional activity, such, for example, as Eycleshymer ('05) describes in the Necturus embryo, for there is no evidence of functional activity in the regenerated limb until it has been liberated from its enveloping membrane during moulting.

The cytoplasm in which the myofibrils are first seen to differentiate (Fig. 22) appears finely granular in structure and is traversed by a delicate cytoplasmic reticulum (*rt*). As soon as the fibrillæ can be distinguished from the cyto-reticulum they appear as heavier fibers

(*mf*) taking a darker stain with Congo red. The very earliest fibrils are, however, not so readily identified on account of their similarity to the cytoplasmic network and their close relations with it; for at the time of their first differeniation the fibrillæ stain but slightly with Congo red, are irregular or wavy in structure, and appear to be in continuity with the cyto-reticulum. These characteristics indicate that the contractile elements in the regenerating limb of the lobster may be derived from the cyto-reticulum, and consequently favor the "network" rather than the "fibrillæ" theory for the origin of striated muscle fibers. It should be added, however, that no such constancy in relation was observed between the cytoplasmic network and the fibrillæ, as would seem to be necessitated by MacCallum's ('98) theory for striated muscle of vertebrates.

As late as the seventh day of regeneration each myofibril (Fig. 23) still retains its individuality as a single structure. During the eighth day, the fibrils began to appear double or in pairs. In Fig. 24 one of the fibrils is still a single structure, but the remaining eight fibrils are in pairs, the members of each pair appearing in cross section as half cylinders. At later stages of differentiation each pair of fibrillæ becomes represented by a group of four fibrils; the number in each group then increases until as many as twenty fibrillæ could be counted in each bundle, which in transverse section may now be recognized as a "Cohnheim's area". As to how the fibrillæ multiply, the evidence from appearances in both cross and longitudinal sections of the regenerating muscle in this invertebrate supports the conclusions of Heidenhain, Eycleshymer, and other investigators for the striated muscle of vertebrates,—that an increase in the number of myofibrillæ arises through longitudinal division of the fibril.

Preceding the first division or splitting there is a marked increase in the diameter of each fibril (compare Figs. 22 and 23). At the same time the cytoplasm immediately surrounding the fibril becomes less granular and is distinguished from the neighboring cytoplasm by its lighter stain (Figs. 23 and 24). The developing fibril is consequently enclosed by a sheath of modified cytoplasm, which is evidently correctly interpreted as representing the beginning of a Cohnheim area of the mature muscle fiber.

Reference has just been made to the fact that each myofibril retains its individuality as a single structure until the seventh day of regeneration. At this time the light and dark bands of the myofibrillæ are already present, but the "Z" line, or membrane of Krause, could not be observed. By the eighth day the "Z" line had become differentiated, the first indication of these lines being found during the early part of the seventh day. It appears, therefore, that the "Z" lines differentiate later than the light and dark bands. In this respect the differentiation of the regenerating crustacean muscle resembles the histogenesis of the striated muscle as found in the 21 mm. pig embryo, where, as described by Bardeen ('00, p. 392), "The fibril bundles are composed of longitudinal fibrils made up of longer deeply staining segments alternating with shorter lighter segments which do not stain. It is only in older embryos that lines through the light areas, corresponding to Krause's membrane, may be clearly distinguished."

The problem of the origin of the multinucleated muscle cell and its sarcolemma in the regenerating tissue of the lobster differs from the same problem in the vertebrates, because in the former there apparently are no myoblasts concerned in muscle formation.

The structure in the adult muscle of the lobster, which is known as the sarcolemma, is a membrane surrounding the muscle fiber and containing elongated nuclei. This membrane is usually regarded as developed ontogenetically from connective tissue elements. No evidence, however, was obtained for a similar origin during regeneration. When the myofibrillæ first appear in regeneration, definite cell membranes cannot be identified, but the sarcolemma first appears in a later stage of differentiation (Fig. 25, *sa*), and seems to arise by a modification of the cytoplasm of the ectodermal cells.

During the differentiation of the myofibrillæ (which are at first placed centrally and later become located eccentrically in the muscle fiber), the nuclei undergo certain characteristic changes in form and structure. The elongation of the ectodermal nuclei, as they proliferate and migrate inward at the joint, has already been described. As the migration advances, the nuclei in the region where the myofibrils differentiate, assume a peripheral position in the developing

muscle fiber and become more spherical in form (Figs. 16, 22, 24, and 25). These nuclei now seem somewhat larger than the earlier ectodermal nuclei, an appearance which may be partly due to the change from an elongated to a rounded shape. The chromatin appears coarsely granular and has a fairly even distribution, the nucleus as a whole taking a lighter stain. In later differentiation the nuclei again become flattened and considerably elongated in the direction of the long axis of the muscle fiber. The present observation indicates that the more peripheral of these nuclei eventually become the nuclei of the sarcolemma, but more evidence is necessary to establish this point.

In the formation of muscle fibers from a syncytium of ectodermal cells, each fiber is from the very first multinucleated. Instead of "each muscle bundle developing from a single cell" (Claus, '86, p. 33), as seems to be the case in the normal development in Branchipus, for example, each bundle is multicellular in origin. Furthermore, the nuclei increase in number, with the growth of the fiber, by mitotic division, the spindle frequently occurring at right angles to the long axis of the fiber. Consequently in regard to the discussed question of direct and indirect division of muscle nuclei, it is evident that in the regenerating muscle of the lobster at least, mitotic division persists even after the differentiation of myofibrillæ has advanced to a considerable dègree.

The conclusion that regenerating striated muscle is ectodermal in origin, draws attention to the problem of the genetic relationship of tissues and the primary germ layers, the question at issue being whether in regeneration a given tissue arises from the same germ layer as in normal development. An adequate discussion of this question in the case of the crustacean muscle, involves, of course, an accurate knowledge of the histogenesis of the tissue under consideration, in both the regenerative and ontogenetic processes.

With regard to regeneration, as a result of the work of Reed ('04) on the crayfish, Ost ('06) on Oniscus, and the present study of the lobster, we have at hand a fairly exact mass of data concerning the genesis of the regenerating crustacean muscle, but as for its origin during normal ontogeny, the evidence does not appear suffi-

ciently conclusive. In the ontogeny of vertebrates, the striated musculature is evidently mesodermal in origin, but whether the same is true in crustacean development, especially in the case of the limb musculature, is not, so far as the writer is aware, clearly established. Consequently it seems obvious that we are not as yet justified in concluding that the ectodermal origin of the regenerating crustacean muscle is a divergence from ontogenetic development. Indeed, as Ost ('06, p. 312) points out, it is not impossible "dass die embryonalen Muskeln von den ectodermalen Sehneneinstülpungen aussprossten und dann Regeneration und Embryonalentwicklung sogar übereinstimmten," a conclusion which is certainly not rendered less improbable when one considers the degree to which the generally accepted conclusions regarding genetic relationships between ectoderm, mesoderm, and certain forms of connective and muscle tissue have been called into question by the work of such investigators as Katschenko ('88), Kölliker ('84), and Platt ('98).

b. *Attachment to the Exoskeleton.*—Among the first investigations on the attachment of the crustacean muscle is Claus's ('86) work on Branchipus and Artemia. Claus concludes that in many cases the muscle fibers are attached directly to the exoskeleton (pp. 22 and 29), a conclusion which involves the assumption that among some invertebrates at least the muscle may be attached to the skeleton without the intervention of a connective tissue tendon, such as are typical of muscle-skeletal attachments among vertebrates. Since 1886 the subject has received considerable attention both for crustacea and insects, with the result that several divergent opinions have arisen. As far as the writer is aware, however, the problem has not been studied from the standpoint of regeneration; consequently data derived from the present investigation of the lobster may not be valueless.

Attention has already been directed to the fact that in the early stages of the regenerating bud and up to the time when the muscle fibrils have become well differentiated, there is no definite boundary between the outer epithelial cells related to the chitin and the internal cell mass in which muscle develops. Cell walls are not evident, and the cytoplasm of the two regions appears syncytially related, the

only noticeable difference being that the epidermal cytoplasm next to the chitin takes a slightly darker stain (Fig. 16). The question now arises, what is the relation of the developing myofibrillæ to the external epidermal cells and how is their attachment to the chitin established?

In studying the regenerating tissue at the stage when the myofibrillæ can just be identified (Fig. 16, six days, six hours), an important fact becomes evident, viz., that fibrils develop in the epidermal layer of cells, simultaneously with the differentiation of the myofibrillæ in the internal cells, apparently without any discontinuity between them. In other words in early differentiation the myofibrillæ can be traced into the layer of epidermal cells, and in some cases at even this early stage (six days, six hours) myofibrils were found continuous even through to the chitin.

The differentiating muscle fibrils early assume a characteristic striation (Fig. 18, *mf*). At the same time more clearly defined differences are becoming evident between the epidermal and muscle cells. The muscle nuclei are more nearly spherical in form, while the epidermal cells take a distinctly heavier stain. Small vacuoles can also be observed at the boundary between the two groups of cells. The striation of the muscle fibrils gradually becomes more distinct. These striations could be traced beneath the inner surface of the epidermis, frequently extending to the level of the proximal ends of the nuclei (Fig. 18). No evidence was obtained, however, that the fibrillæ are ever striated entirely through the epidermis to the chitin.

A definite boundary (basement membrane) now becomes apparent between the epidermal and muscle cells (Fig. 19, *em*). There is an interesting variation in the general level of the boundary or inner surface of the epidermis in the region of muscle attachment. The epidermis between the attachment of two or more muscles, frequently projects inward in rounded elevations or broad papillæ. The result is that in the immediate vicinity of the muscle attachment the inner surface of the epidermis appears to follow the fiber for some distance toward the chitin, thus forming a sort of pit, through the bottom of which passes the muscle fiber (Figs. 19 and 20). Since this elevation of the epidermis increases in the later stages of the regenerating

limb, it is probably due to the rapid growth of the epidermal cells, which are consequently forced inward between the muscle fibers. After the moult, the regenerating limb expands and the elevations then disappear as the epidermis spreads out to cover the now larger surface of the limb.

The muscle fibers appear to be continuous throughout the epidermis to the chitin (Figs. 18, 19, and 20). Frequently just before the fibers reach the chitin they spread out in a brush-like manner and fuse with the chitin (Fig. 20). In this fusion the fibrils frequently terminate on knob-like thickenings or inward projections of the chitin (Fig. 19). These chitinogenous processes were found only in connection with the attachment of the regenerating muscle fibers.

In later stages of differentiation, the epidermal nuclei adjacent to the muscle attachment frequently become considerably lengthened in the direction of the long axis of the muscle fiber. In addition to the fibrils concerned with muscle attachment, there are other fibrils which later differentiate in the cytoplasm of the epidermal cells (Fig. 19, *ef*). These latter fibrils do not appear to be associated with muscle attachment. In structure they are much finer and are distinguished from muscle fibrils by their stain reaction. With Mallory's connective tissue stain they appear light blue, whereas the fibrils concerned with muscle attachment take a dark red or purple stain similar to the striated part of the myofibril.

On the inner surface of the fully developed epidermis of the crustacean there occurs a very thin layer or lamella of tissue which has been termed the "Grenzlamelle" (Schneider). The fact that a flat nucleus is occasionally found in this "border lamella" has favored the interpretation that it is a connective tissue formation and consequently mesodermal in origin. Other investigators (Claus, for example) have regarded this layer or "basement membrane," as merely a differentiation of the inner surface of the epidermal cells. In regard to this question it is clear that in the early regeneration of the lobster's limb there is no definite limiting membrane between the epidermal and muscle cells. The first limiting membrane to appear during the differentiation of the epidermal cells is evidently

epidermal in origin and to that extent is ectodermal. This earlier membrane seems similar to the basement membrane which McMurrich ('96) describes on the epithelium of the mid gut of isopods, and which he concludes "was formed from the epithelial cells and not by the mesoderm" (p. 89). It is to be observed, however, that at a later stage of development (Fig. 21) a thin nucleated layer does appear on the inner surface of the epidermis, which is perhaps to be regarded as the true "Grenzlamelle." The origin of this latter layer has not been satisfactorily determined. It was noticed that it is first apparent in the region of the developing blood sinuses, where it appears to serve as a vascular epithelium.

c. *Discussion and Conclusions.*—The study of the attachment of the arthropod muscle is attended with considerable technical difficulty. That the question is still an open one is indicated by the diverse opinions existing at the present time. The chief points at issue are involved in the solution of the questions whether the muscle fibers are attached (1) directly to the chitin of the exoskeleton, or (2) indirectly by means of intervening epidermal cells; and in the latter case, whether (3) in addition to the epidermal cells there is also an intervention of connective tissue between the muscle fibers and the epidermis, in a manner somewhat analogous to the muscle attachment typical among vertebrates.

The literature upon this subject is becoming very extensive, especially for insects, where the question of muscle attachment has been studied by Henneguy ('06), Holmgren ('02), Lecaillon ('07), Riley ('08), Snethlage ('05),[5] and others. Without reviewing this growing literature, we may advantageously confine our discussion to the present observations on the lobster and their bearing on the problem among crustacea.

The third of the different modes of muscle attachment just considered involves the union of three sets of fibrils,—the fibrils of the muscle, of connective tissue, and of the epidermal cells. Schneider ('02) is inclined to regard this method of attachment as typical for crustacea, and his description of the jaw muscle of the crayfish states

[5]Unfortunately, I have been able to have access only to abstracts of Snethlage's work, "Ueber die Frage vom Muskelansatze, etc., bei den Arthropoden."

that "Das Bindegewebe ist an den Muskelenden als typisches Fasergewebe entwickelt, das sich vom faserigen Zellengewebe durch reichliche Entwicklung extracellulärer fibrillärer Bindesubstanz, im Umkreis spindeliger Bindezellen scharf unterscheidet. Die Myofibrillen einerseits und die Deckzellenfibrillen andererseits senken sich in eine dicke Lamelle ein, in der Bindefibrillen in dichter Anordung, von spärlicher Grundsubstanz verkittet, entsprechend den Myo- und Stützfibrillen verlaufen" (p. 494). On the other hand, Claus ('86) in his study of Branchipus finds that "An vielen Stellen heften sich aber die Muskelsehnen nicht direct mittelst Connectivfäden am Integument an, sondern gehen in einer durch solche suspendirte Lamelle über, welche sich der Integumentfläche parallel als Basalplatte unterhalb jener ausbreitet" (p. 29).

The critical question here is in regard to the continuity of the muscle fibers within the epidermal cells. If the muscle fibers can be traced below the level of the inner surface of the epidermis, independent of any connective tissue elements, evidently the third mode of attachment may be eliminated from further consideration. That such is certainly the case in the regenerating musculature of the lobster's limb has already been indicated, and the crucial fact remains that this relation of the myofibrillæ was already evident at a stage of development before there was any differentiation of connective tissue elements or of a "Grenzlamelle." Consequently, at this time at least, the muscle fibrils are found passing into the epidermis unaccompanied by any connective tissue elements. The apparently close relation, which is later established between the muscle fibers and the connective tissue, may perhaps be not incorrectly regarded as a secondary development resulting from a later differentiation of connective tissue around the already formed muscle fibrils, rather than as a primary functional relation. It is of interest to observe that even in the fully developed lobster, as admitted by Dahlgren and Kepner ('08), the connective tissue elements are in some places "so small as to be apparently absent, and it would seem possible that in some attachments they were absent altogether and the muscle joined directly with the epithelium" (p. 66).

In considering the two remaining modes of attachment, certain

aspects of the problem, as they present themselves in other animal forms, disappear in approaching the subject from the standpoint of regeneration. For example, the question whether the fibrils by which muscle attachment is accomplished are inter- or intra-cellular with reference to the epidermal cells, loses much of its significance here on account of the syncytial structure of the cytoplasm. Genetically, there is perhaps no distinction to be drawn between the purely epidermal fibrils and the myofibrillæ, for if the conclusions regarding the origin of the muscle are correct, both groups of fibrils are derivatives of the cytoplasm of epidermal cells, and are consequently also both ectodermal. Structurally, however, they have diverged in their differentiation, in conformity with their differences in function. The striation of the muscle fibril can frequently be traced for some distance into the epidermal cytoplasm, but it was not observed that the striation of the fibrils ever continues through to the chitin; it appears that the peripheral ends of the original myofibrillæ diffentiate as tensile rather than as contractile structures. But even the non-contractile elements thus directly concerned with the attachment of the muscle to the chitin can be distinguished from the purely supportive fibrils of the epidermal cells by (a) their staining reaction with Congo red and Mallory's stain; (b) their frequently larger size; (c) their evident continuity with the contractile part of the muscle fiber; and (d) their relations with the exoskeleton where they terminate in characteristic end-plates or chitinogenous processes.

The conclusions which these observations support may, therefore, be summarized as follows:

1. The myofibrillæ differentiate in the cytoplasm of a syncytial mass of cells, derived from the epidermis and consequently ectodermal in origin.

2. The original fibrillæ ultimately differentiate throughout their whole length into true striated muscle elements, except in the region of skeletal attachment, where the peripheral ends of the fibrils remain unstriated, and serve as tensile structures directly uniting the contractile elements with the chitin of the exoskeleton.

3. In later development connective tissue elements may form over the inner surface of the epidermis and around the muscle fibers, and

purely supportive fibrils differentiate within the epidermal cells, but these structures are secondary in their relation to muscle attachment.

2. *Nerve Fibers.*—In studying the regeneration of nerve fibers we are at once involved in the intricate questions of the origin of the neurilemma, and of the axis cylinder with its neurofibrillæ. Complete agreement regarding ectodermal or mesodermal origin of these structures has not yet been attained. Regarding the neurilemma, for example, the conclusions of one group of investigators may be summarized in the statement that sheath cells "are true connective tissue cells from the locality through which the nerve fiber has passed in its development" (Dahlgren and Kepner, '08, p. 188). On the other hand, the ectodermal origin of the sheath cells appears to be strongly supported by experiments in which it has been shown that in the tadpole at least "the source of the sheath cells, both of the motor and sensory nerves is in the ganglion crest" (Harrison, '08, p. 393). Although the present observations on the lobster are, on account of a lack of material especially prepared for neurological study, not sufficiently extensive to render them very important in relation to these fundamental problems, still they may be of some value.

In early stages of the regenerating limb bud there is found a cord or column of cells extending from the tip of the bud to the end of the old nerve trunk. As the limb segments are formed, this cord of cells is joined by similar strands from the various segments. Within these cords the axis cylinders of the nerve fibers develop, and from the cells composing the cord the sheath cells are differentiated. The important question is as to the origin of these cords of cells; have they migrated outward from the sheath cells of the old nerve trunk, or are they derived from the ectodermal cells of the regenerating bud?

The present observations support the latter conclusion. Among the old sheath cells no evidence of cell division was obtained, nor was there observed any extensive proliferation or outward migration of the sheath cells from the old nerve trunk. On the other hand, cells from the first-formed ectodermal plate migrate inward toward the injured nerve, as has already been described in the early

stages of the regenerating bud (Fig. 9). At later stages this inward migration of ectodermal cells is quite marked in certain regions (Fig. 28, *n*), where they evidently contribute to the formation of the cords of cells just described. The distal end of each cord always contains a larger number of cells than its more proximal end, giving it a club shape, with the smaller end joined to the old nerve. Occasionally mitotic figures were found, but they always occurred among the more distal cells of the cords. The nuclei of these distal cells were generally large and spherical in form, but in passing proximally toward the old nerve trunk, there was a gradual transition from the spherical nuclei to the long flat nuclei of the sheath cells, indicating a direct differentiation of the neurilemma from these ectodermal cells.

These observations on the lobster, together with the results of Reed's ('04) on the crayfish, and Steele's ('07) on the nerve endings in the ommatidia of the eye, furnish collective evidence for the participation of ectodermal cells in the regeneration of the nerve fibers in crustacea. It remains to be determined, however, whether this conclusion can be extended to arthropods in general. For it must not be overlooked that different results have been obtained by Ost ('06) in his study of the regenerating antenna of Oniscus, in which form he finds that the neurilemma regenerates "durch Nachschieben vom proximalen Ende her . . . Die Regeneration des Antennennerven von Oniscus geht also nach meinen Beobachtungen durch direktes Auswachsen junger Nervenfasern aus dem alten Stumpf vor sich" (p. 313). It is noteworthy that Ost was unable to obtain any evidence of cell division among the sheath cells, for he states, "Mitosen oder sonstige Teilungsvorgänge konnte ich an diesen Kernen freilich nie beobachten,"—a result corresponding with the conditions found in the regenerating lobster's limb, *i. e.*, in the region of the old nerve trunk.

By the fifth day of regeneration (five days, ten hours) the differentiation of an axis cylinder within the cord of cells just described, had advanced sufficiently to present delicate neurofibrillæ.[6] These

[6]The nerve fiber thus appears to develop earlier than the muscle, for in all cases regenerating nerve fibers were found before myofibrillæ could be detected.

fibrillæ were not of equal size and were distributed in an interesting manner. In some of the preparations, the larger or heavier fibrils were mostly at the periphery of the axis cylinder, whereas the finer ones were nearer the center (Fig. 29). Consequently at certain stages of regeneration the nerve fiber could be structurally analyzed into four parts: (a) a central core of very delicate fibrillæ; (b) a peripheral layer, not sharply marked off from (a) but containing heavier fibrils; (c) the external sheath of nucleated cells or future neurilemmma, and (d) a sheath of cytoplasm between (b) and (c) relatively free from neurofibrillæ. In later stages of differentiation the heavier fibrils predominate (Fig. 30). At the time when the neurofibrillæ are beginning to appear, the cytoplasm of the nucleated sheath, as compared with that of the axis cylinder, is more coarsely granular. In some regions there also appears to be a membrane present between the nucleated sheath and axis cylinder.

No conclusive evidence was obtained as to whether the axis cylinder is formed in situ from the sheath cells, or whether it is an outgrowth from the axis cylinder of the old nerve cell. In either case, however, if the present observation is correct, that the nerve fiber develops within a cord of cells proliferated from the ectoderm of the various new limb segments, it points to the sheath cells as one of the factors involved in determining the final distribution of nerve fibers within the regenerated limb. It was not determined whether the neurofibrillæ differentiate distally or proximally, or whether they appear simultaneously throughout the regenerating nerve fiber. The fact that in at least certain stages of differentiation the finer fibrillæ occur at he center, and the heavier fibrils at the periphery of the fiber, apparently lends support to the conclusion that the neurofibrillæ are being derived from the cytoplasm of the axis cylinder.

3. *Connective Tissue.*—The connective or supporting tissue of the crustacean limb consists mostly of broad loose bands or sheets extending between opposite walls of the exoskeleton, around the extremities of each segment, and in certain regions filling almost the entire segment. In addition to these sheets and bands of connective tissue there is also a thin layer of simple flat epithelium found covering the inner surface of the epidermal cells (here known as "Grenzlamelle") and surrounding the muscle bundles.

Let us consider first the broad sheets of tissue extending from one wall to the other within the limb segment. As the segments of the regenerating limb become well differentiated, there occurs an extensive inward migration of ectodermal cells in those regions where there later develop the bands of connective tissue characteristic of the adult limb. This migration is especially well defined in the regenerating meropodite. The epidermal cells in this region migrate inward from the epidermal wall and form a broad sheet extending across the central cavity of the segment (Fig. 26, *ic*). The nuclei become elongated, and distinct fibrils arise in the cytoplasm. At later stages (Fig. 27, eleven days, ten hours) the cytoplasm, with the exception of the epidermal cells next the exoskeleton, becomes vacuolated. These intercellular spaces then become filled with blood plasm (Fig. 27, *b*), and eventually there is thus formed the vascular and connective tissue characteristic of the fully developed limb.

It seems evident, therefore, that in at least certain regions of the regenerating limb a supportive and apparently true connective tissue may be derived from ectodermal cells. Nor is this divergent from conditions found in normal development among crustacea, for Claus ('86) in his work on the embryology of Branchipus, concludes that the broad connective tissue bands which unite the opposite surfaces of the integument, "sind Erzeugnisse der Chitinogenzellen der Hypodermis", and quotes Braun to the effect that in the crayfish it is "nicht möglich, eine scharfe Grenze zwischen den Erzeugnissen von Chitinogenzellen und den mesodermalen Bindegewebebildungen festzustellen" (pp. 22-23).

We may now briefly consider the tissue characteristics of the so-called "Grenzlamelle." In the description of the regenerating muscle, reference has been made to the fact that a "Grenzlamelle" is not present under the epidermis at the time when myofibrillæ begin to differentiate. It is only after the muscle fibers and the epidermis have become well developed that an epithelial layer is formed over the inner surface of the epidermal cells. In various parts of the limb this "Grenzlamelle" is apparently the only tissue between the blood sinuses and the epidermis, and consequently in at least certain regions it seems to serve as a vascular epithelium (cf. Williams, '07, p. 155).

As to the origin of the regenerating "Grenzlamelle," the present observations fail to furnish conclusive evidence. The fact, however, that the regenerating bud becomes filled with ectodermal cells involved in the regeneration of muscles and nerves, and the lack of clear evidence of cell proliferation from the old connective tissue, suggests an ectodermal origin. Of interest in this connection are Claus's observations on the normal development of Branchipus:— "Was man als solche (connective tissue) beim ersten Blick in Anspruch zu nehmen geneigt ist, erweist sich wenigstens an vielen Stellen nach genauer Betrachtung als eine Art innere Cuticula, die aus der Basis des Epithels durch Erhärtung des Protoplasmus entstanden ist" (p. 21). On the other hand, Schneider ('02) from a comparative point of view is unable to support this conclusion: "Nach Claus stammen die Grenzlamellen und die Muskelsehnen mindestens zum Teil vom Epiderm, dessen Deckzellen die basalen ausscheiden sollen. Dieser Ansicht kann in Rücksicht auf die Verhältnisse bei anderen Arthropoden, wo die Sehnen und Lamellen unzweifelhaft Bindegewebsbildungen sind, nicht beigestimmt werden, wenn auch die Bildung von Seiten des Bindegewebes nicht bekannt ist" (p. 466).

VIII. The Direction of Differentiation.

The question of the direction in which the process of differentiation proceeds in both regeneration and normal ontogeny is a subject which has given rise to diverse opinions. In elaborating certain theories of form regulation, Holmes ('04) assumed that in a regenerating appendage, differentiation proceeds from the base toward the tip, whereas Child, at least in an earlier paper ('06), was inclined to regard the reverse as generally true.[7] Zeleny ('07) found in the regenerating antennule of Mancasellus that there are two distinct periods of development, in the first of which the process of differentiation "begins at the base and travels irregularly outward", while in the second period, the process is in the reverse order, *i. e.*, beginning with the terminal segment and differentiating inwards (p.

[7]Holmes ('07) and Child ('08) have later restated their theories as not being inconsistent with differentiation in either direction.

325). Haseman ('07) concluded that in the regenerating cheliped of the crayfish, differentiation proceeded in a disto-proximal direction. Haseman, appreciating the importance of determining whether changes in external form could be safely taken as an index of internal changes, gave some attention to the internal differentiation of tissues. But as a rule conclusions upon this subject have been largely based upon evidence derived from modifications in external structure, rather than from internal cytological and histological changes in the segment tissues. In view of this fact, the following study has been made with the purpose of obtaining further data upon the direction of differentiation among the internal regenerating tissues.

The results to be described were derived from the study of serial sections of somewhat over a hundred regenerating chelæ at various stages of development. In tracing the progress of differentiation it becomes necessary to rely on such criteria as are most clearly defined. In the present case the following structural characters have been chosen on account of their ready identification by means of selective stains: (1) the formation of chitinogenous muscle plates or tendons; (2) the differentiation of myofibrillæ; and (3) the striation of the muscle fibrils.

In the description of the differentiation of the regenerating limb, it will be recalled that distal to the breaking joint there are five segments. Each segment contains a pair of muscles controlling the extensor and flexor movements of the next distal segment (see Fig. 37). It is important to note that in each pair of muscles the flexor is the larger, and that of the four pairs of muscles, it is the first and third pairs (counting from the distal end) which contain the largest muscles, *i. e.*, the muscles which lie in the propodite and meropodite. Each muscle takes its origin from the exoskeletal wall of the segment in which it lies, and is inserted upon the chitinogenous plate extending proximally from the next distal segment. The differentiation of these chitinogenous plates will first be considered.

1. *The Chitinogenous Plates or Muscle Tendons.*—The differentiation of chitin within the joint invagination is indicated by a characteristic brick-red stain reaction with Congo red, or bright blue with Mallory's connective tissue stain. The developing chitin is thus

sharply contrasted with any surrounding tissue. Using this stain reaction as an index of differentiation, serial sections were studied and graphic reconstructions made of different stages in the regeneration of the limb (Figs. 31-36).

a. *Two Days and Twenty-two Hours after Operation* (*Fig.* 31).—Although externally at this time there was little, if any, indication of segmentation, internally there was found a well defined invagination of epidermal cells, within which a differentiation of chitin (r^1) was already evident. This invagination marks the beginning of the first and second distal segments. It is here that the chitin plate is formed for the flexor muscle of the dactyl, which, it is to be observed, is normally not only much larger than the opposing extensor, but is also the largest muscle of the limb.[8]

b. *Four Days and Six Hours* (*Fig.* 32).—The differentiation of additional chitin plates is not evident until on the fourth day of regeneration. At this time two more invaginations were beginning to develop chitin. Of these two invaginations the one representing the extensor for the dactyl (e^1) showed as yet but a slight differentiation of this tissue. The other invagination (r^3) represents the flexor for the meropodite, and shows a well-defined plate of chitin.

In regard to the latter invagination (r^3) there are two important points to be emphasized. First, the invagination is not in the next or third proximal segment, but in the fourth segment or meropodite; and second, of the two muscles in the meropodite, the invagination represents the flexor.[9]

[8]This invagination occurs at one side of the apex of the bud, so that at first the dactyl is relatively larger than the index,—a relation, it is interesting to note, which corresponds with the condition found in the larval stages of development, but is reversed in the adult. (Emmel, '06[2]).

[9]In the external segmentation of the limb at this stage, the groove between the second and third segments is quite as well marked, if not more so, than the groove between the third and fourth segments. This agrees with Haseman's ('07) observations on the cheliped, and with my own earlier description ('06) for the lobster, that the external segmentation appears to proceed in a disto-proximal direction; but the crucial fact that in the present case it is the tendon in the fourth segment instead of in the third segment which differentiates next after the second segment, demonstrates that the external segmentation cannot, therefore, be safely relied upon alone as a correct index of internal differentiation.

The first point becomes especially interesting when it is considered that according to the requirements of the theory that differentiation proceeds in a disto-proximal direction, the next chitin muscle plate to develop after those of the propodite should have been in the third segment or carpopodite instead of the fourth segment or meropodite. Serial sections of the carpopodite were carefully examined, but there was no evidence that any chitinization had yet taken place for the tendon plates in this segment.

c. *Four Days and Twenty-two Hours* (*Fig.* 33).—At this stage a fourth chitinous tendon (r^2) has become evident. This tendon is in the third segment and is the one involved in the development of the flexor muscle. The extensor (e^1) and flexor (r^1) in the propodite, and the flexor (r^3) in the meropodite, show an advance in differentiation as compared with the preceding stage, but otherwise there is no evidence of chitin differentiation in any of the remaining muscles. Consequently at this stage only four chitinogenous plates or tendons are present; two in the propodite and one in each of the next two proximal segments. The important point is that in each case it is the tendon for the flexor muscle which differentiates first in each segment.

d. *Five Days and Six Hours* (*Fig.* 34).—The tendon for a second extensor muscle (e^3) is now beginning to differentiate. Here again the next tendon to develop is not for the extensor in the carpopodite, but for the one in the meropodite. At this time two other chitinous tendons (e^4) are also just becoming evident in the fifth segment or ischiopodite. The muscles correlated with these last two tendons, it will be recalled, are both extensors.

e. *Five Days and Nine Hours* (*Fig.* 35).—The chitinous tendon (e^2) for the extensor of the carpopodite is now present. This introduces, therefore, the last of the eight muscle tendons to differentiate.

f. *Ten Days and Two Hours* (*Fig.* 36).—By this time the eight chitinogenous tendons and muscles of the regenerating limb have become fully developed. The animal is now about to undergo the process of moulting, in which the regenerated limb will be liberated from its membranous sac and become a functional appendage.

In summarizing these observations it may be stated that the chitin-

ogenous plates or tendons for each of the eight muscles distal of the breaking joint were found to be differentiated in the following order: (1) for the flexor muscle in the second limb segment; (2) the flexor in the third segment, together with the extensor in the second segment; (3) the flexor in the third segment, and at the same time the two extensors in the fifth segment; (4) and last, the extensor in the third segment. From these results it seems evident that the direction of differentiation cannot be described as being any more a disto-proximal one than the reverse. The important point disclosed is that in each segment the tendon for the flexor, or larger muscle of the pair, is differentiated first; and further, of these flexors the larger ones are the first to develop.

2. *Differentiation of Myofibrillæ.*—The results of the microscopical study of the earliest differentiation of the myofibrillæ in each pair of regenerating muscles are presented in the following table. In this table the first column gives the time of regeneration. The signs + and 0 indicate, respectively, the presence or absence of myofibrils.

TABLE SHOWING THE SEQUENCE OF DIFFERENTIATION OF MYOFIBRILLAE IN EACH OF THE EIGHT LIMB MUSCLES DISTAL TO THE BREAKING JOINT.

Series No.	Regeneration.	Second Segment.		Third Segment.		Fourth Segment.		Fifth Segment.	
		Flex.	Exten.	Flex.	Exten.	Flex.	Exten.	Exten.	Exten
29	5 da. 9 hrs.	+	0	0	0	0	0	0	0
30	5 da. 10 hrs.	+	0	0	0	+*	0	0	0
31	5 da. 22 hrs.	+	+	+	+*	+	+*	0	0
33	6 da. 6 hrs.	+	+	+	+	+	+	+	+

*Differentiation just beginning.

In this table it will be seen that the first muscle fibrils to be differentiated were those of the flexor in the second segment; the next to appear were those of the flexor in the fourth segment. Up to this time no muscle fibrillæ were found in either the third segment or in the extensors in the second and fourth segments. The flexors in the fifth segment were the last to develop. Here again, differentia-

tion does not proceed definitely in either a proximal or a distal direction, but appears rather to be correlated with the size and function of the muscles concerned.

3. *Differentiation of Striæ in the Myofibrillæ.*—The series were also studied to ascertain the time at which myofibrils in each muscle showed striations. The following table represents the results obtained. The signs + and 0 indicate, respectively, the presence and absence of striation.

TABLE SHOWING THE SEQUENCE IN THE DIFFERENTIATION OF STRIAE IN THE REGENERATING LIMB MUSCLES.

Series No	Regeneration.	Second Segment.		Third Segment.		Fourth Segment.		Fifth Segment.	
		Flex.	Exten.	Flex.	Exten.	Flex.	Exten.	Exten.	Exten.
34	6 da. 22 hrs.	+*	+	0	0	+	0	0	0
35	7 da. 2 hrs.	+	+	+	+	+	+	+	+†

*Striations were not found until after the myofibrillæ had begun to be differentiated in all the segments.

†Differentiation of striæ just becoming evident.

The time intervening between the appearance of the first striæ and the striation of all the muscles is apparently much shorter than the time between the first differentiation of myofibrils and their complete development for all the muscles. Nevertheless, striation does not occur simultaneously throughout the limb. The striæ differentiate first in the flexor muscles in the second and fourth segments, and later they appear in the remaining limb muscles.

4. *Conclusions.*—The results of the present study of the differentiation of the chitinogenous muscle tendons, the myofibrillæ and their striation justify two conclusions:

First, that the differentiation of the regenerating musculature proceeds in neither a definite disto-proximal direction, nor the reverse.

Second, that whatever sequence there may be in this differentiation is correlated rather with the size and functional relations of the various muscles concerned, the larger muscles differentiating first.

Reference has already been made to the recent discussions of the direction of differentiation by Child ('06, '08), Holmes ('04, '07), Zeleny ('07), and Haseman ('07). Among these investigators Haseman has studied the form most closely related to the lobster. From his observations upon the regenerating appendages of the crayfish, he concludes that differentiation in the chela proceeds from the tip toward the base, while the reverse is true for the walking legs.

In view of the results of these investigators Zeleny has discussed three possibilities regarding the direction of differentiation in the development of an appendage.

1. That "all the parts may appear at once"; (2) that "the progression may be outwardly directed", or (3) "it may be inwardly directed". Zeleny points out that the first lacks evidence, that the second has been more especially favored by Pflüger and Holmes in connection with certain general theories of development, and the third by Morgan, Driesch, and Child. From his own study Zeleny concludes that the second method may predominate in some animals, the third in others, and further that both modes may predominate at different times in the same animal, as appears to be the case in the antennule of Mancasellus macrourus. It is evident that the present data from the lobster fail to conform with any of the three modes of differentiation. But the evidence does support a fourth proposition, viz., that sequence of differentiation may be influenced or determined by the relative size and functional rôle of the regenerating structures. Consequently, differentiation in the regenerating appendage is not necessarily either "centrifugal" nor "centripetal," the new structures, on the contrary, differentiating rather according to size and functional relations, in a manner perhaps similar to the formation of organs in embryonic development,—a most striking illustration of which is furnished by Sutton's ('83) rule for the differentiation of the epiphyses of the long bones in human osteology: "The centers of ossification appear earliest for those epiphyses which bear the largest relative proportion to the shafts of the bones to which they belong" (p. 480).

IX. Summary.

1. Two new structures are recorded in connection with the breaking joint of the chela of lobsters.

a. A muscle crossing the joint, but degenerating during the fifth larval stage.

b. Valves in the venous blood sinus which prevent excessive hemorrhage after the autotomy of the limb. The valves persist throughout life.

The following conclusions concern the regeneration of the limb after autotomy.

2. The first layer of cells, aside from the blood clot, to cover the wound is formed by the migration of epidermal cells.

3. The nuclei of these migrating cells enlarge and become somewhat wedge-shaped. The karyosomes disintegrate into fine granules which tend to collect at the proximal end of the nucleus. Thus the nuclear contents appear polarized into an inner zone of darkly stained chromatin, and an outer zone relatively free from chromatin granules.

4. The significance of this polarization is not clear, but its regular occurrence, and is reversed on opposite sides of the limb, together with the frequent expansion of the higher pole, and the association of axial chromation masses with the darker pole, indicate that it is a phenomenon characteristic of certain stages in the regenerative activity of the epidermal cell.

5. During these nuclear activities the cytoplasm loses its supportive fibrillæ and reticular structure, and becomes finely granular. Although definite cell membranes are never evident, there are more or less clearly defined cytoplasmic units around each nucleus.

6. Mitotic cell division begins after the formation of the first epidermal plate over the wound. The formation of this plate seems sufficiently accounted for by the volumetric increase of the cytoplasm, together with the enlargement and wider separation of the nuclei of the migrating cells.

7. The wall of the regenerating bud, and a large part, if not the entire core of cells filling its interior, are derived from epidermal cells. Since there is no conclusive evidence of either cell division or migration among the old muscle and connective tissue cells, these tissues appear to contribute little, if anything, to the formation of the new limb bud.

8. a. Regenerating striated muscle is ectodermal in origin.

b. The myofibrillæ appear genetically related to the cyto-reticulum. Each fibril is early surrounded by a sheath of modified cytoplasm, multiplies by longitudinal splitting, and in its differentiation the "z" line or membrane of Krause, becomes evident after the formation of the light and dark bands.

c. The muscle fiber or cell is multinuclear when first formed. Later the nuclei come to be peripheral in position while the fibrillæ lie to the side of the fiber nearest the center of the muscle bundle. The nuclei multiply by mitotic division, and with the development of the sarcolemma they become flattened and elongated in the long axis of the fibers.

d. The myofibrillæ differentiate throughout their whole length into true striated muscle elements, except in the region of skeletal attachment. There the peripheral ends of the fibrils remain unstriated, and serve as tensile elements.

e. The connective tissue over the inner surface of the epidermis, and the purely supporting fibrillæ within the epidermal cells develop later, but these structures appear to be secondary in their relation to muscle attachment.

9. a. The present observations indicate that the neurilemma is derived from the regenerating epidermal cells.

b. During certain stages in the differentiation of the axis cylinder the finer neurofibrillæ are central and the coarser fibrils more peripheral in position.

10. In at least certain regions of the regenerating limb, supporting and apparently true connective tissue differentiates from epidermal cells.

11. a. In the development of the chitinogenous muscle plates and the myofibrillæ and their striæ, differentiation appears to be neither directly "centrifugal" nor "centripetal" in direction.

b. On the contrary, the facts warrant the statement that whatever sequence there may be in differentiation, it is correlated with the size and functional relations of the muscles concerned, rather than with any distal or proximal relation of these structures to the organism.

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XI. DESCRIPTION OF PLATES.

Unless otherwise indicated, all figures are from camera drawings made from longitudinal sections of the chelæ of fourth stage lobsters.

List of Abbreviations.

a, artery.
a^1, a^2, arteries passing through the breaking joint.
b, blood plasm.
bc, blood corpuscles.
bcl, blood clot.
bk, breaking joint.
bs, basipodite.
c, carpopodite.
ch, chitin.
ch^1, new chitin.
cm, connective tissue membrane.
ct, connective tissue.
d, dactylopodite.
e, epidermis.
e^1, migrating epidermal cells.
e^1, e^2, e^3, e^4, extensor mucles in the propodite, carpopodite, meropodite, and ischiopodite, respectively.
ec, regenerating and proliferating epidermal cells.
ef, supportive fibrillæ.
em, epidermal membrane.
en, regenerating epidermal nuclei.
i, ischiopodite.

ic, inward proliferation of epidermal cells to form connective tissue.
in, invagination of epidermal cells at the joints
m, muscle crossing the breaking joint.
ma, muscle attachment.
mb, muscle in the basipodite.
me, meropodite.
mf, myofibrillæ.
mn, muscle nuclei.
mp, chitinogenous muscle plate or tendon.
mt, cells undergoing mitotic division.
n, nerve.
n^1, n^2, nerves passing through the breaking joint.
nf, neurofibrillæ.
ng, nuclei of the "Grenzlamelle."
nn, nuclei of the neurilemma.
p, propodite.
r^1, r^2, r^3, flexor muscles in the propodite, carpopodite, and meropodite, respectively.
rt, cyto-reticulum.
s, septum of connective tissue dividing venous blood sinus into two channels.
sa, sarcolemma.
v, valves in venous blood channels.
v, valves in venous blood channels; v^1, v^2, inner and outer channels, respectively.
vc, venous blood channel.
vs, venous sinus; vs^1, vs^2, respectively distant and proximal of the breaking joint.

PLATE I.

Structure of the Chela in the Region of Amputation.

Graphic reconstructions of the breaking joint and related segments of the left chela, to show the anatomical relations of the blood vessels, muscles, nerves, epidermis, and connective tissue. The drawings are incomplete in one particular because the finer meshwork of connective tissue in which the arteries and nerves are suspended is not represented in its entirety.

Fig. 1 represents the outer third, and

Fig. 2 the inner third of the breaking joint and segments. The sides of the figures nearest each other are vertical. × 263.

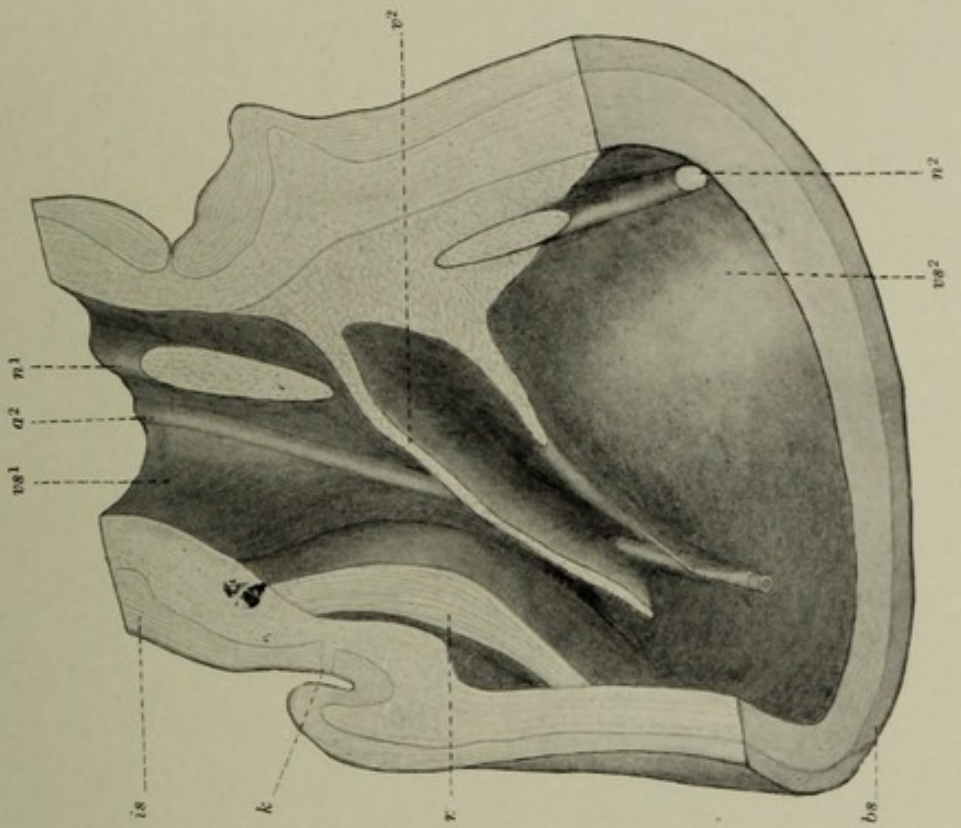

FIG. 2.

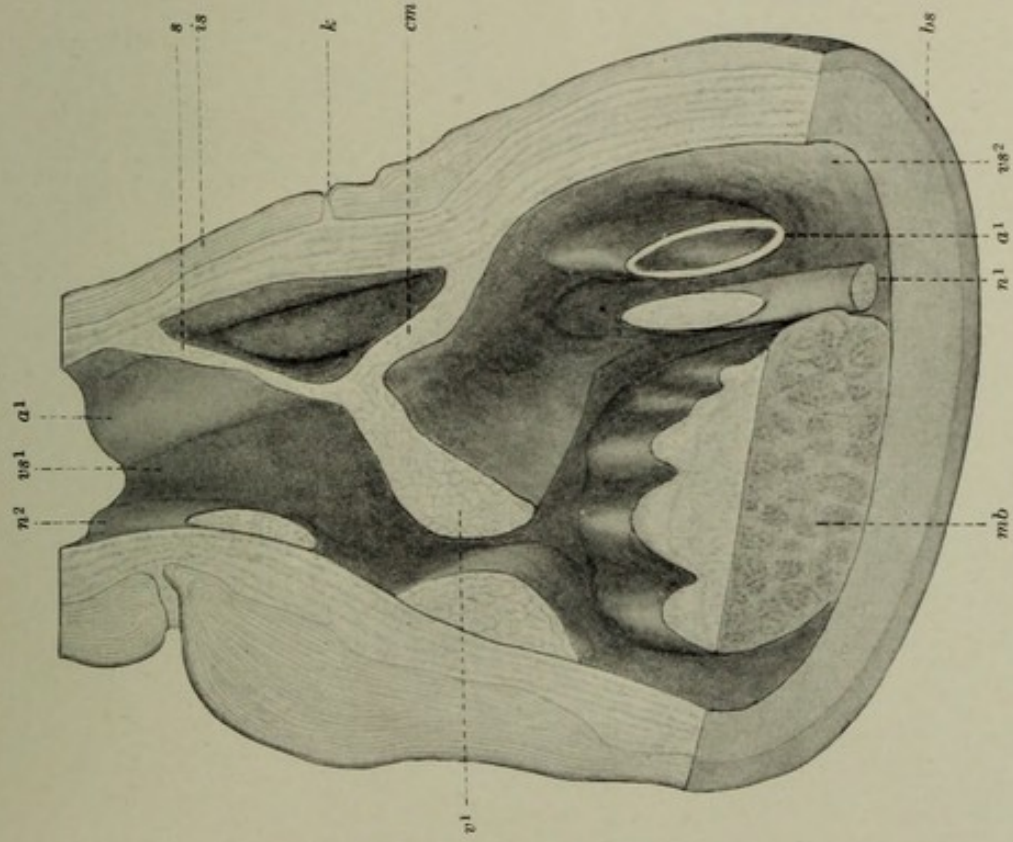

FIG. 1.


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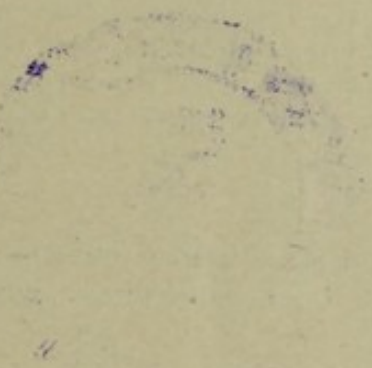

PLATE II.

Structure of the Chela in the Region of Amputation.

Fig. 3. Section of the breaking joint from the region midway between the parts represented in Figs. 1 and 2, to show the relations of the epirdermis, nerve trunk, and connective tissue membranes preceding autotomy. × 368.

Fig. 4. Showing relation of tissues after the autotomy of the limb, and the function of the valve (v^2) in closing the venous blood channel (vc). × 368.

Fig. 5. Section showing the relations of the muscle (m) discovered at the breaking joint (bk). × 204.

Fig. 6. In the fifth stage lobster this muscle has almost entirely degenerated, only remnants (md) of the former muscle being present. × 210.

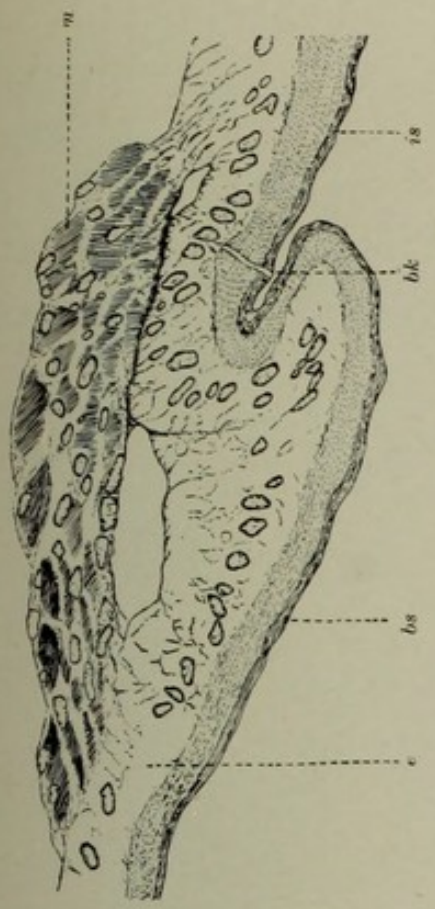

Fig. 5.

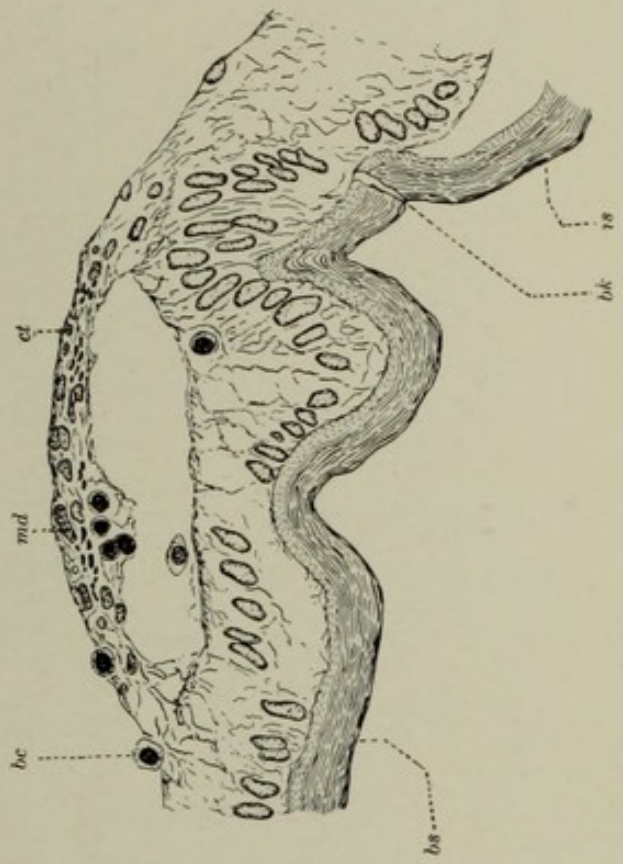

Fig. 6.

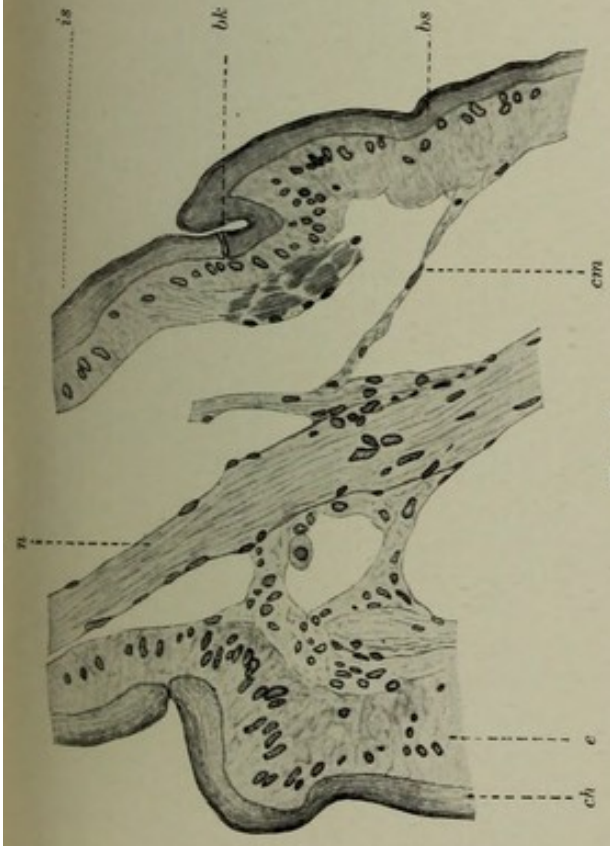

Fig. 3.

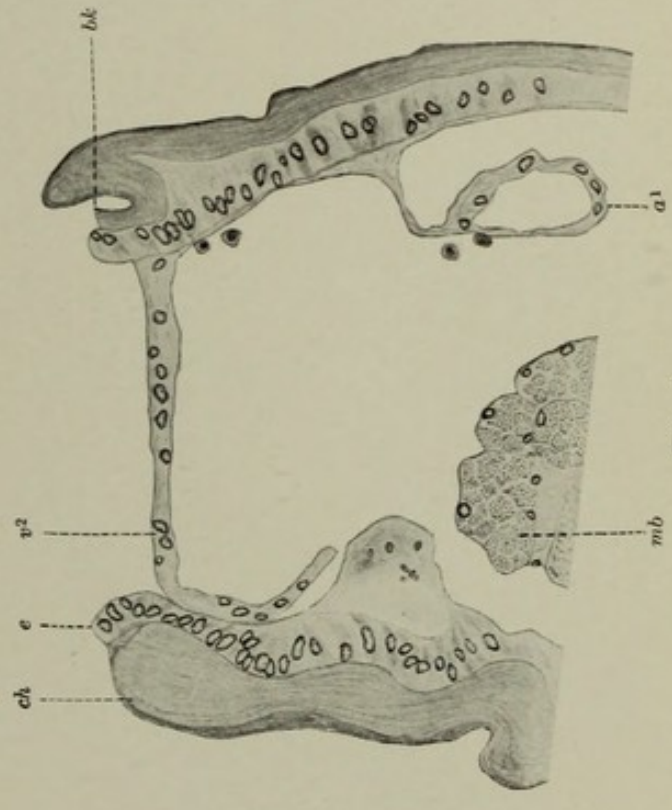

Fig. 4.


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PLATE III.

Successive Stages in the Regeneration of the Limb.

Fig. 7. Regeneration, 1 day. Epidermal cells (e^1) beginning to migrate across the wound beneath the blood clot (*bcl*). × 240.

Fig. 8. Regeneration, 1 day, 14 hours. Migrating epidermal cells have formed a complete plate or disc over the wound. Valve (*v*) of the venous blood channel now assuming its normal position. × 240.

Fig. 9. Regeneration, 2 days, 2 hours. The beginning of cell multiplication (*mt*) in the first formed epidermal plate. × 240.

Fig. 10. Regeneration, 2 days, 22 hours. Epidermal cells (*ec'*) at the apex of the bud are migrating inward to form the flexor muscle of the claw. × 240.

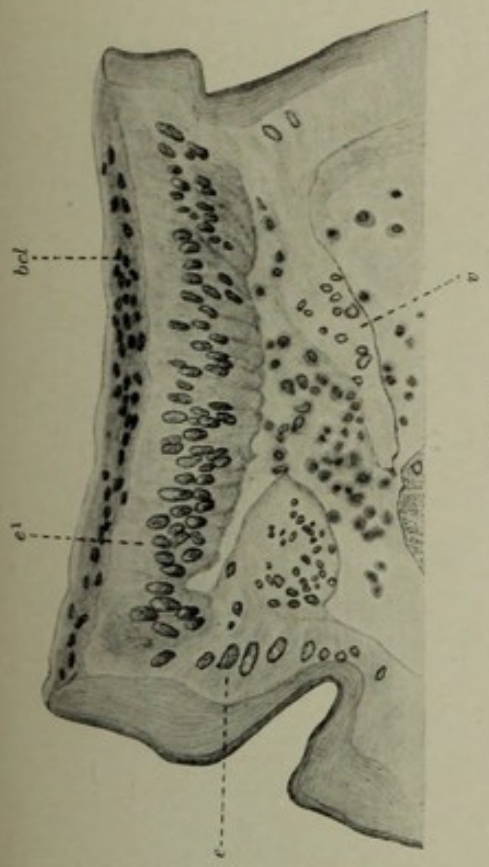

FIG. 8.

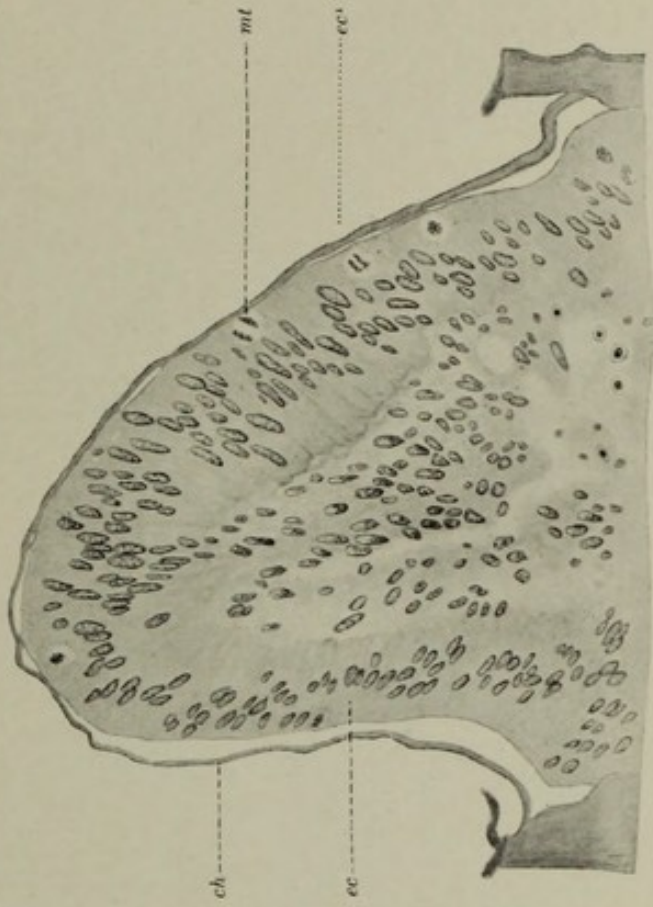

FIG. 10.

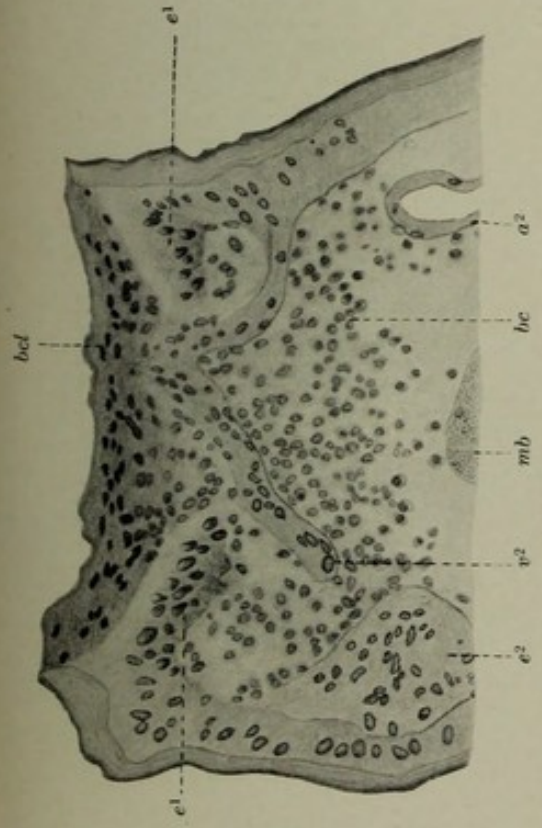

FIG. 7.

FIG. 9.

PLATE IV.

Nuclear Changes.

Changes in epidermal nuclei during regeneration. The groups of nuclei in Figs. 11-13 taken from the same region of the limb, *i. e.*, on the same side of the limb just below the breaking joint; in each case (*c*) and (*d*) are nearer the center of the wound than (*a*) and (*b*).

Fig. 11. Normal nuclei before the amputation of the limb. × 2000.

Fig. 12. Regeneration, 14 hours. × 2000.

Fig. 13. Regeneration, 24 hours. × 2000.

Fig. 14. Regeneration, 2 days, 2 hours. Nuclei undergoing mitosis; *a* (apparently), prophase; *b*, anaphase. × 2000.

Fig. 15. Regeneration, 5 days, 10 hours. Nuclei in the epidermal wall of the regenerating limb bud, in the region of the invagination (*in*) for the flexor muscle in the meropodite. × 1250.

FIG. 11.

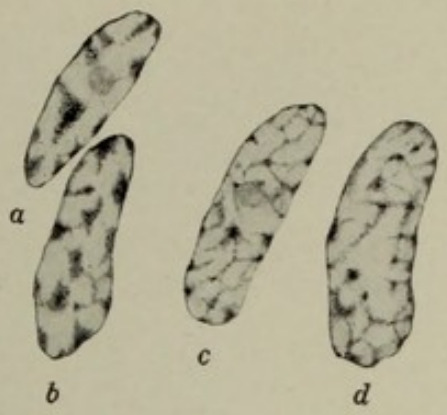

FIG. 12.

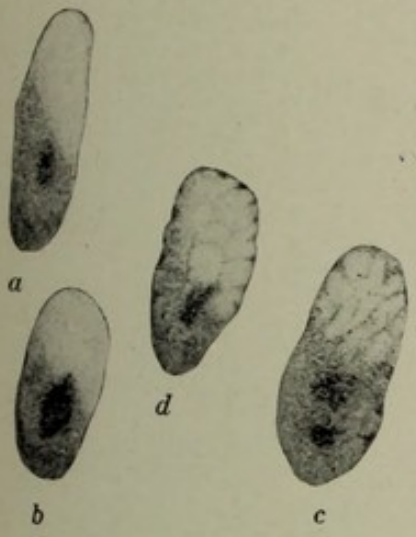

FIG. 13.

FIG. 14.

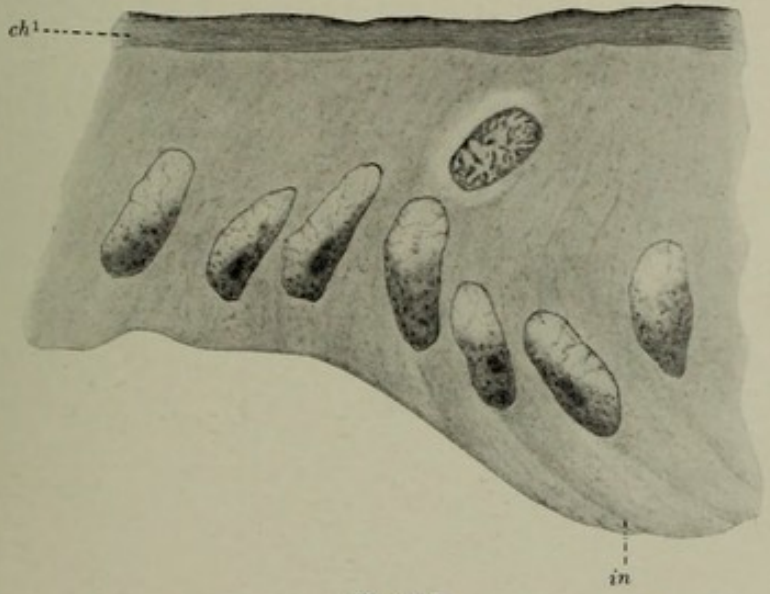

FIG. 15.

PLATE V.

Regeneration of Striated Muscle.

Fig. 16. Regeneration, 6 days, 6 hours. Myofibrillæ (*mf*) just beginning to differentiate for the flexor of the dactylopodite. × 334.

Fig. 17. Regeneration, 7 days, 6 hours. Showing the great elongation of epidermal nuclei during their invagination for the formation of the muscles,—in this case the flexor is the meropodite. × 1250. (Cf. with Fig. 15.)

Fig. 18. Regeneration, 12 days, 10 hours. Shows striation of muscle fibrils (*mf*) and their attachment to the chitin of exoskeleton. × 334.

Fig. 19. Showing skeletal attachment of regenerating muscle fibrils. (From the regenerating chela of a two-year-old lobster.) The myofibrillæ appear to be attached to the chitin (*ch'*). × 292.

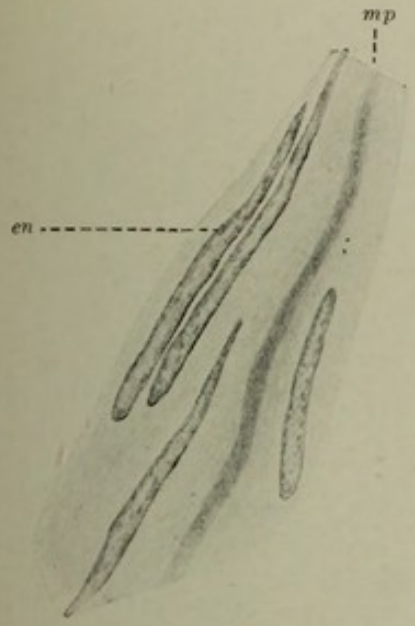

FIG. 17.

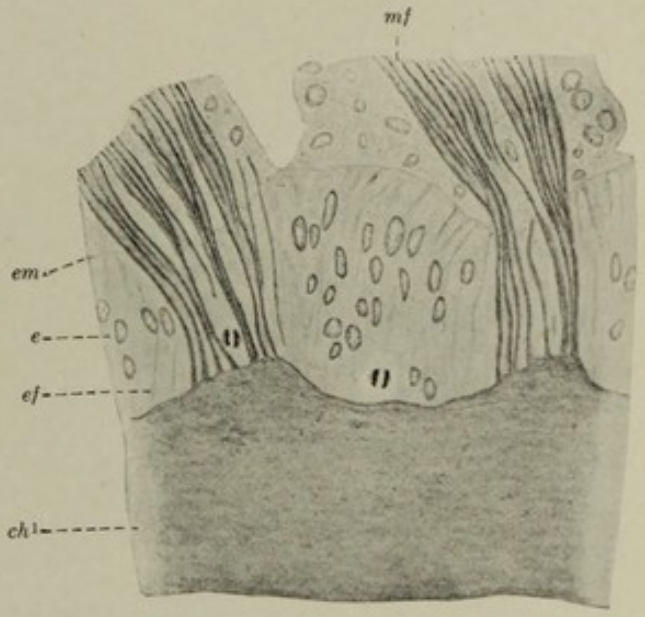

FIG. 19.

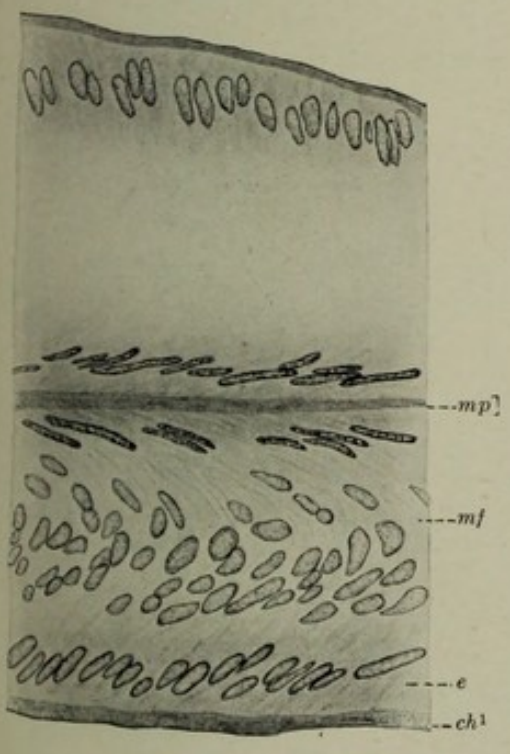

FIG. 16.

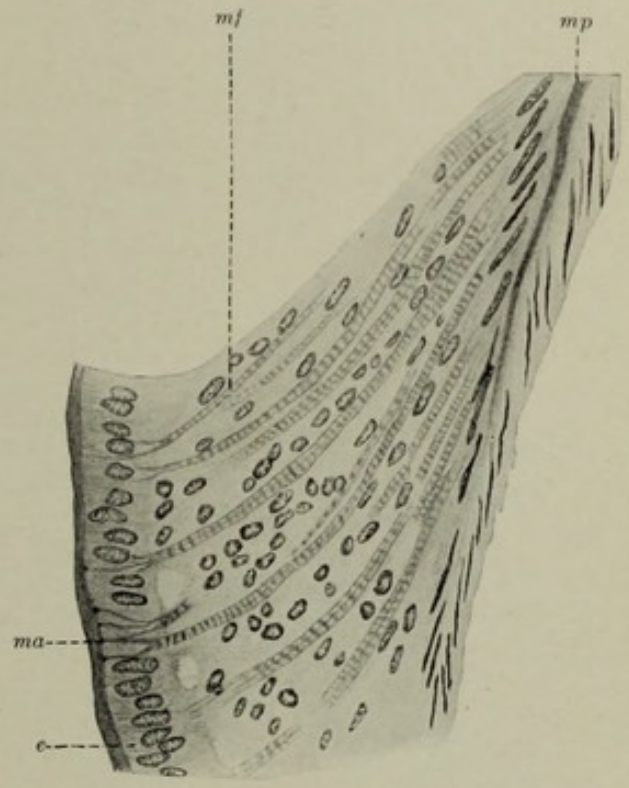

FIG. 18.


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PLATE VI.

Regeneration of Striated Muscle.

Fig. 20. Regeneration, 10 days, 6 hours. Showing the muscle fibrils forming brush-like end pieces in their attachment to the chitin. Epidermal membrane (*em*) just beginning to differentiate. × 1250.

Fig. 21. Completely regenerated epidermis in the chela of a sixth stage lobster just after moulting, showing a nucleus (*ng*) of the "grenzlamelle," and the relation of the "grenzlamelle" to the epidermis and muscle fibers. × 750.

Fig. 22. Regeneration, 6 days, 2 hours. Showing the close relation of the myofibrillæ (*mf*) to the cytoplasmic reticulum (*rt*) during early differentiation. × 1250.

Fig. 23. Regeneration, 7 days, 6 hours. Myofibrillæ (*mf*) surrounded by a sheath of modified cytoplasm (transverse section). × 1250.

Fig. 24. Regeneration, 8 days, 10 hours. Transverse section of myofibrillæ (*mf*), showing the first longitudinal splitting of the fibrils. × 1250.

Fig. 25. Regeneration, 11 days, 10 hours. Transverse section of a fully formed muscle fiber showing membrane or sarcolemma (*sa*), nuclei, and Cohnheim's areas. × 1250.

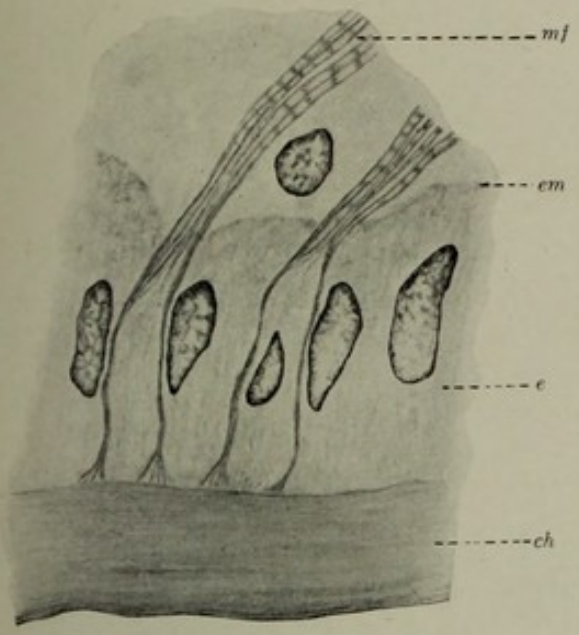

FIG. 20.

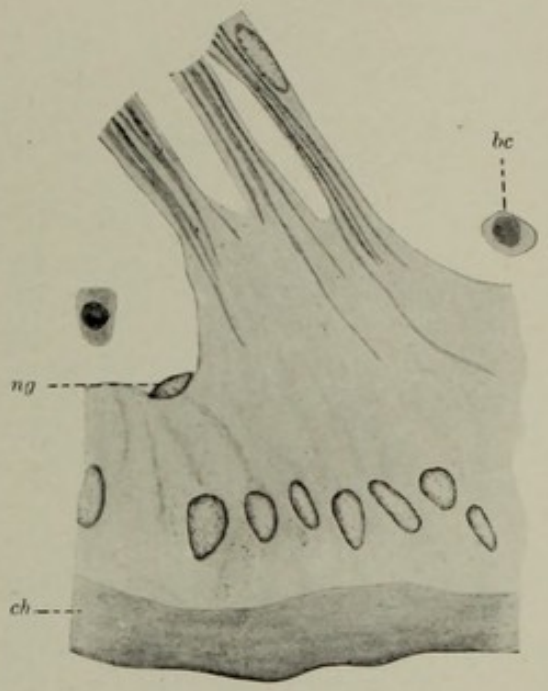

FIG. 21.

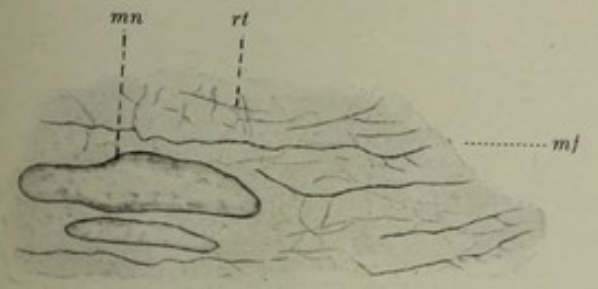

FIG. 22.

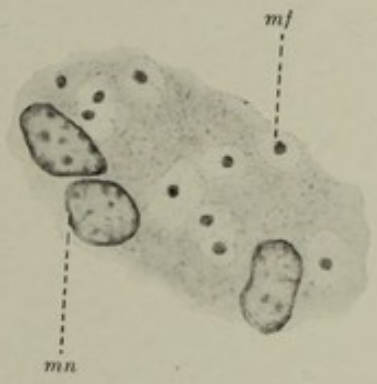

FIG. 23.

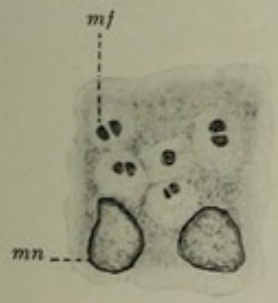

FIG. 24.

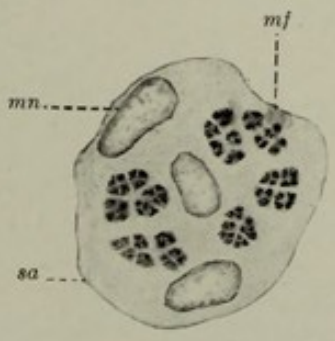

FIG. 25.


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PLATE VII.

Regeneration of Connective Tissue and Nerves.

Fig. 26. Regeneration, 10 days, 2 hours. Shows inward proliferation of epidermal cells to form a sheet of connective tissue (*ic*) across the segment (meropodite). × 417.

Fig. 27. Regeneration, 11 days, 10 hours. A later stage in the differentiation of the connective tissue (in the meropodite) showing vacuolation and infiltration of blood plasm (*b*). × 334.

Fig. 28. Regeneration, 5 days, 10 hours. Proliferation of epidermal cells (*c*) in the formation of a nerve fiber (*n*). × 582.

Fig. 29. A stage in the differentiation of the nerve fiber showing a nucleated sheath and axial core of delicate neurofibrillæ (*nf*) in the axis cylinder. At the periphery of this core of fibrillæ may be observed two heavier fibrillæ. × 750.

Fig. 30. A stage of the regenerating nerve fiber in which the heavier neurofibrillæ predominate. × 750. (Figures 29 and 30 were made from sections of the regenerating chela of a two-year-old lobster in which the neurofibrillæ were more sharply defined than in preparations from younger specimens.)

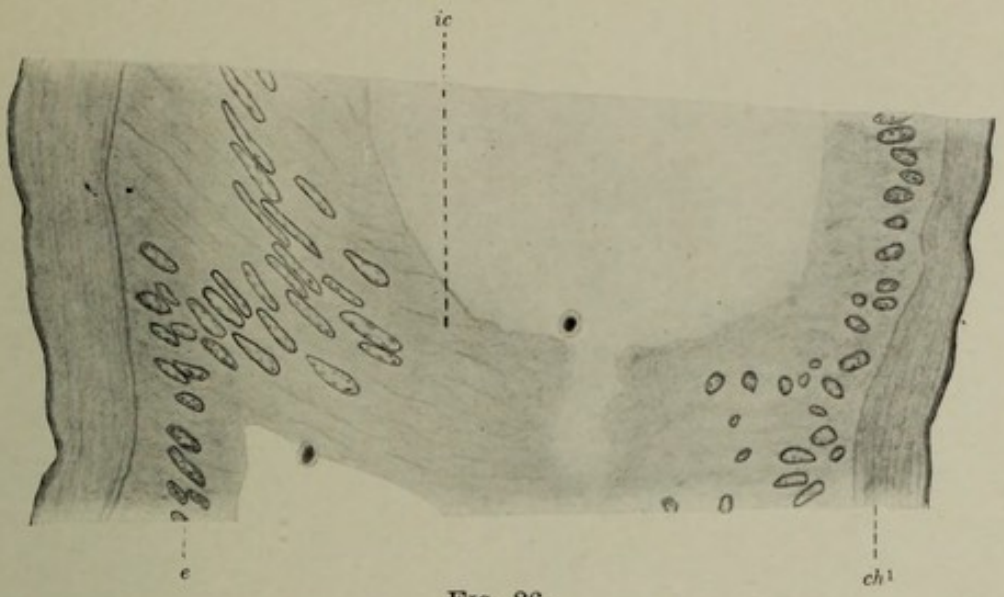

FIG. 26.

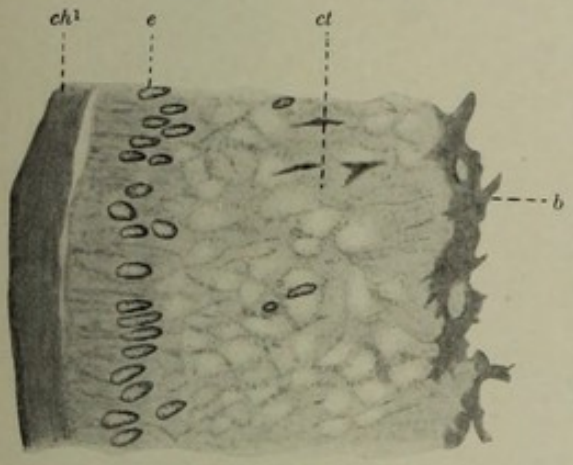

FIG. 27.

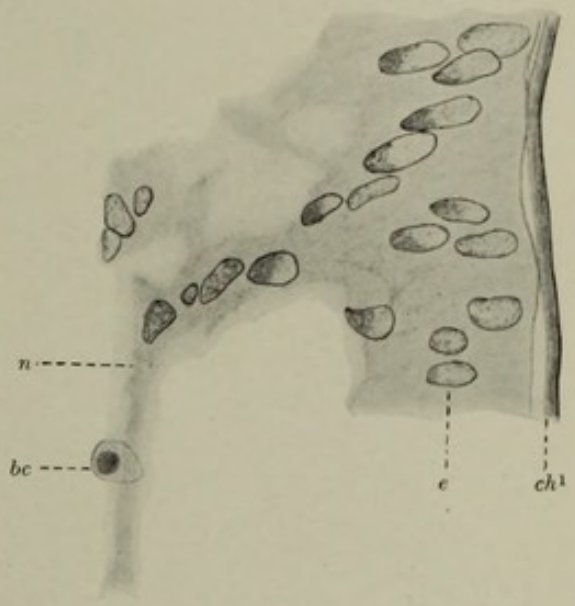

FIG. 28.

FIG. 30.

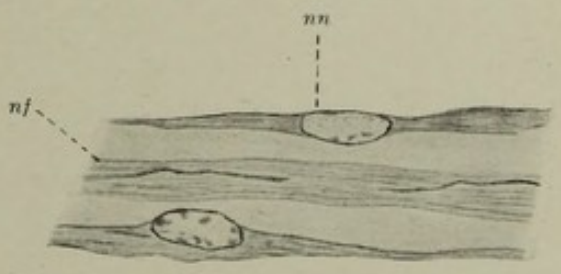

FIG. 29.

PLATE VIII.

The Direction of Differentiation.

The direction of differentiation of the eight chitinogenous muscle plates or tendons of the limb. Figs. 31-36 are reconstructions from serial sections.

Fig. 31. Regeneration, 2 days, 22 hours. × 60.

Fig. 32. Regeneration, 4 days, 6 hours. × 60.

Fig. 33. Regeneration, 4 days, 22 hours. × 60.

Fig. 34. Regeneration, 5 days, 6 hours. × 60.

Fig. 35. Regeneration, 5 days, 9 hours. × 60.

Fig. 36. Regeneration, 10 days, 2 hours. × 54.

Fig. 37. Diagrammatic drawing of a fully developed chela showing the relative size and position of the eight limb muscles distal of the breaking joint (*bk*). × 4-9.

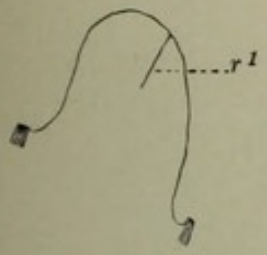

FIG. 31.

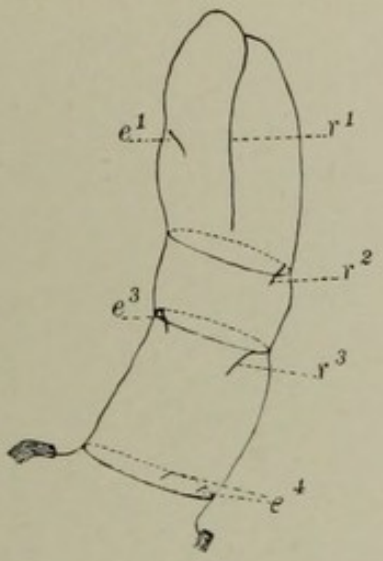

FIG. 34.

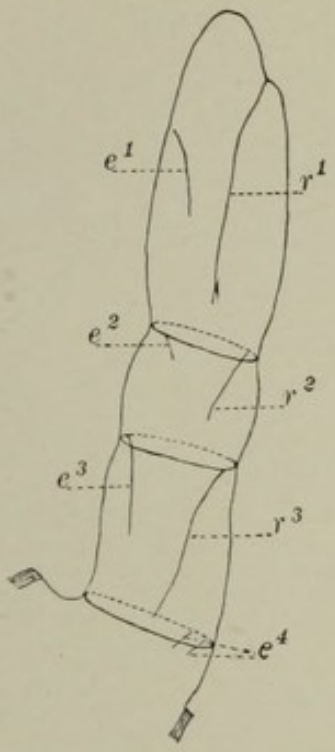

FIG. 35.

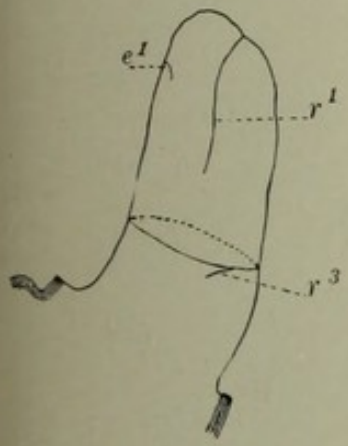

FIG. 32.

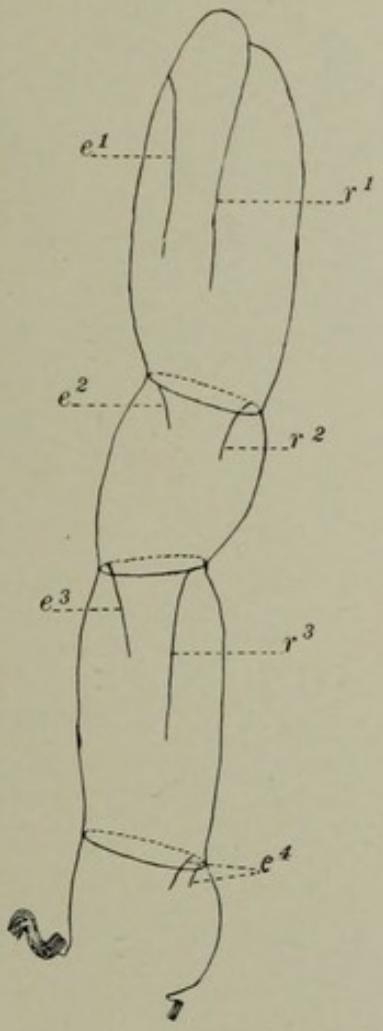

FIG. 36.

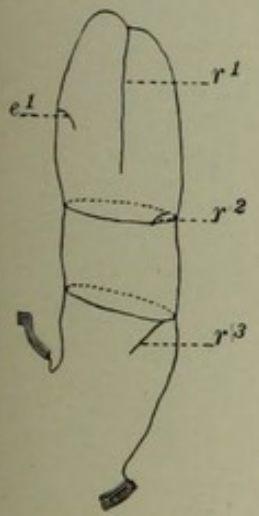

FIG. 33.

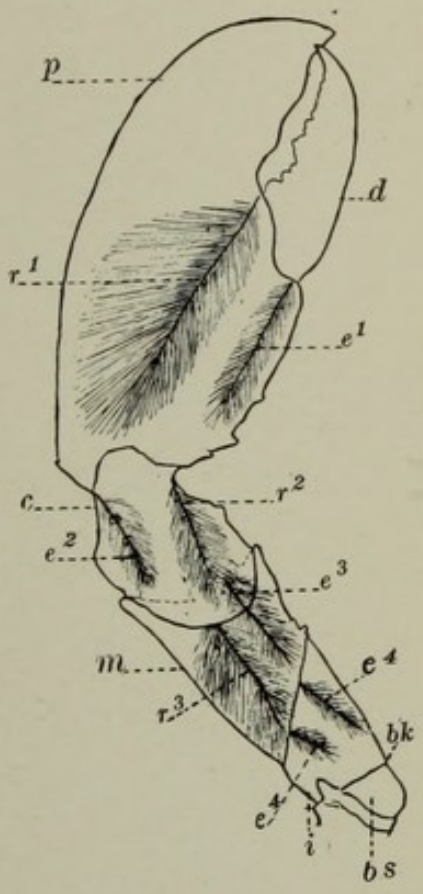

FIG 37.


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