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Resumen por el autor, Robert H. Bowen

Sobre ciertos rasgos de la espermatogénesis de los anfibios e insectos

Un estudio comparativo de las espermátidas de Plethodon, Lixus, Rhomaleum y Ceutophilus ha confirmado la opinión de que el acrosoma del espermatozoide se origina en relación con el aparato de Golgi. En Plethodon se ha demostrado que el aparato citado y el idiozoma, después de producir el acrosoma, son expulsados y pasan a la base de la cabeza del espermatozoide donde puede observarse durante algún tiempo. Nunca presenta una connexión orgánica con los centriolos situados en su vecindad, ni tampoco juega ningún papel en la formación del segmento intermedio del espermatozoide, conforme se ha supuesto. En Lixus y Ceutophilus el acrosoma se origina de un modo muy semejante al de los hemípteros. En el saltamontes Rhomaleum, por otra parte, los corpúsculos de Golgi de la espermátida nunca se fusionan para formar un solo acroblasto como sucede en las formas precedentes, sino que cada elemento de Golgi parece contribuir una porción individual y separada, formándose el acrosoma en virtud de la fusión de estas numerosas partes.

Translation by José F. Nonidez
Cornell Medical College, New York

AUTHOR'S ABSTRACT OF THIS PAPER ISSUED
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ON CERTAIN FEATURES OF SPERMATOGENESIS IN AMPHIBIA AND INSECTS

ROBERT H. BOWEN

Department of Zoology, Columbia University

TWO PLATES (FORTY-SIX FIGURES)

In a recent paper (Bowen, '20) on insect spermatogenesis I was able to demonstrate a relationship between the acrosome and the Golgi apparatus, and from this and facts previously published by other workers, I suggested that "The acrosome of the animal sperm is probably universally formed in connection with the Golgi apparatus, and has nothing to do with spindle fibers or other cell parts." At that time the evidence from other groups was very scanty indeed, the work of Sjoevall, Schitz, Gatenby, and Duesberg on various molluscs and mammals furnishing almost the only indications of the correctness of this suggestion. On the other hand, there were several well-known cases in which the current descriptions seemed to present serious difficulties, if not a direct contradiction. I decided, therefore, to examine some of these cases more carefully, together with material from other groups in which the Golgi apparatus had not yet been studied. In working up the material, a number of unexpected features were brought to light and it seemed of value to look more carefully into some of the cytoplasmic constituents which had not been accurately followed heretofore. This, however, called for the collection of much more material and further experimenting with fixation and staining—tasks which could not be immediately undertaken. I have accordingly decided to publish the major facts relating to acrosome formation, together with certain other features of interest in the spermatogenesis of several Amphibia and Insecta, leaving the details for a later study. A review of technical methods and acknowledgment of much assistance in the collection and identification of material will be for the present postponed.

AMPHIBIA

Accounts of the formation of the acrosome and the middle-piece in the urodele sperm have long presented a most puzzling contradiction. According to Meves ('97), in Salamandra, the acrosome ('Sphaerenblaeschen') arises by the fusion of many small, clear vesicles which appear in the idiosome ('Sphaerensubstanz') of the spermatid. The vesicular acrosome thus formed becomes the apical body of the sperm, while the remnant ('der kleine Rest') of the sphere substance disappears completely. The 'middle-piece,' on the other hand, is formed by the proximal centriole alone, which penetrates the nuclear membrane and thus comes to lie inside the nucleus. But McGregor ('99), who has made a similar study of Amphiuma, gives a quite different interpretation of these details. According to McGregor, the acrosome arises from the sphere (idiosome) in a manner essentially similar to that described by Meves, except that the remnant of the sphere does not disintegrate. Instead it migrates with the centrioles to the opposite pole of the nucleus, where it forms the major part of the middle-piece, in which the small, spherical proximal centriole becomes embedded. McGregor also describes the penetration of the middle-piece into the nucleus, both authors thus agreeing that the middle-piece is intranuclear. With respect to the fate of the sphere remnant (Golgi remnant of my nomenclature) and the origin of the middle-piece, there was thus a discrepancy which has never been cleared up. It was with the idea of ascertaining the true relations of the Golgi apparatus (plus idiosome), acrosome, and centrioles that this work was undertaken. The material for this study has been drawn from a single species, Plethodon cinereus Green, though I hope eventually to examine other urodeles in a comparative way.

The cytoplasmic structures so far made out in the primary spermatocytes are of at least three categories, the Golgi apparatus-idiosome complex, the mitochondria, and a number of darkly stained granules of doubtful nature, but possibly to be considered as chromatoid bodies. The mitochondria, present in rather limited amount, occur as more or less scattered rodlets of very small diameter, similar to those figured in Geotriton by

Terni ('14). The idiosome and Golgi apparatus have been often described by earlier workers. Together they form much the most conspicuous object in the spermatocytes. The idiosome appears as a more or less irregularly spherical mass, staining rather more darkly than the general cytoplasm. To its surface are applied a large number of small, separate Golgi rodlets, each shaped somewhat like a banana (fig. 1), and seemingly never interconnected to form a 'network.' These rodlets, now definitely shown to be Golgi elements, have of course been long known under a variety of names, e.g., the 'Archoplasmaschleifen' of Hermann, the 'Centralkapsel' and 'Pseudochromosomen' of Heidenhain, and the 'formazioni periidiozomiche' of Terni.

During the maturation divisions the idiosome goes to pieces and its exact behavior during the division period is unknown. The Golgi rodlets undergo a great loss in staining capacity, and appear to be simultaneously fragmented, so that with the usual methods they cannot be satisfactorily followed in the division stages. It is certain, however, that the Golgi pieces (dictyosomes) are collected about the poles of the spindle (fig. 2) as described in invertebrates by several workers, although the exact method by which this distribution is achieved has not yet been clearly made out. At the close of the first maturation division the idiosome is reconstituted with the Golgi rodlets applied to its surface as before (fig. 3). Similarly, in the spermatids the same structure is built up soon after the second maturation division is completed. In my preparations, however, the Golgi rodlets, as distinct elements, are soon lost, and the outer part of the acroblast (as the Golgi apparatus plus idiosome may now be called) tends to impregnate rather uniformly with the various Golgi methods (figs. 5 and 6).

The acroblast lies at first some little distance from the nucleus; but presently it moves over into contact with the nuclear membrane and a small clear vesicle makes its appearance on one side of the acroblast (fig. 5). This is the 'Sphaerenblaeschen' of Meves and the acrosome of McGregor. In general appearance it closely resembles the homologous structure which I have described in the hemipteran spermatid (Bowen, '20), and for which I have

adopted the term acrosome. The acrosome increases in size, becoming a large, clear vesicle, to the surface of which the remains of the acroblast are attached (figs. 6 and 7). The centrioles presently become conspicuous, being sharply stained with Fe-hematoxylin. They are always located in the neighborhood of the acroblast (figs. 6, 7, and 8); indeed, they seem often to be resting on its surface. The relation seems, however, to be purely a topographical one.

When the acrosome has reached a certain stage, it becomes applied closely to the nuclear membrane, and gradually the cell wall is drawn down over it, creating the impression that the acrosome has actually protruded through the cell wall (fig. 8). Throughout this period of fixation, the acroblast remains close to the acrosome (figs. 8 and 9), but the connection between the two has obviously become very loose. In fact, the acroblast or, as it may now be called, the Golgi remnant, becomes more or less spread out or broken up into separate masses (fig. 9), and in Fe-hematoxylin preparations is usually not to be made out at all. This loss in staining capacity is obviously the source of Meves' erroneous description of the disappearance of the 'Sphaerensubstanz.'

The Golgi remnant, together with the centrioles, lingers for a time in the immediate vicinity of the acrosome, and then both are shifted to the opposite pole of the nucleus establishing the future long axis of the sperm. Everything indicates that McGregor was correct in holding that the separation of acrosome and centrioles is due to the active migration of the centrioles, rather than of the acrosome, as Meves contended.

The spermatid nucleus meanwhile becomes very irregular in shape and then begins to draw out to form the head of the sperm with the acrosome at one end and the centrioles at the other (fig. 11). The Golgi remnant lies in the cytoplasm at the base of the head close to the centrioles (figs. 11 and 12), becoming oftentimes more or less broken up and diffuse. It is easily demonstrated by the special methods for the Golgi apparatus, but not by the usual Fe-hematoxylin techniques. The Golgi remnant remains in this same position for a long time; indeed,

long after the stage shown by McGregor in his figure 32, in which it has supposedly been incorporated with the proximal centriole to form the middle-piece of the sperm. Its ultimate fate is unknown, preparations of late stages in sperm formation not yet having been successfully impregnated. Meanwhile the proximal centriole (in particular) increases progressively in size and staining capacity (compare figs. 11, 12, and 13) and becomes applied very closely to the nuclear membrane. At first it is spherical, but as the head elongates and becomes narrower, the sphere begins to draw out (fig. 14) and it becomes eventually a long rod (fig. 19)—the (centrosomal) middle-piece of the sperm. In this process I have been quite unable to find any evidence of an actual penetration of the nuclear wall by the 'middle-piece,' as described by both Meves and McGregor. The centriole seems rather to be merely in close contact with the nucleus, as is the case in many other animal sperms.

With respect to the origin of the middle-piece, I am, therefore, in general agreement with Meves. The unusual topographical relation existing between the Golgi remnant and the centrioles was doubtless the cause of McGregor's error in the derivation of the middle-piece, coupled also with the poor staining conditions at this period. There is also a possibility that some large, darkly staining granules of doubtful relations, which occur in the spermatids, may have been confused by him as derivatives of the cast-off 'sphere.'

Finally, the conditions in the completed middle-piece described by McGregor remain to be explained. Since, according to this author, in the formation of the middle-piece the proximal centriole merely becomes embedded in a mass of sphere material, it should be possible to separate these two components by differential staining. This was in fact apparently accomplished (McGregor's fig. 32), the sphere material staining less heavily than the centriole. My own observations of the proximal centriole (so-called) suggest, however, a quite different interpretation of this appearance. As was noted by McGregor, the proximal centriole in the earlier spermatid stages often appears

bipartite or dumb-bell shaped,[1] the two parts being typically equal in size. Meves figures much the same thing, though he described the formation as a curved rod. This same division of the proximal centriole occurs in Plethodon (figs. 7 and 10), the separation of the two parts being sometimes very marked. To one of the halves the axial filament is attached. When the nucleus begins to draw out, this division of the proximal centriole is apparently lost in many cases—sometimes, no doubt, by the recombination or fusion of the two parts and at other times by the orientation of the centrioles. At the same time, the part to which the axial filament is attached increases very rapidly in size, while the other part remains relatively small and inconspicuous. However, with a little searching, examples can be found at any of the intermediate stages in which the two parts of the proximal centriole can be clearly separated (figs. 13, 14, and 15). That the two bodies in these cases are actually the equivalent of the apparently single proximal centrioles of other cases can be demonstrated by comparison of adjacent spermatids in which the parts can or cannot be separated (figs. 13 and 14). It seems probable, as Professor Wilson has suggested to me, that in McGregor's preparations the large centriole was extracted more than the smaller one, giving rise to the interpretation already noted. The

[1] It should be pointed out that the origin of this bipartite condition has not yet been adequately worked out. I have followed the usual custom in considering the two parts as formed by the division of the proximal centriole, my own preparations not permitting a detailed study of the very early spermatid centrioles. Doctor Wilson has, however, called my attention to the fact that generally in the differentiation of the sperm a ring arises from only a portion of the distal centriole, while the remainder later comes into close relation with the proximal centriole. It is possible that something of the same kind obtains in the case of the urodele. Thus we might consider the portion of the so-called 'proximal' centriole to which the axial filament is directly connected as the central part of the ring centriole, while the other portion would actually be the proximal centriole itself. This would explain the apparent transfer of the insertion of the axial filament from the distal to the proximal centriole as required by McGregor's description, and would fall in line with the usual accounts of sperm formation which derive the axial filament from the distal centriole. Further study of this possibility is contemplated. However, in view of the uncertainty attaching to these speculations, I have thought it best to follow the traditional accounts for the present purely as a matter of convenience in description.

variability in staining behavior of the centrioles in Plethodon leads me to look upon this as a very plausible explanation.

In the late stages this small centriole seems to fuse with the free end of the much elongated middle-piece proper, from one end of which the axial filament arises (compare fig. 15). The function of this small centriole is not yet understood, but a possible explanation has suggested itself, which I hope eventually to prove or disprove definitely. It will be recalled that the tail of the urodele sperm bears a long, undulating membrane, the base of which is inserted along the axial filament of the tail, while the free edge is marked by a definite filament somewhat smaller in size than the main tail filament. The axial filament proper undoubtedly arises from the large centrosomal middle-piece. The exact origin of the membrane filament has not, however, been satisfactorily explained. I would like to suggest, on the basis of observations already made, the possibility of the origin of this filament from the second, eventually much the smaller, of the original halves of the proximal (?) centriole. Unfortunately, this filament is so delicate that its free portion can be made out in its earlier stages only with some difficulty, while the insertion can never be satisfactorily followed. It frequently happens, however, that the developing sperms are cut obliquely through the base of the head, and in such sections one can sometimes see what appears to be a fine filament arising from the small centriole and passing backward through the ring centriole (figs. 16 and 17). (See also fig. 18, in which the ring was not included in the section.) Should further study bear out such an interpretation, the origin of the membrane filament would fall in line with the facts already known as to the origin in general of vibratile filaments. It is possible, nevertheless, that the small centriole plays a rôle in the late stages during the drawing out of the ring centriole; for I have constantly observed a small darkly staining mass at the base of the tail filament which seems to originate from the centrosomal middle-piece (fig. 19). This mass subsequently moves out along the filament toward the free end of the elongating ring centriole, but its fate and function are alike unknown.

I have also found in the spermatids a collection of granules which seem to be preserved best in the absence of acetic acid. During the formation of the acrosome these granules are arranged in a circle around the acroblast and lie close to the nuclear membrane. This arrangement is a constant and striking one (fig. 4). The nature and fate of these granules are not known. Possibly they are related to the chromatoid bodies of other animals. They may be related to the granules previously mentioned as occurring in the spermatocytes. A more critical study of these granules will be undertaken on new material.

Summary

1. The acrosome of the urodele sperm arises as in many other animals from the Golgi apparatus plus idiosome (acroblast), which is later cast off and remains for some time in the cytoplasm near the base of the sperm nucleus.

2. The middle-piece of the sperm is derived from the so-called proximal centriole, and is not, as McGregor claimed, "chiefly derived from *the remnant of the sphere.*"

3. The origin of the filament on the free edge of the undulating membrane of the sperm tail from one part of the originally bipartite proximal (?) centriole is suggested as a possibility.

COLEOPTERA

My observations on the Coleoptera are confined to a single species, Lixus concavus Lec., which was examined merely to check up the origin of the acrosome in the light of my previous work on Hemiptera. In this beetle, the Golgi apparatus is present in the spermatocytes in the form of numerous scattered Golgi bodies (fig. 27) similar in a general way to those which I have described in the pentatomids. I have not followed out the preliminary history of these Golgi bodies or their distribution in the maturation divisions. In the early spermatids, however, before the mitochondria have condensed to form the nebenkern, the Golgi bodies are easily demonstrated, scattered irregularly in the cytoplasm (fig. 28). They gradually draw together (fig. 29), and eventually fuse to form a single mass (fig. 30), the acro-

blast, exactly as in Hemiptera. This lies at first in the angle between nucleus and nebenkern, whence it moves to an anterior position (fig. 30). Later it appears again at the base of the head (figs. 31 and 34), so that it probably undergoes a migration similar to that which occurs in Hemiptera. The acroblast in this beetle impregnates in a very peculiar manner. Instead of forming a thimble-shaped mass with a heavily impregnated periphery as in the pentatomids (Bowen, '20), it is apparently shaped like a disc, only the periphery of which is blackened by osmic acid. That this is a ring, not an optical section of some solid surface, is easily demonstrated by viewing the acroblast from different angles (compare figs. 30 and 31).

From the acroblast the acrosome arises by a process of differentiation similar in all probability to that in the Hemiptera and Amphibia. In this case, however, the acrosome is so very small that it cannot be satisfactorily demonstrated until the time approaches for the casting off of the acroblast, when it can be made out as a small vesicle applied closely to the nuclear membrane (fig. 36). Then the acroblast separates from the acrosome proper (figs. 37 and 38), and passing rapidly back along the tail (fig. 39), remains visible as a conspicuous blackened ring for a considerable period. Its fate is doubtless similar to that in the Hemiptera. At the time the acroblast is cast off, the head of the sperm is usually bent rather sharply over, so that its major axis is nearly at right angles to that of the tail filament. It is accordingly difficult to say whether the acrosome is deposited directly in place and becomes subsequently anterior by the straightening out of the head; or whether there is an active migration of the acrosome to its definitive position, as in the pentatomids. Probably both factors play a part. At all events, the head eventually straightens out, and the acrosome can be made out as a minute body applied to its tip (fig. 40). Subsequently the head becomes much drawn out, as in other insect sperms.

I have also been able to make some observations on the nebenkern which form an interesting supplement to my recent discussion of this subject (Bowen, '22 b). In Lixus the nebenkern goes through a process of condensation quite similar to that

which I have described in Brochymena, and already outlined in part by Shaffer ('17) in Passalus, and Holmgren ('02) in Silpha. The chromophilic substance (Bowen, '22 b) seems to form a plate-work very like that in the Hemiptera, and I can find no trace whatever of a 'spireme' such as Gatenby ('18) claims to have demonstrated in Tenebrio. At all events, in the later stages of condensation, the chromophilic substance is arranged exactly as in the pentatomids, and cross-sections of the nebenkern (figs. 32 and 33*A* and *B*) could hardly be distinguished from similar sections of Brochymena. (Compare Bowen, '22 b, figs. 21*A* and *B*, also fig. 13.) In one respect, however, there is a fundamental difference in the behavior of the nebenkern. In the Hemiptera the chromophilic substance disappears completely and the nebenkern is divided into two parts during the initial stage in its drawing out; while in the Coleoptera the drawing out of the nebenkern has progressed much farther before these same results are attained. Thus, in figure 34, a stage in the final condensation of the chromophilic substance is shown, which is quite comparable to my figure 16 (Bowen, '22 b) of Brochymena; while in an adjacent spermatid (fig. 35) the chromophilic substance has completely (?) disappeared.

In the paper already referred to, I attempted to show that the division of the nebenkern always waits on the final disappearance of the chromophilic substance. The observations of Holmgren on Silpha indicated that this was the case in the beetles, but Shaffer's account of Passalus left the point somewhat in doubt. I have, therefore, reexamined this point more carefully in Lixus, and I find the facts exactly as described by me in the Hemiptera. Cross-sections of the nebenkern through the chromophilic substance and also above and below it demonstrate conclusively that the nebenkern is divided only in the region from which the chromophilic substance has been withdrawn (figs. 33*A* and *B*, cross-sections of the nebenkern at a stage approximately like fig. 34. Compare these cross-sections with figs. 21*A* and *B* from Brochymena, Bowen, '22 b).

The halves of the nebenkern ultimately spin out to form the tail sheaths so characteristic of insect sperms, and I can find no

evidence of a degeneration of the mitochondrial elements such as Holmgren described in Silpha. The central substance (Bowen, '22 b) was not demonstrable in my preparations, so that I have been unable to check Holmgren's account of this feature.

Summary

1. The acrosome arises in connection with the Golgi apparatus plus idiosome, in a manner similar to that described in several other animals.

2. The nebenkern passes through a series of condensation phenomena similar to those of the Hemiptera, and is completely divided only with the disappearance of the chromophilic substance.

ORTHOPTERA

The development of the acrosome in various groups of Orthoptera has been studied by many workers with very discordant results, a short résumé of which I have given in another place (Bowen, '22 a). Most of these cases, however, have been easily interpreted on the basis of conditions found in the Hemiptera, but in the case of the grasshopper the facts are as yet by no means clear. It was with the hope of clearing up the whole problem of the acrosome in Orthoptera that this study was undertaken. Thus far I have had opportunity to examine only a few species from the families Acrididae and Tettigoniidae.

Family Acrididae

Although the grasshoppers have been studied by many workers there has been a uniform failure to make out any details whatever concerning the acrosome. Indeed, Buchner seems to have been the only one who even noted its occurrence. According to his latest account (Buchner, '15), the acrosome is derived from the 'proximal' centriole by a process of division—an origin which is obviously quite different from that to be expected on the basis of my hypothesis. As a matter of fact, Buchner failed to make out most of the essential stages, due to the fact that the cyto-

plasmic elements in the grasshopper seem to be most difficult to preserve and stain satisfactorily. My observations thus far are based on two species, Rhomaleum micropterum Beauv., and Dissosteira carolina Linn.

The Golgi apparatus in the grasshoppers is present in the primary spermatocytes in the form of many scattered Golgi bodies not essentially unlike those which I have described in the Hemiptera. The division stages have not yet been worked out, but at the close of the second maturation division the Golgi elements can be demonstrated clearly, being scattered about in the cytoplasm around the nucleus and mitochondria (fig. 20). The latter consist of a group of rather imperfect threads which have been derived from the second maturation division in a manner somewhat similar to that noted in Hemiptera. These threads soon condense to form the typical spherical nebenkern of insects.

The separate Golgi bodies are not easily demonstrated with clearness, but it is certain, especially from later stages, that each one is made up of two substances, one of which is more readily stained than the other which it partially encloses. (Compare with the Hemiptera which I have described fully (Bowen, '22 a).) In one respect, however, the Golgi bodies differ in behavior very decidedly from other cases which have been described. They remain separate for the most part as distinct bodies (figs. 23 and 24), and never fuse to form the massive acroblast so characteristic of the animal spermatid. Occasionally, it would appear, two or three fuse together to form a larger aggregate, but in general this does not occur. These Golgi bodies tend now to collect near the nuclear membrane, particularly on one side of the nebenkern (figs. 21 and 23), and they remain, generally speaking, in this vicinity until the nebenkern begins to elongate (fig. 24). Then they begin to migrate back along the tail (fig. 25), and are probably cast out in the protoplasmic mass sloughed off the tail at the close of sperm formation.

In the later spermatid stages and especially just prior to the backward migration of the Golgi bodies, an intensely staining globule can be made out in contact with the nuclear membrane not far from the centriole (figs. 23 and 24). This is at first difficult to

distinguish with certainty, but it seems to increase in size, and when the Golgi bodies clear away from the nucleus it is a very conspicuous object. This globule is the acrosome. It now migrates around to the opposite side of the head (nucleus) (fig. 25), always retaining its close contact with the nuclear wall. As it moves it becomes differentiated into a basal, plate-like portion which rests on the nucleus, and a small knob directed toward the cell wall (fig. 25), the whole reminding one of a collar-button. As the head begins to elongate in the manner characteristic of the insect sperm, the basal portion of the acrosome becomes drawn out into two rod-like lugs which extend back over the surface of the nucleus for a short distance and apparently serve as a means of anchorage for the acrosome (fig. 26). The distal portion becomes drawn out as a little ball on the end of a short rodlet. Very frequently this can be divided into two—a duplex condition which is possibly the characteristic one, though visible only when the sperm head is favorably turned. As the head draws out, the acrosome retains, for a time, this rod-and-ball structure; its ultimate history has not been studied.

Unfortunately, the exact method by which the acrosome is produced has not been made out. The separate Golgi bodies are themselves so small that in my preparations I was not able to follow the history of each individual. It seems probable, however, that from each one is differentiated its small proportionate share, and by the deposition of many such parts the acrosome is gradually built up. Instead of the Golgi bodies' fusing to form a single acroblast from which the acrosome is differentiated in toto as in most animals, each Golgi body is an acroblast in itself and the acrosome arises as a fusion product of the portions contributed by each such 'acroblast.' This all fits in with the facts which I have made out in the Hemiptera. In the pentatomids I was able to show that occasionally the Golgi elements fail to fuse on time, and in such cases each partial acroblast produces its own acrosome of a size proportionate to the available material (Bowen, '22 a, fig. 54). Thus, what may be an occasional accident in the bug becomes in the grasshopper the customary procedure.

In addition to these observations on the acrosome, I have been able also to add some facts corroborative of the nebenkern history which I have described in the Hemiptera (Bowen, '22 b). Thus I have found that the nebenkern passes through a process of differentiation, which, in the beginning at least, is strikingly like that in Brochymena, and I have been unable to make out the thread-like formation figured by Giglio-Tos and Granata ('08). As in the Hemiptera, the chromophilic substance gradually condenses and eventually disappears entirely, figure 21 being a cross-section of the nebenkern just before its final disappearance. Following this, the nebenkern divides. The condensation of the chromophilic substance in the grasshopper nebenkern takes place so rapidly that the nebenkern divides some time before it begins to elongate, the two halves rounding up in a very characteristic manner (fig. 23). Another case is thus added to those already described, which show that the complete division of the nebenkern follows immediately upon the disappearance of the chromophilic substance. With respect to the condition of the chromophilic substance at the time the nebenkern begins to elongate, an interesting series is thus formed by the grasshopper, the bug, and the beetle (and possibly the butterfly).

Finally, in Cajal preparations, I was able to demonstrate in the nebenkern (as in the Hemiptera) the occurrence of a material which I have called the 'central substance.' This stuff in the grasshopper nebenkern is by no means as regular in its arrangement as in Euschistus, for example. Instead, it forms a very indefinite mixture of granular (and thread-like?) elements which combine to make an exceedingly complicated picture (fig. 22). When the nebenkern halves elongate this central substance is drawn out also, and seems eventually to form a delicate thread-like core for the mitochrondrial tail sheaths. The vesicles which I have described on the tail sheaths in Hemiptera appear likewise in the grasshoppers, but are by no means so conspicuous. Their general history seems not unlike that in the Hemiptera.

FAMILY TETTIGONIIDAE

In contrast to the unusual method of acrosome formation found in the grasshoppers, among many of their relatives the process would appear to be a very simple one, quite like that in the Hemiptera. Thus far I have examined carefully only Ceuthophilus maculatus Harris, but the condition in this form is probably similar to that in other crickets and the locustids, where the published accounts are very unsatisfactory indeed (see Bowen, '22 a, for a short résumé). In Ceuthophilus I have not yet obtained satisfactory Golgi preparations of the spermatocytes and earliest spermatid stages. However, during the early steps in the condensation of the chromophilic substance, the acroblast, in the form of a single compact sphere, appears in the cytoplasm in its accustomed location in the angle between nucleus and nebenkern (fig. 41), indicating an origin from the fusion of scattered Golgi elements as in the beetle and the bug.

The differentiation of the acrosome begins immediately, and a small vesicle soon appears in connection with the acroblast quite as in the Hemiptera (fig. 42). The vesicle is sometimes clear and transparent (fig. 43), but very frequently it takes the stain intensely (fig. 42), by which it is rendered more conspicuous. I have noticed a similar tendency in Murgantia after certain staining technique. The position of the acroblast-acrosome complex with reference to the nucleus is not constant as in the Hemiptera, and the acrosome often does not touch the nuclear wall at all.

Shortly before the nucleus begins to elongate to form the sperm head, the acrosomal vesicle becomes applied to the anterior surface of the nucleus (fig. 43), the acroblast still maintaining its original attachment to the acrosome. Presently the acrosome flattens out on the surface of the nuclear membrane (fig. 44), and its connection with the acroblast is severed (fig. 45). The latter quickly migrates to the base of the head (fig. 46) and thence moves backward along the tail exactly as in other insects, in all of which its ultimate fate is probably the same. The acrosome, already in its definitive position, rounds out to form a

knob-like apical piece (figs. 45 and 46), the further history of which has not been studied.

Aside from the mitochondria, the spermatids of Ceuthophilus contain two cytoplasmic components of interest. The first of these appears in the early spermatids as a small flocculent mass, more or less granular in texture and blackening with osmic acid (modified Kopsch method) (fig. 41). Later this mass seems to move backward along the tail (fig. 42), but it no longer impregnates clearly and cannot be followed satisfactorily. It seems to correspond to the granular mass of similar behavior which I have described in the Hemiptera under the name of spermatid remnant (Bowen, '22 a). In Ceuthophilus, however, this material seems to be traceable at least back to the maturation divisions, and a more complete study of its history and significance is now being attempted.

The other cytoplasmic component referred to above is a body apparently homologous with the 'formation juxta-nucléaire' described in Gryllotalpa by Voinov ('16). According to this author, the late primary spermatocytes contain four of these bodies which are distributed to the spermatids by a regular division process, each spermatid always receiving one juxtanuclear body. The last contention seems to hold true in Ceuthophilus, but I have been unable thus far to substantiate the earlier history as outlined by Voinov, and in my preparations this body first becomes clearly demonstrable in the differentiating spermatids. In shape, Voinov describes it as a flattened, oval body the periphery of which stains very darkly. In Ceuthophilus also it has the same disc-like shape, but whether the whole periphery stains heavily (after Benda and various Fe-hematoxylin methods) or only a portion of it, giving rise to a crescentic appearance, is not certain (figs. 43 and 45). It is clear from my figures, and in agreement with Voinov, that the juxtanuclear body has nothing to do with the formation of the acrosome. It finally passes out along the tail together with the Golgi remnant, with which it probably shares a similar fate. As to the nature of this body, nothing definite can be stated. Its fate recalls that of the chromatoid body, but in other respects there is little ground for com-

parison. Possibly it is related to the micro-mitosome described by Gatenby ('17) in Lepidoptera. Although we lack any definite knowledge as to what this juxtanuclear body may be, attention may nevertheless be called to the fact that it seems to be in no way related to the Golgi apparatus. The suggestion of Duesberg ('20) (based on Voinov's account), that these bodies represent Golgi elements, is, therefore, almost certainly incorrect.

Finally, a word may be added concerning the mitochondria in the spermatid, especially in view of the wide divergence of Vejdovský's account (in a related genus, Diestrammena) from more recent work on other insects. In the first place, the development of the vacuoles on the periphery of the nebenkern (fig. 41), as described by Vejdovský, is obviously the first step in the condensation of the chromophilic substance as already noted in the Hemiptera, etc. (Bowen, '22). The chromophilic substance gradually condenses (fig. 42), though always in a somewhat irregular way, and eventually disappears during the early stages in the elongation of the nebenkern. The exact nature of the 'pattern' which it forms is not clear in my preparations.

The central substance, correctly described by Vejdovský in the intermediate stages (see his figures 182, 183, and 184), seems not to arise from the chromophilic material as he believed, but to be differentiated de novo in the unstained area of the nebenkern, as I have described in the pentatomids and the grasshopper.

The formation of vesicles on the mitochondrial tail sheaths is very striking in Ceuthophilus, and due to the general failure of the chromophobic substance to stain, it is not easy to follow their differentiation. Hence the error of Vejdovský in describing them as fatty degeneration products of the central substance, and I can find no support whatever for his assertion that the mitochondrial structures are entirely lost from the sperm. The vesicles in Ceuthophilus are, it is true, very confusing if one has not become familiar with them in more typical cases, for the intervening portions of the sheaths are often not demonstrated (fig. 43), and one would be readily misled as to the actual meaning of the vesicles themselves. Somewhat later stages leave little doubt that the sheaths actually spin out along the tail filament

as in Hemiptera (Bowen, '22 a, fig. 132). During the early development of the tail vesicles the central substance can be demonstrated with Fe-hematoxylin and it can be seen to retain its threadlike distribution even in the region of the vesicles. It would accordingly seem that the central substance cannot play the rôle in the development of the vesicles which the conditions in Hemiptera led me to suggest as a possibility (Bowen, '22 b).

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EXPLANATION OF PLATES

All of the figures have been outlined as far as possible with the camera lucida, those of plate 1 at an initial enlargement of approximately 2875 diameters, those of plate 2 at approximately 3800 diameters. At so great enlargements it has, of course, been necessary to correct the outlines extensively and to add much of the finer detail free hand. In reproducing, the figures have been reduced uniformly, those of plate 1 to an enlargement of 2300 diameters, those of plate 2 to 3350 diameters. In every case the method employed in the preparation of the original object has been indicated.

ABBREVIATIONS

A, acrosome
a, acroblast
G, Golgi remnant
j, juxtanuclear body
n, nucleus
s, spermatid remnant

PLATE 1

EXPLANATION OF FIGURES

Figures 1 to 19 are from Plethodon cinereus; figures 20 and 22 are from Dissosteira carolina; the others are from Rhomaleum micropterum.

1 Late primary spermatocyte. (Flemming without acetic-hematoxylin.)

2 Metaphase first spermatocyte division. (Cajal's formol-uranium nitrate.)

3 Secondary spermatocyte. (Flemming without acetic-hematoxylin.)

4 Spermatid nucleus showing group of granules encircling the acroblast. (Cajal-gold chloride-hematoxylin.)

5 Spermatid. Early formation of acrosome from acroblast. (Modified Kopsch.)

6 Spermatid. Later stage in formation of acrosome. (Cajal-gold chloride–hematoxylin.)

7 Spermatid. Final stage in formation of acrosome. (Cajal-gold chloride-hematoxylin.)

8 Spermatid. Fixation of acrosome. (Champy-hematoxylin.)

9 Spermatid. Casting off of the acroblast (Golgi remnant). (Cajal.)

10 Spermatid. Showing proximal (?) centriole divided into two equal parts. (Champy-hematoxylin.)

11 and 12 Spermatids. Progressive stages in the elongation of the nucleus. Figure 12 shows only a portion of the nucleus. (Cajal-gold chloride-hematoxylin.)

13 and 14 Basal end of two adjacent spermatid heads in successive stages of differentiation, to show separation and fusion of the components of the proximal (?) centriole. (Champy-hematoxylin.)

15 Basal end of spermatid nucleus, to show continued separation of the components of the proximal (?) centriole. (Champy-hematoxylin.)

16 to 18 Oblique sections through the basal end of the spermatid head, to show possible origin of the membrane filament. (Champy-hematoxylin.)

19 Late stage in the differentiation of the spermatid. Elongation of the ring centriole. (Champy-hematoxylin.)

20 Second maturation division. Late telophase. (Cajal.)

21 Cross-section through the nebenkern just prior to the complete disappearance of the chromophilic substance. (Flemming without acetic-hematoxylin.)

22 Spermatid. Development of the central substance in the nebenkern. (Cajal.)

23 and 24 Spermatids. Stages in the development of the acrosome. (Flemming without acetic-hematoxylin.)

25 and 26 Spermatids. Later stages in the transformation of the acrosome. (Flemming without acetic-hematoxylin.)

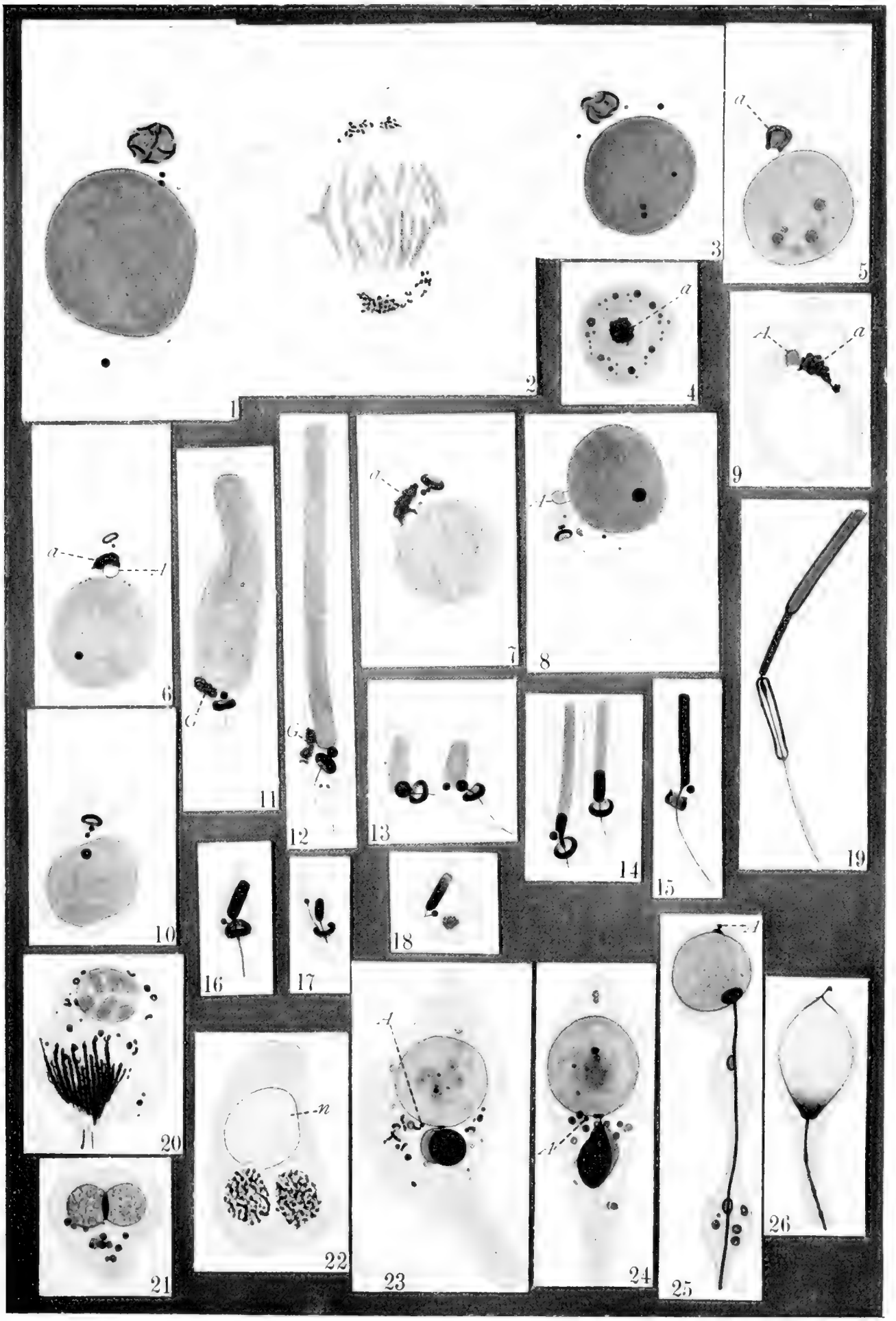
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PLATE 2

EXPLANATION OF FIGURES

Figures 27 to 40 are from Lixus concavus; figures 41 to 46 are from Ceuthophilus maculatus.

27 Spermatocyte (or late spermatogonium). (Modified Kopsch.)

28 to 30 Spermatids. Successive stages in the fusion of Golgi bodies to form the acroblast. (Modified Kopsch.)

31 Spermatid. (Modified Kopsch.)

32 Cross-section of slightly elongated nebenkern. (Benda.)

33 Cross-sections of the nebenkern at approximately the stage of figure 34. *A*, through the chromophilic substance; *B*, above (or below) the chromophilic substance. (Benda.)

34 and 35 Spermatids, just prior to, and at the time of, disappearance of the chromophilic substance. (Flemming-hematoxylin.)

36 to 38 Late spermatids. Casting off of the acroblast. (Benda.)

39 Late spermatid. Golgi remnant passing backward along the tail. (Modified Kopsch.)

40 Spermatid, showing acrosome in final position. (Benda.)

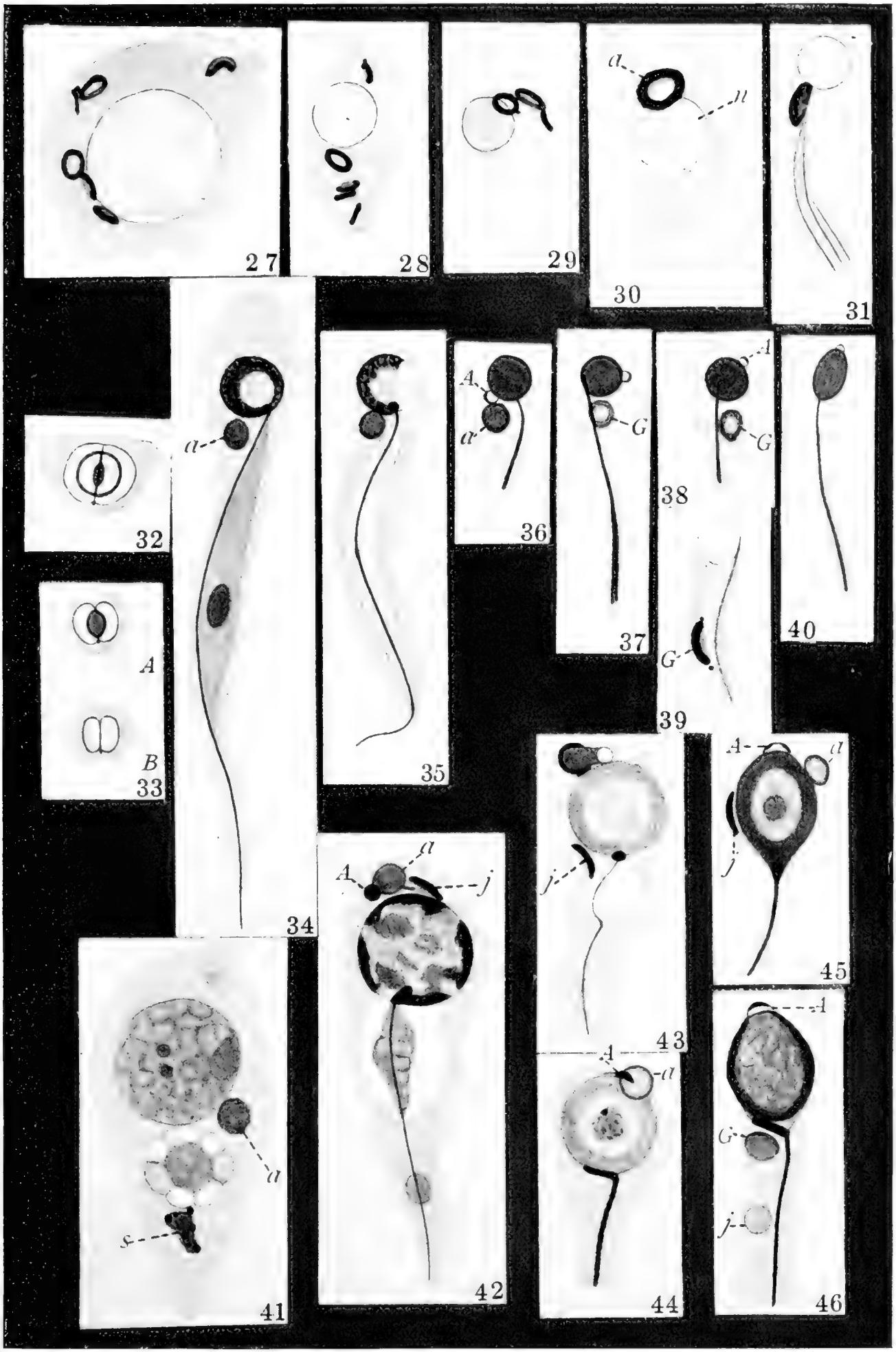
41 Early spermatid. (Modified Kopsch.)

42 and 43 Spermatids. Development of the acrosome. (Flemming without acetic-hematoxylin.)

44 Spermatid. Fixation of the acrosome. (Benda.)

45 Spermatid. Casting off of the acroblast. (Benda.)

46 Spermatid. Backward migration of the Golgi remnant and the juxtanuclear body. (Flemming without acetic-hematoxylin.)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Resumen por el autor, E. V. Cowdry

El material reticular como indicador de la reversión fisiológica de la polaridad secretora de las células de la tiroides del conejillo de indias

En las glándulas tiroideas de los conejillos de indias normales el material reticular no se encuentra invariablemente entre los núcleos y la cavidad de los folículos, como se ha supuesto generalmente, sino que en algunos casos experimenta una activa emigración hacia el polo opuesto de la célula, la cual como demuestran otras pruebas, indica la existencia de una reversión de la polaridad fisiológica mediante la cual la secreción pasa directamente a la circulación en vez de acumularse primero en los folículos.

Translation by José F. Nonidez
Cornell Medical College, New York

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THE RETICULAR MATERIAL AS AN INDICATOR OF PHYSIOLOGIC REVERSAL IN SECRETORY POLARITY IN THE THYROID CELLS OF THE GUINEA-PIG

E. V. COWDRY

Anatomical Laboratory of the Peking Union Medical College and the Laboratories of the Rockefeller Institute for Medical Research, New York

TWO PLATES (FOURTEEN FIGURES)

It can easily be observed by the application of Da Fano's ('20, p. 157) modification of Cajal's silver-impregnation method to the thyroid glands of guinea-pigs that the reticular material is not always restricted to the zone of cytoplasm between the nucleus and the follicular cavity, as has been supposed by all previous workers (Negri, '00, p. 61; '00 a, p. 178; Holmgren, '01, p. 314, and Kolster, '13, p. 128); but is found, in about one cell in every five hundred, in the opposite pole near the peripheral blood vessels. Detailed reviews of the literature relating to the material are given by Duesberg ('14) and Cajal ('14).

A group of follicular cells is reproduced in figure 1 to illustrate the average position assumed by the blackened reticular material between the nucleus and the lumen. Reversal may take place in single isolated cells, as shown at (*A*) in figure 2, or in large or small groups of cells, as is illustrated in figures 3 and 4. On the other hand, the reticular material may be found to be unusually close to the lumen, as shown in figures 6 and 7. In very rare cases, in which the epithelium is several layers in thickness, the reticular material is extremely variable in position (fig. 8) and no polarity can be distinguished.

In the acinus cells of the pancreas of the guinea-pig (fig. 9) it is wholly different, because here the reticular material is, as far as I can ascertain, invariably located between the nucleus and the discharging pole of the cell, with strands sometimes extending up on either side of the nucleus, and has been recorded in this

position by all investigators (Negri, '00; v. Bergen, '04, p. 533; Bensley, '11, p. 364; Kolster, '13; Cajal, '14, p. 169, and others). The same generalization also holds for the salivary glands and, to the best of my knowledge, for all glands in which the secretion invariably passes in the same direction, at any rate under normal conditions.

By means of a new method, Bensley ('16, p. 48) has shown that the thyroid is peculiar in another respect by confirming the suspicion, which physiologists have for some time entertained, that the secretion may change its direction and pass immediately into the peripheral blood vessels without first being stored in the follicular cavity. The technique consists of staining with Brasilin in phosphotungstic acid after formalin-Zenker fixation and of counterstaining with wasserblau in phosphomolybdic acid. This brings to light the true secretion antecedents in the form of tiny vacuoles which contain a dilute solution similar in its properties to the colloid of the follicular lumen, but less concentrated. Since the droplets always occur in the outer poles of the cells, he concludes that the secretion "is destined to direct transport into the vascular channels, and that the thyroid cell presents a true reversal of polarity in accord with its endocrine function." He believes that only when "the rate of secretion is in excess of body needs, the indirect mode of secretion comes in, and the product of secretion is condensed and stored in the intrafollicular cavity." By way of further evidence, he calls to mind the fact that in exocrine glands fat droplets "practically always make their appearance in the anti-secretory pole of the cell." In the thyroid they are confined to the end of the cell next to the lumen, which would indicate that the secretion is discharged from the opposite pole, remote from the lumen, in accordance with his hypothesis. Still other evidence may be cited.

Norris's ('18, p. 462) suggestion that Bensley's hypothesis of a reversal is unnecessary because the interfollicular spaces may represent the primitive lumina, so that the secretion has always passed in this direction, is not substantiated. The presence of the reticular material in so many cells between the nucleus and the follicular lumen, as I have described it in the guinea-pig or

as Cajal ('14, p. 215) records it in other gland cells, between the nucleus and the polo mundial, shows that the follicular cavities are to be considered from the phylogenetic point of view as being at one time in communication with the exterior (outside world). If further proof is needed that these spaces represent the lumen or duct of the ancestral gland, it is offered by the observation (Cowdry, '21), that the cells bordering them are uniformly flagellated in the dogfish.

We have, then, a remarkable coincidence. Apparently in the thyroid gland alone is there variability in the position of the reticular material, and in the thyroid gland alone have we clear evidence of physiologic reversal in the direction of secretion. In my opinion, the two phenomena are related. Since the position of the reticular material, like that of the centrosome, is a clue to the polarity of the cell, preparations made by Da Fano's method will indicate the functional polarity, or, in other words, the direction in which secretion is taking place at the moment the preparations are made. This cannot be so safely inferred from a consideration of the height of the epithelium and the general appearance of the follicles as seen in routine hematoxylin and eosin preparations. Neither is the amount of colloid to be considered as a reliable clue, since it may have been produced during a storage phase which preceded the examination of the gland by a considerable interval of time.

The relatively small percentage of reversals in the position of the reticular in the guinea-pigs which I have examined suggests that the balance in production of secretion is in favor of storage rather than of immediate discharge. Examination by Bensley's method, which is probably more delicate since it is also more direct, would probably have failed to reveal any considerable amount of secretion antecedent near the perifollicular net. In the opossums, which he studied, in which the discharge into the perifollicular spaces is probably more marked, I would predict that the reticular material is often to be found between the nucleus and the discharging pole. Judging from his figures, it would appear that the nuclei have been forced toward the lumen by the accumulation of secretion antecedents near the periphery.

My contention regarding oscillation in secretion in the thyroid receives some support from a study of the parathyroid glands in which the reticular material has only thus far been recorded by Kolmer ('17, p. 272).[1] He illustrates it in the form of a rather dense and well-circumscribed network of a ring-like shape. Since he finds that it is located at one pole of the nucleus (as in columnar epithelia), he concludes that the cells possess two poles and are cylindrical in shape, not roughly cubical as is generally supposed to be the case, and, further, that they are disposed in rows or end to end. My preparations of the parathyroid glands of guinea-pigs reveal a blackened network which is usually somewhat more diffuse (figs. 10, 11, and 12). Quite frequently it is circumnuclear in position, as is illustrated in one cell in figure 12. But when the cells become grouped, the reticular material comes to occupy a position between the nucleus and what may be considered to be the discharging pole. This condition is evident in rare cases when the cells form follicle-like cavities, as shown in particular by figure 13. The arrangement of cells in figure 12 is also suggestive. From this we may conclude that here also the reticular material acts as an indicator of polarity assumed under normal but unknown conditions.

Special interest attaches to the parathyroid glands because their secretion is active, but unknown, and because true secretion antecedents remain to be discovered within their cells. Perhaps they may be more easily detected in these cells which assume a certain measure of polarity, though it would be unsafe to say that cells not definitely grouped are any less active physiologically. The same amount of secretion antecedent in a polarized cell is condensed into a relatively small area of the cytoplasm, whereas in an unpolarized cell it is presumably spread throughout a much larger extent of peripheral cytoplasm for transport in all directions. Just as in the thyroid, the first clues will probably be found in hyperactive glands (if such can be produced). But we have to remember that under normal conditions the secretion antecedents may be present in such minute amounts or may be

[1] Kolmer ('16, p. 507) also mentions the reticular material in the thyroid gland, but does not describe its position within the cell.

discharged so rapidly from the cells that they escape observation. It is conceivable that the creation of passive congestion by ligating the venous drainage may cause a retention of secretion in appreciable amounts.

With this idea of the reticular material as an indicator of secretory polarity in mind, I think that a careful study of the hypophysis may indicate into which channels the secretion is actually being discharged at the time the preparations are made. The occurrence of this material in the cells of the anterior lobe has already been recorded by Gemelli ('00) and by Tello ('12). More recently Addison ('17, p. 448), attempting to confirm and extend Gemelli's work, has described a close-meshed reticulum which is much larger in the basophiles than in the acidophiles. He is so reticent regarding its nature that he prefers to call it the "macula, until further information is forthcoming." My preliminary studies with the guinea-pig have failed to establish anything approaching an orderly arrangement (fig. 14). In order to obtain decisive results, the silver preparations will have to be combined with vascular injections, and will have to be reconstructed in serial sections, because the relations of the cells are so intricate.

Cajal ('14, p. 214) devotes several pages to a general consideration of the phenomena of polarization of the Golgi apparatus (or reticular material), mentioning particularly cases of ontogenetic reversal in its position in nerve cells. It is interesting to note also that Tello's ('13, p. 145) investigations on mammary-gland carcinomata bring to light a progressive depolarization in its position as the tissue assumes the characteristics of a neoplasm.

Two interesting cases of experimental reversal are described in the stomach and in the kidney which show the sensitivity of the reversal mechanism.

D'Agata ('10, p. 518) found that the position of the reticular material in the gastric cells is reversed sixteen hours after operating on the stomach of triton. His figures show clearly that the material has taken up a position between the nucleus and the proximal border of the cell; that is to say, it is now in the pole of the cell which is generally considered to be the antisecretory. If

our hypothesis is correct, this reversal would mean the temporary passage of material from the lumen in the direction of the peripheral blood vessels. Absorption from the lumen, which normally takes place to some extent, is probably accelerated as a result of the operation. We would not expect to be able to induce so simply experimental reversal in the position of the reticular material in the acinus cells of the pancreas or in the salivary glands, because absorption, with the accompanying tendency to the production of a current in an unusual direction, is in them at a minimum.

Basile ('14, p. 3) discovered that unilateral nephrectomy produces definite changes, possibly compensatory in nature, in the reticular material of the remaining kidney. From its normal position between the nucleus and the lumen it migrates, in both the tubuli contorti and recti, to a new position between the nucleus and the base of the cell. This would, in my judgment, indicate the predominance of absorption over excretion and would seem to pave the way for a study of the physiology of the kidney from a new angle.

But we are laboring more or less blindly because so little is known regarding the true nature of the reticular material. The chief difficulty is that we have thus far been unable to study it directly in living cells. It is a simple matter to give a long list of chemicals which destroy it during fixation, and to enumerate stains, like resorcin-fuchsin, which have been known in rare instances to color it, without mentioning any positive reaction of value. By virtue of its affinity for silver (methods of Golgi and Cajal) and of the fact that it blackens after prolonged treatment with osmic acid (method of Kopsch), it may be studied closely in fixed tissues. Though both of these methods are more or less unsatisfactory, the results which they yield are always mutually confirmatory; so that one is justified in concluding that by their characteristic morphology and location, the blackened networks reveal the existence of some unknown and hitherto unrecognized material in the living cell. This conclusion is supported by the further observation that fixation in several fluids, such as Zenker, under certain conditions, and a mixture of formalin, potassium

bichromate, and mercuric chloride, as recommended by Bensley ('10, p. 192), brings to light, in most tissues, a system of clear canals which bear a very close resemblance in shape and position to the blackened networks (compare figs. 5 and 1); thus there are in reality three methods, two positive and one negative, which yield the same results.

We infer that this unknown reticular material is of considerable physiologic importance in the living cell; because our methods show that the blackened networks (or clear canals) are very widely distributed in animal cells and that, in some cases, they undergo characteristic and progressive alterations during development and in different physiologic and pathologic conditions.

We also infer that the reticular material is fluid in consistency because there is no indication of the existence of a rigid framework in follicular cells teased out in salt solution, and the distribution and movement of cytoplasmic granules, like mitochondria do not seem to be restricted. Centrifuging fragments of thyroid for upwards of forty-five minutes at 3,500 revolutions per minute does not displace it, so that its specific gravity cannot differ greatly from the remainder of the cytoplasm. Further evidence regarding its fluidity I have presented elsewhere ('21, p. 7).

It has been suggested that in other glands, like the pancreas, the reticular material plays a definite part in secretion. Saguchi ('20, p. 393) is willing to go so far as to say that "the intracellular network consists of secreting material which is to be extruded either directly into the lumen or indirectly into the intercellular capillary." This is difficult to deny in the thyroid or elsewhere because the entire cytoplasm, to say nothing of the nucleus, is either directly or indirectly involved. If our inference regarding its fluidity is correct, many chemical reactions of diverse character may take place in it and along its changing surfaces. It is probably quite heterogeneous in composition and one of the most active areas of the cytoplasm.

SUMMARY

In the thyroid glands of normal guinea-pigs the reticular material is not invariably found between the nuclei and the follicular lumen, as is generally supposed, but in some cases undergoes an active migration to the opposite pole of the cell, which, together with other evidence at hand, indicates the existence of a reversal in physiologic polarity whereby the secretion is discharged directly into the circulation, instead of being first stored within the follicles.

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DESCRIPTION OF FIGURES

All the drawings have been made with a 1.5-mm. Zeiss apochromatic objective, no. 10 Spencer ocular and camera lucida, and are reproduced without reduction.

PLATE 1

EXPLANATION OF FIGURES

1 Group of follicular cells showing the usual form and position of the reticular material from a preparation made by Cajal's uranium and silver nitrate method, which blackens it.

2 Follicular cells prepared by Da Fano's cobalt nitrate silver method. At (*A*) one large cell presents an apparent reversal in polarity, since the blackened reticular material is on the opposite side of the nucleus remote from the follicular cavity.

3 A row of cells in all of which the position of the reticular material is reversed, from a preparation made by the same method.

4 Follicular cells showing different degrees of reversal in position as one passes from left to right, from another guinea-pig treated by the same method.

5 Follicular cells fixed in Zenker's fluid and stained with iron hematoxylin, showing a system of clear canals in the same position as the blackened material illustrated in figure 1.

6 Cells prepared by Da Fano's cobalt nitrate silver method, showing reticular material which has moved up unusually close to the follicular cavity and which exhibits vesicle-like enlargements. Compare this with its average position and form illustrated in figure 1.

7 The same, without the swellings.

8 Three layers of epithelial cells bordering the follicular lumen and showing no sign of polarity as indicated by the variable location of the blackened reticular material, same method.

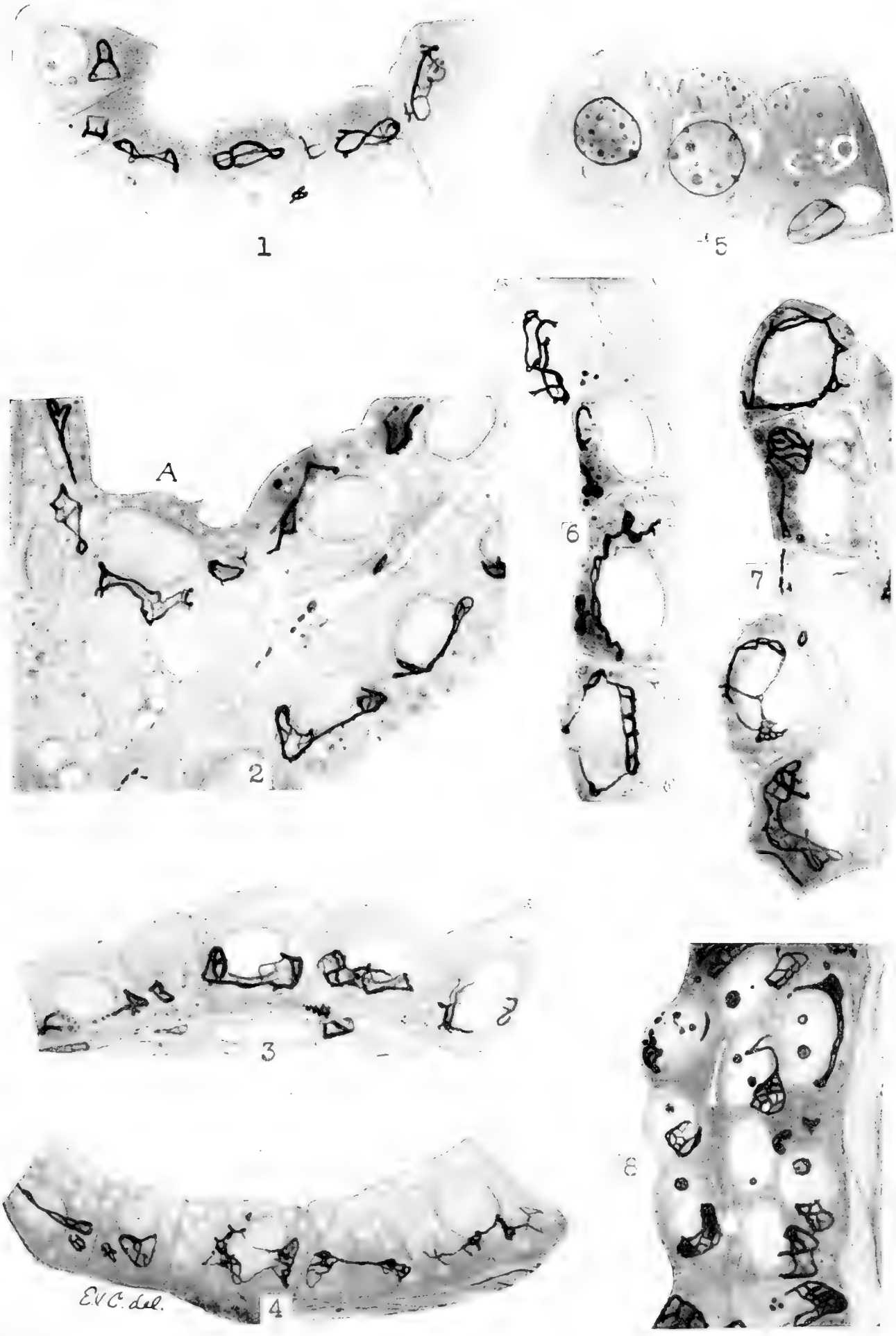
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PLATE 2

EXPLANATION OF FIGURES

9 A pancreatic acinus showing perfectly definite secretory polarity with the blackened reticular material placed in each cell between the nucleus and the lumen, same method.

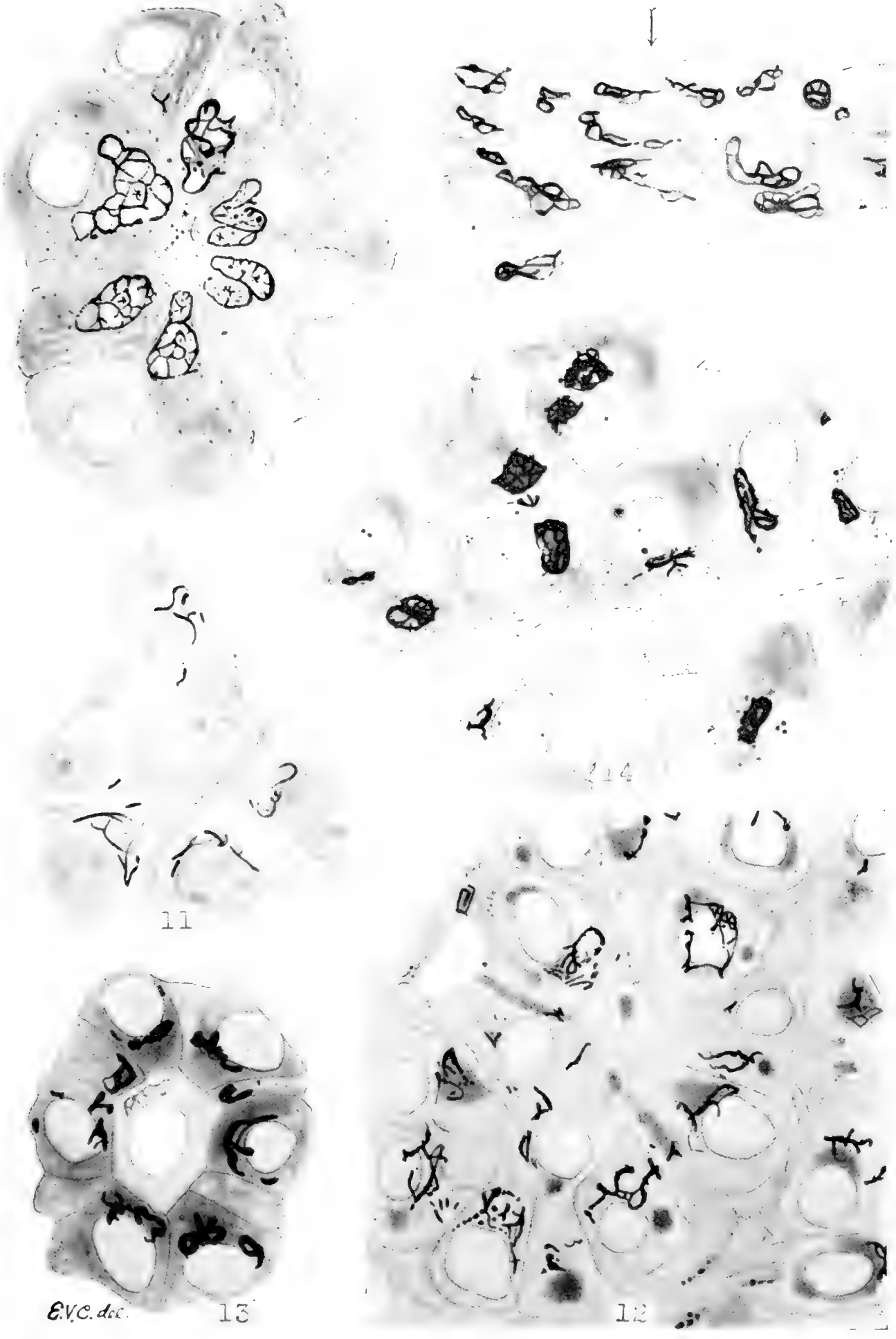
10 Group of parathyroid cells from a piece of tissue centrifuged in the direction of the arrow in normal saline for forty-five minutes, at about 4,500 revolutions per minute, same method.

11 Parathyroid cells fixed in Bensley's acetic-osmic-bichromate mixture and impregnated with 2 per cent osmic acid according to the method of Kolatchev as suggested by Ono. The reticular material is blackened, but is not very definitely oriented.

12 Parathyroid cells prepared by Da Fano's cobalt nitrate silver method in which the blackened reticular material shows but slight evidence of polar arrangement.

13 Cluster of parathyroid cells from the same centrifuged tissue as figure 10, showing a grouping of the cells with definite orientation of the reticular material between the nucleus and the lumen.

14 A small group of cells from the anterior lobe of the hypophysis, also prepared by Da Fano's method, and showing how variable is the position of the reticular material in the cytoplasm. There appears to be no conformity in its location even in adjacent cells.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Resumen por el autor, Warren H. Lewis

El endotelio en cultivo de tejidos

Los cultivos fueron hechos del modo corriente utilizando el medio Locke-Lewis. El hígado del embrión de pollo de siete dias consiste de higado y células endoteliales; cada uno de estos dos tipos emigra hacia el cubreobjetos de una manera característica. El endotelio forma un retículo laxo de células alargadas más o menos adherentes entre sí por medio de sus extremos y procesos. El carácter de este retículo varía considerablemente, pero los procesos celulares no son tan abundantes como en las células mesenquimatosas multipolares. En la periferia, y también en una forma de degeneración, las células pueden aislarse completamente unas de otras. Estos hechos indican que el retículo es adherente en vezde ser sincicial.

En los cultivos jóvenes las mitocondrias aparecen principalmente en forma de filamentos con un número variable de bastones y gránulos. A medida que envejecen dichos cultivos las mitocondrias tienden a disponerse radialmente a partir del centriolo y centrosfera en vías de crecimiento. También tienden a fragmentarse en bastones y gránulos. Su carácter y cantidad varían considerablemente. Gránulos y vacuolas que presentan una marcada afinidad con el rojo neutro (gránulos de degeneración) se acumulan gradualmente en las células y presentan una tendencia a amontonarse alrededor del centriolo y centrosfera dilatada. Las células binucleadas son comunes. En los cultivos más viejos, en los cuales los cambios degenerativos son evidentes, los núcleos a menudo se hacen irregulares y llenos de dentellones, dividiéndose en varios núcleos más pequeños El ectoplasma a veces presenta fibrillas longitudinales semejantes a las de las fibras musculares lisas depués de la fijación. Dichas fibrillas no pueden distinguirse en la célula viva.

Translation by José F. Nonidez
Cornell Medical College, New York

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ENDOTHELIUM IN TISSUE CULTURES

WARREN H. LEWIS

Carnegie Laboratory of Embryology, Johns Hopkins Medical School

FIVE PLATES (TWENTY-FOUR FIGURES)

INTRODUCTION

It is but natural that investigators in the field of tissue culture should have had and should still have more or less difficulty in identifying the various types of cells that grow out from explants of embryonic tissues. Most explants contain several types of cells that migrate out into the medium, some of which are comparatively easy to identify.

Nerve fibers from the central nervous system have been observed by Harrison ('07, '10), Ingebritsen ('13), and Levi ('16 a), and those from the sympathetic system by Lewis and Lewis ('12 a), and Matsumoto ('20). They are always quite characteristic. Endodermal membranes from the cells lining the alimentary tract and allantois were not at first identified as such (Lewis and Lewis, '11; Lambert, '12), but later their origin was established (Lewis and Lewis, '12 b) and their recognition is now simple. Ectoderm from the skin (Lambert, '12) and the amnion (Lewis and Lewis, '12 b) grows out in the form of a membrane or sheet, as does also the pigmented epithelium from the retina (Luna, '17, fig. 1; Smith, '20). In the frog, likewise, the epithelial cells grow out as membranes (Uhlenhuth, '14, '15; Matsomoto, '18). Liver cells form membranes similar to the endoderm and are easily identified (Lynch, '21). The thyroid-gland cells grow out either as tubules or membranes (Carrel and Burrows, '11 a, '11 b). Renal epithelium from the tubules likewise grows out in the form of membranes or sheets (Lewis and Lewis, '12 b) and sometimes as tubules (Reinhoff[1]). Blood-

[1] Unpublished.

cells and wandering cells from the spleen (Foot, '12), bone-marrow (Erdmann, '17), lymph nodes (Lewis and Webster, '21 a, '21 b), and thymus (Pappenheimer, '13) seem always to migrate out as isolated cells and to remain so. Skeletal muscle has been observed by M. R. Lewis ('15, '19), and Lewis and Lewis ('17 a), and premuscle by Congdon ('15). This tissue is usually easy to recognize by its elongated multinuclear fibers and peculiar cytoplasm. Heart muscle has been studied by Burrows ('10, '12), Congdon ('15), and more especially by Levi ('16b, '19). Some of the figures of heart muscle shown by the last-named investigator correspond to our own unpublished ones, but this is not the case with those of Burrows and of Congdon, except possibly Congdon's figure 5. Smooth muscle resembles more the ordinary mesenchyme than either skeletal muscle or heart muscle and has been observed by Lewis and Lewis ('17 b) and by M. R. Lewis ('20 a).

We have been using in a rather loose fashion the terms mesenchyme, connective tissue, and fibroblast to designate the reticular radiating outgrowths in tissue cultures, because we were not sure of the exact identity of the tissue. I think most investigators working with tissue cultures have felt, as we do, that in using these terms they were probably dealing with several types of cells, namely, mesenchyme (including reticulum), fibroblasts, and other types of fixed connective-tissue cells, endothelium, and mesothelium. The literature is full of figures of these mesenchymal tissues, and among them are some which are probably endothelium. Most explants contain capillaries with endothelial cells and in many cultures these cells probably migrate out, although they may not be recognized as endothelium.

The present study is devoted to a consideration of the endothelial cells that migrate out from the explants of embryonic chick liver. It is well known that the liver, after ninety-six hours of incubation contains but two types of cells, the liver cells proper and the endothelium of the sinusoids (Minot, '00). It seemed to offer, therefore, an excellent opportunity for the study of endothelial outgrowths, as no difficulty should be encountered in distinguishing the liver-cell membrane from the loose

reticular outgrowths (Lynch, '21). I have regarded these reticular outgrowths as endothelium. They differ in character from the reticular outgrowths of ordinary subcutaneous mesenchyme, as can be seen by comparing figures 1 and 2 of subcutaneous mesenchyme with figures 3 to 12 of endothelium.

MATERIAL AND METHODS

The explants were all from the livers of chick embryos of five to ten days' incubation. The cultures were made in the usual manner with Locke's solution (NaCl, 0.9 gram; $CaCl_2$, 0.024 gram; KCl, 0.042 gram; $NaHCO_3$, 0.02 gram; dextrose, 0.5 gram, H_2O, 100 cc.), 80 cc., plus chicken bouillon, 20 cc. The chicken bouillon was made according to the usual bacteriological method, except that freshly killed chicken was used in the place of beef. The hydrogen-ion concentration of the solutions varied from pH 6.4 to pH 7.6, but was usually about pH 6.8 to pH 7.2.

The figures are all from untouched photographs. The cultures from which they were taken were first subjected to a weak solution of janus green or neutral red, or the two in combination, until the mitochondria or the granules (and vacuoles) or both were stained. The cultures were than fixed with iodine vapor by placing a small flake of pure iodine on the bottom of the hollow-ground slide. The iodine vapor rapidly tinted the cells a beautiful yellow-brown. The mitochondria when stained with janus green became a very dark brown (almost black) and the neutral red granules became a dark red-brown. These colors are very advantageous for photography.

GENERAL CHARACTERISTICS

The endothelial cells migrate out from the liver explants onto the cover-glass in the form of a loose reticulum of elongated cells that are more or less adherent to one another by their extremities and processes. The character of this reticulum varies considerably in different cultures and in different regions of the same culture, as will be seen by comparing figures 3 to 12. Near the explant and in the middle of the outgrowth the cells are elongated and slender, while at the periphery, where the crowding

is less, they tend to flatten out in various directions. This is often still more marked in the isolated cells near the periphery. The explanation of this is probably not in a longitudinal polarity that disappears as the cells move peripheralward, but in the interplay of surface tension and of the differential adhesiveness of the cells for each other and for the cover-glass. The cells have apparently a greater adhesiveness for the cover-glass than for each other, and at the periphery, where they have more space, they are drawn out more and more on the cover-glass by the tension pull of the surface film of the fluid medium which lies against the cover-glass, until a balance between capillary attraction and cohesion is reached. Tait ('18) has recently described the spreading-out of certain blood-cells on glass as due to capillary attraction or, in other words, to surface-tension phenomena. The endothelial cells are evidently sticky and semifluid and are subject to the same physical forces as the blood-cells described by Tait. There may also be alterations, both local and general, in the consistency of the cytoplasm, which would favor the more extensive spreading-out of the cell. The consistency or viscosity of protoplasm is probably a variable character, as shown by Chambers ('17) and Seifriz ('20). The migration of the cells probably depends on alternating changes in the consistency of the protoplasm with changes in surface tension, as suggested by Loeb ('20, '21) for the amoeboid movements of the blood-cells of Limulus.

In the many cultures examined we have never seen spread-out endothelial cells from the liver with long, slender, fine-branched processes such as are shown in figures 1 and 2 from subcutaneous tissue. Figures 7 and 8 show the slender more elongated type of cell, and figures 3, 4, 9, and 11, the more common type, which possesses a broad, thin, lateral expansion. Figures 5 and 6, a more unusual variety, show the cells forming a somewhat membranous or sheet-like outgrowth resembling the mesothelium-like membranes in cultures from the heart and intestine. They are different, however, and mesothelial membranes have not been observed in these liver cultures.

The contrast between the endothelial cells and the liver cells is always quite marked, as shown in figures 5 and 12. Sometimes the endothelial cells have exceedingly long and slender processes which extend out from the ends of the cells parallel to each other and to the long axes of the cells.

It is very difficult to determine whether the reticulum formed by the endothelial cells is a syncytium or not. In most places it is impossible to decide between actual fusion and mere adhesion. Occasionally it is clearly evident that the connections are only adhesions and that each cell preserves its individuality. This together with the fact that cells at the periphery readily become isolated from one another, would lead one to believe that actual continuity does not exist at any time. Again, under certain conditions, cells retract their processes and tend to round up, losing all connection with neighboring cells; this also would indicate adhesion and not fusion. The difficulty of determining the exact status of the exceedingly thin processes that extend out onto neighboring cells, especially where the cells are closely packed together, has led us in the past to consider that we were dealing with a syncytium, but we are inclined now to doubt this, both for endothelium and mesenchymal reticuli in general.

CELL STRUCTURES

Mitochondria. In young cultures the mitochondria are mostly in the form of threads of varying lengths and forms and often with no definite orientation. In addition there are often rods and granules (fig. 13). Sometimes, even on the first day, they may show a more or less radial arrangement about the centriole region. The conditions of the mitochondria in different cultures of the same age, and even in different cells of the same culture, vary so tremendously that it is difficult to give a general description of their appearance (figs. 13 to 20). No two cells are ever exactly alike. As the culture ages the mitochondria tend to become more or less radially arranged about the centriole and enlarging centrosphere (figs. 14, 15) and the threads tend to break up into rods and granules until finally the cells contain a large (giant) centrosphere and mitochondrial granules (figs.

16, 19). The process is essentially the same as that already shown for fibroblasts and mesenchymal cells (W. H. Lewis, '19, '20).

I have before me seventy photographs (at 1450 diameters) of endothelial cells from this series, which were taken especially for the mitochondria, and it is difficult to select therefrom a few that will adequately convey to the reader an idea of the great variability displayed by these cells. In some cultures varying numbers of cells, as early as the third day, contained only granular mitochondria. This condition, however, may be delayed; some cells, even as late as the tenth day of cultivation, still showed thread-like, as well as granular and rod-shaped, mitochondria. Most of the cells, even up to the tenth day, contained all three types, granules, rods, and threads, varying in length thickness, and form. Variations in the amount of mitochondrial substance were sometimes quite marked. Presumably the mitochondrial complex is much more constant under the normal conditions within the embryo, and the great variations in the cultures are to be attributed to the variations in the abnormal conditions of the cultures. They do not appear to be due to differences in hydrogen-ion concentration from pH 6.4 to pH 7.6, nor to the age of the embryo, since great variations may occur in two successive cultures of the same series or even in the same culture. The most probable factor seems to be in the thickness of the medium drop and the accompanying evaporation in the air chamber, with consequent variations in the oxygen supply to the cells, dependent on the thickness of the fluid separating them from the air in the chamber of the hollow slide.

Granules and vacuoles. Granules and vacuoles, which have a marked affinity for neutral red, methylene blue, and brilliant cresyl blue, gradually accumulate in these cells and vary as much as they do in the fibroblasts and mesenchyme cells (Lewis and Lewis, '15; W. H. Lewis, '19, '20). The rate at which they make their appearance varies in different cultures; as do likewise the relative proportions and the size of the granules and vacuoles (figs. 21 to 23). We are still of the opinion that these granules and vacuoles bear a relation to degenerative changes

going on within the cells (W. H. Lewis, '19). Recent work has tended to confirm this view. Mrs. Lewis ('20 b, '20 c) has shown that the addition of bacillus typhosus to the cultures causes a very rapid formation of similar vacuoles, and still more recently she has found that with the absence of dextrose from culture media vacuolization of the cells is more rapid and extensive than when dextrose is added. Miss Prigosen ('20, '21), working in our laboratory, found that the cells in film preparations of the subcutaneous tissue of chick embryos showed a rapid accumulation of granules and vacuoles that had a great affinity for neutral red.

Centriole and centrosphere. The centriole was not observed in the living cells, but in the fixed material could often be recognized near the nucleus as a small granule about which the mitochondria and neutral red granules tended to accumulate, the former often assuming a more or less radial arrangement. In older cultures there frequently develops about the centriole a centrosphere which gradually enlarges (figs. 14, 15, 16, 19, 20, 21). This area varies in character; it may be quite homogeneous, as in figures 16, 19, 20, or granular, as in figures, 14, 15. The enlargement of the centrosphere has not been followed in detail in these cells, but the process seems to be similar to that previously described for the mesenchyme cells (W. H. Lewis, '19, '20).

Nucleus. The nuclei are oval in form, long and narrow in the elongated cells, and short and plump in the spread-out flattened cells. The contour is usually smooth, but not infrequently in the older cultures it is uneven, being more or less irregular and indented. This appears to be the first indication of the process of budding and amitosis which results in the splitting of the nucleus into several, sometimes four or more, smaller nuclei (fig. 23). This process seems to take place only in the older cultures where other degenerative changes are evident. When a culture shows such changes there are usually many cells thus affected exhibiting various stages of the process. I have not actually followed this amitotic division in the living cells, but the evidence from the fixed material is very conclusive. In addition

to this degenerative fragmentation of the nuclei, more normal binucleate cells are not uncommon in cultures of all ages.

Binucleate cells. Macklin ('16) found that binucleate cells were more numerous in cultures from the hearts of chick embryos of five days' than in those of eight days' incubation and that they were more numerous in the two-day- than in the one-day-old cultures. He found, furthermore, that the average binucleate cell was approximately twice as large as the mononucleate, and that each nucleus was very similar in size, shape, and general appearance to the nuclei of the mononucleate cell—findings which my own observations have confirmed. I have further noted that there is a very decided increase in the mitochondrial content. Figure 10 shows a very large binucleate cell—a giant compared to its neighbors. Each nucleus seems to be as large as normal, while the cytoplasm is more than twice that of the largest mononuclear. The mitochondria are of the same general character (granules and rods) as those in the neighboring cells, but are almost, if not fully, fourfold in number.

Nucleolus. The nucleoli vary in number from one to four and also vary in position, size, and form. In some of the older cultures the nucleoli are extruded from the nucleus, usually from one end (fig. 24). The exact details of this process have not as yet been carefully followed. The nucleolus in some way reaches the end of the nucleus and lies against its membrane. Disintegration then takes place in this region, the nuclear membrane disappears, a vacuole-like area develops in the adjoining cytoplasm, and the nucleolus eventually comes to lie within it.

Cytoplasm. In the living cultures both ectoplasm and endoplasm appeared homogeneous. In the fixed material the ectoplasm was sometimes homogeneous and sometimes striated (figs. 16 and 17). These striae were not seen in the living cells, but formed as the cytoplasm coagulated under the influence of the iodine vapors. I do not believe they correspond to preformed structures. They varied in size and ran out into processes often to their very tips. This would seem to indicate that the peculiarity of the ectoplasm which causes it to coagulate into fibrillae is a molecular thing associated with tension, due to the

surface tension pull. This striation was similar to, but not so marked as that seen in the smooth-muscle cells in cultures, and it suggests that there is an unusual amount of contractile substance in endothelium which is interesting in connection with recent physiological work on the contraction of capillaries by Dale, Krogh, and Hooker.

The endoplasm became very finely granular on coagulation and in it were embedded most of the mitochondria and granules.

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PLATE 1

EXPLANATION OF FIGURES

1 Culture 708. Subcutaneous tissue, 8-day chick embryo; 2-day culture; pH 7.6; janus green, iodine. × 146.

2 Same culture. × 480.

3 Culture 667. Endothelium from liver, 7-day chick embryo; 3-day culture; janus green, iodine. × 146.

4 Same culture. × 480.

5 Culture 666. Endothelium from liver, 5-day chick embryo; 4-day culture; pH 6.5; janus green, iodine. × 146.

6 Same culture. × 480.

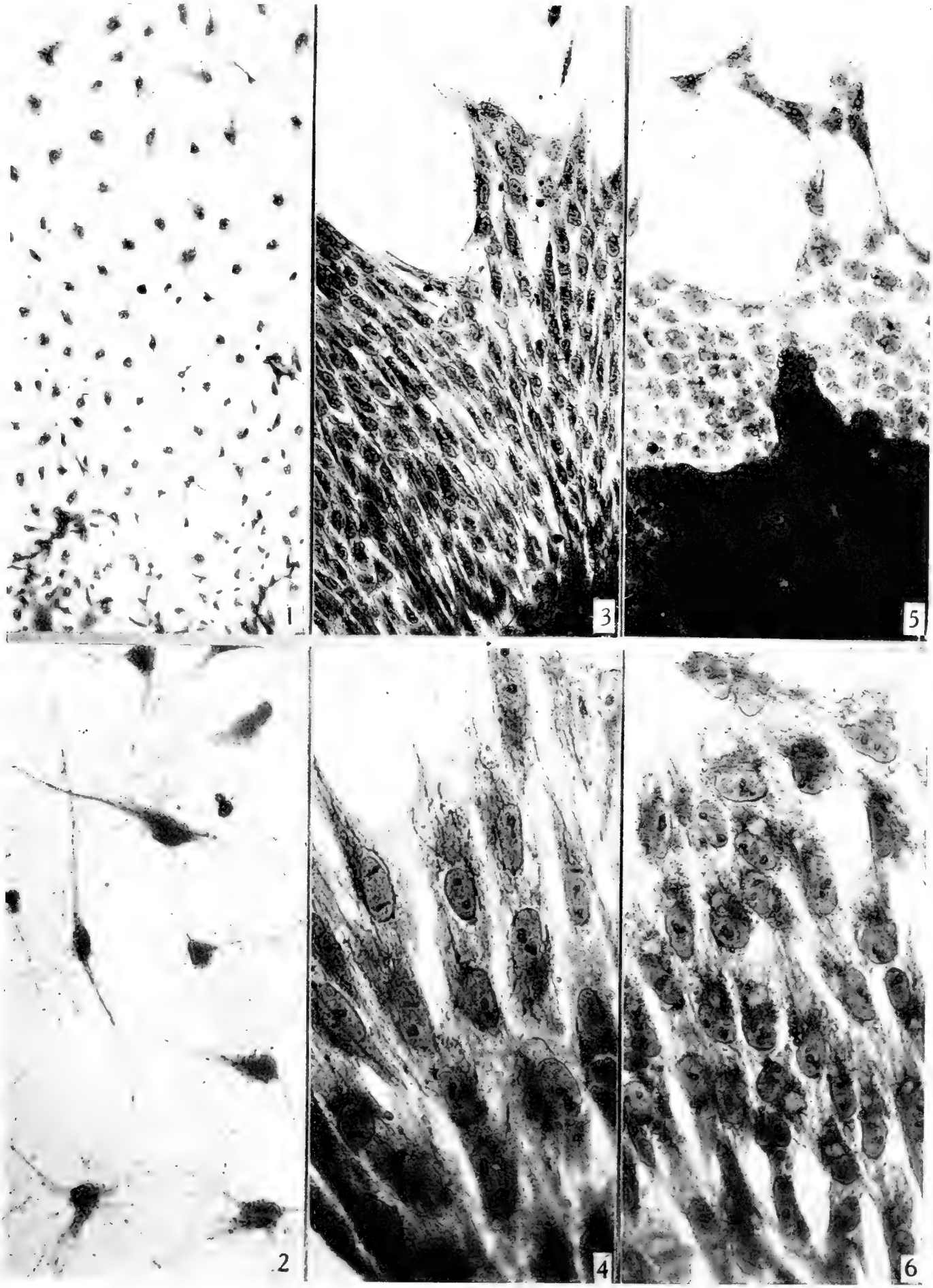
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PLATE 2

EXPLANATION OF FIGURES

7 Culture 681. Endothelium from liver, 8-day chick embryo; 7-day culture; janus green, iodine. × 146.

8 Same culture. × 480.

9 Culture 636. Endothelium from liver, 10-day chick embryo; 1-day culture; janus green, neutral red, iodine. × 480.

10 Culture 678. Endothelium from liver, 8-day chick embryo; 4-day culture; janus green, iodine. × 480.

11 Culture 717. Endothelium from liver, 8-day chick embryo; 6-day culture; pH 7.4; janus green, iodine. × 480.

12 Culture 683. Endothelium and liver cells from liver, 8-day chick embryo; 6-day culture; pH 6.4; janus green, neutral red, iodine. × 480.

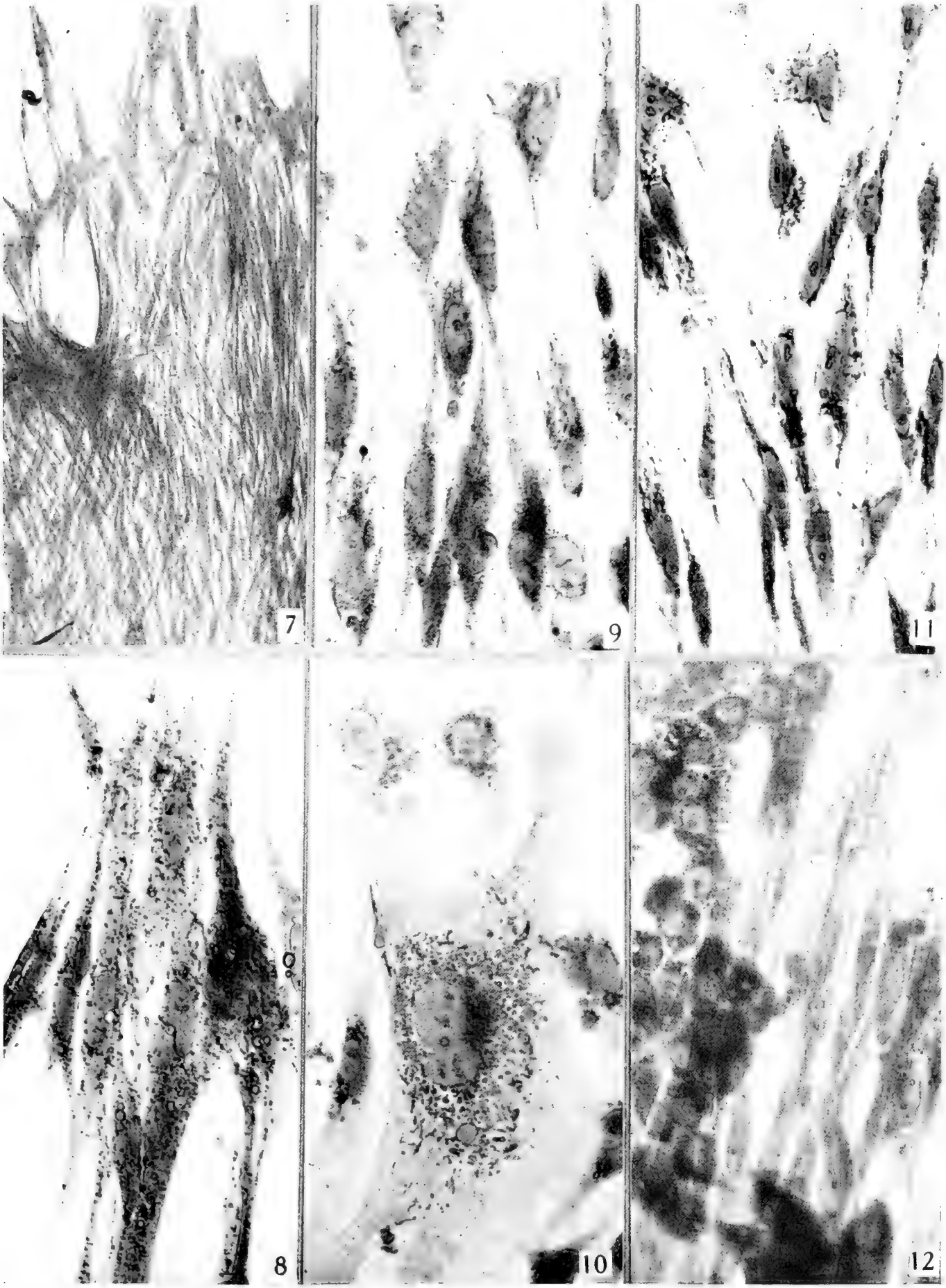
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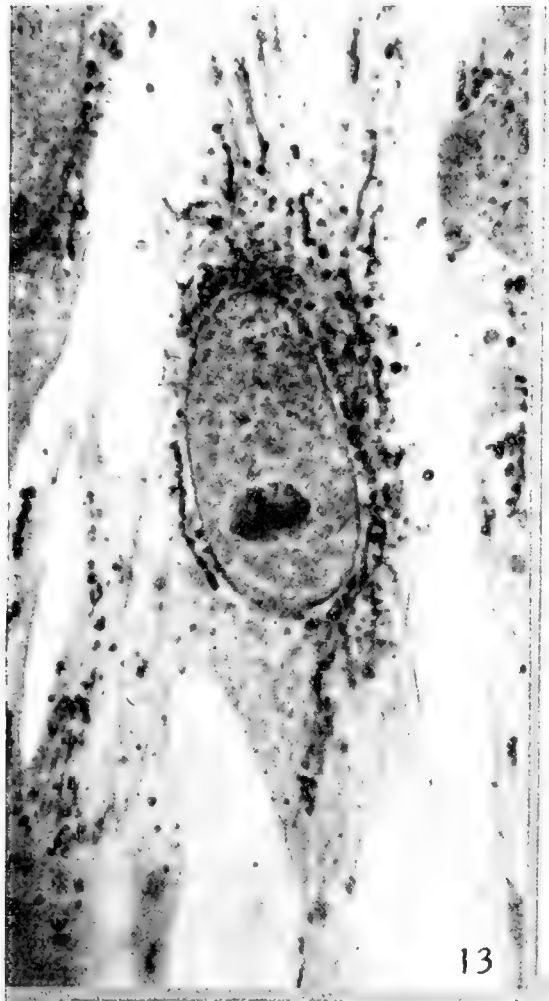
PLATE 3

EXPLANATION OF FIGURES

13 Culture 636 (same culture as fig. 9). Endothelial cell from mid part of outgrowth. Liver, 10-day chick embryo. 1-day culture. Mitochondria, threads, rods, granules; few neutral red granules. Janus green, neutral red, iodine. × 1450.

14 and 15 Culture 717 (same culture as fig. 11). Endothelial cells from liver, 8-day chick embryo; 6-day culture; pH 7.4 Variations in shape of cells and arrangement of mitochondria. Janus green, iodine. × 1450.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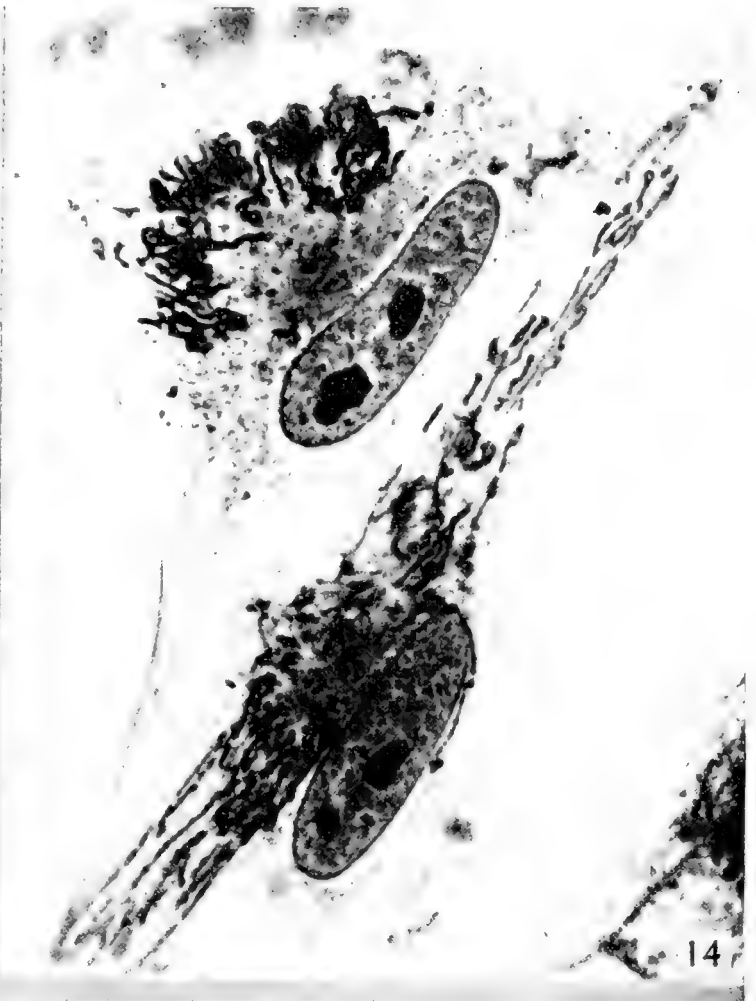
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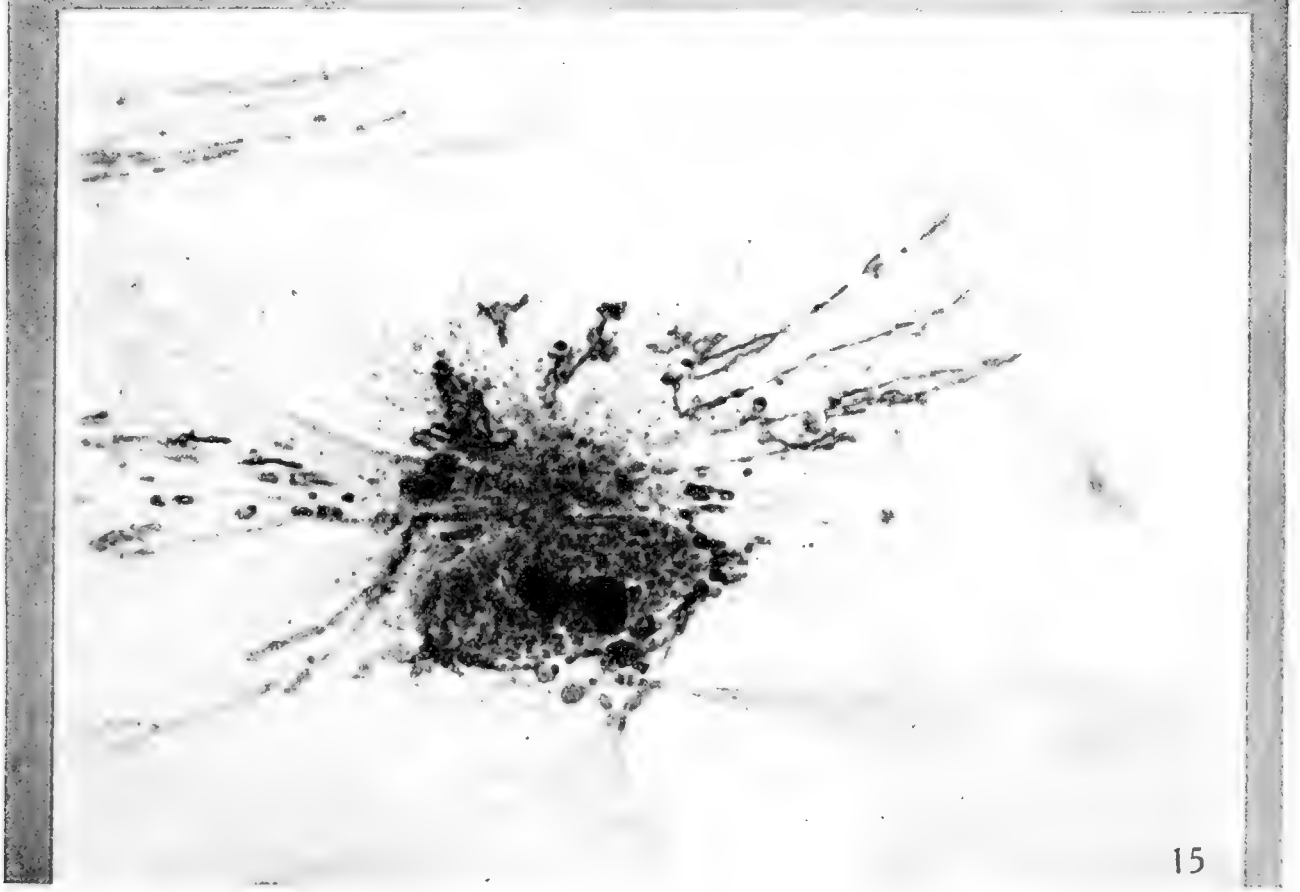
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PLATE 4

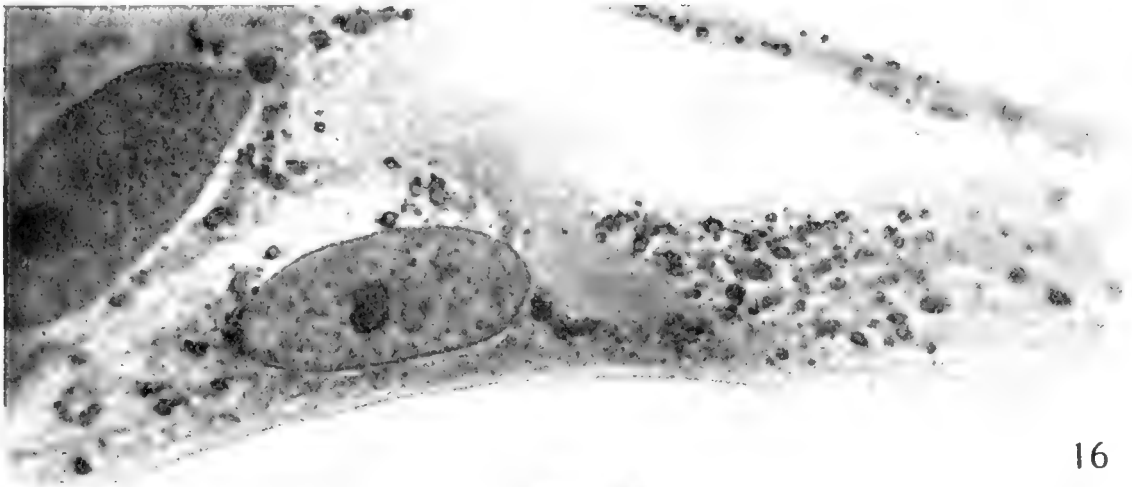
EXPLANATION OF FIGURES

16 Culture 613. Endothelial cell from liver, 7-day chick embryo; 5-day culture. Mitochondrial vesicles, degeneration vacuoles, large centrosphere, cytoplasmic striae. Janus green, neutral red, iodine. × 1450.

17 Culture 612. Endothelial cell from liver, 9-day chick embryo; 3-day culture. Marked striation of cytoplasm. Janus green, neutral red, iodine. × 1030.

18 Culture 716. Endothelial cell from liver, 8-day chick embryo; 6-day culture; pH 7.4. Branching mitochondria. Janus green, iodine. × 1450.

19 and 20 Culture 666 (same culture as figs. 5 and 6). Endothelial cells from liver, 5-day chick embryo; 4-day culture. Fig. 19, mitochondrial granules and vesicles, large centrosphere. Fig. 20, mitochondrial threads, rods, granules, large centrosphere. Janus green, iodine. × 1450.



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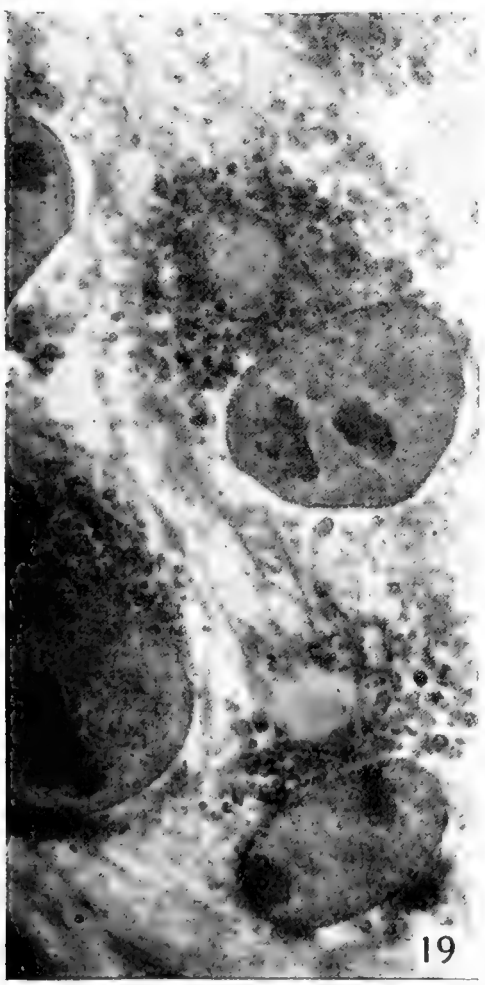

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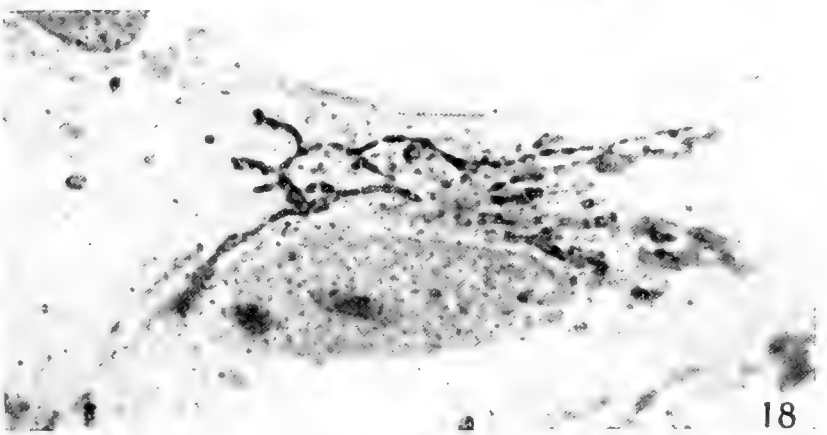

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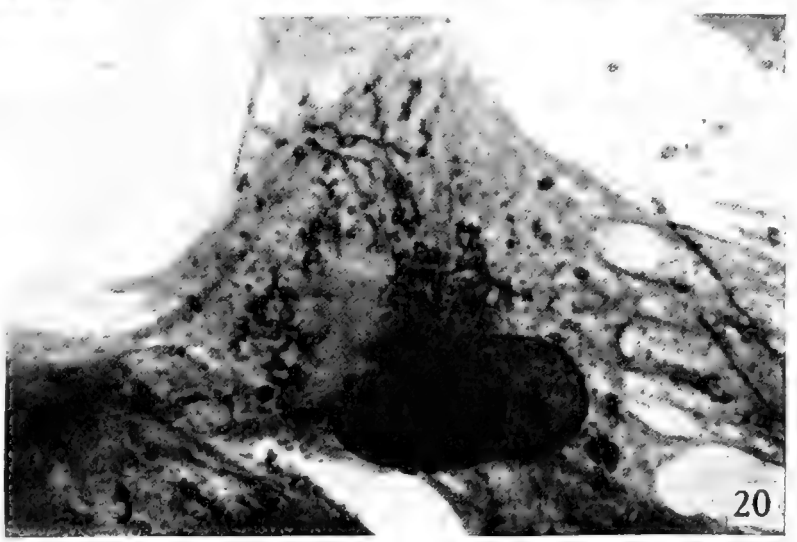

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PLATE 5

EXPLANATION OF FIGURES

21 Culture 675. Endothelial cells from liver, 7-day chick embryo; 7-day culture. Numerous large degeneration vacuoles, mitochondrial granules, large centrosphere Janus green, iodine. × 480.

22 Culture 694. Endothelial cells from liver, 7-day chick embryo; 7-day culture. Large vacuoles (dark in figure), pale long mitochondria. Janus green, neutral red, iodine. × 1450.

23 and 24 Culture 676. Endothelial cells from liver, 6-day chick embryo; 10-day culture; pH 6.6. Fig. 23, fragmentation of nucleus, vacuoles, mitochondrial threads, rods, granules. Fig. 24, extrusion of nucleolus. Janus green, iodine. × 1450.


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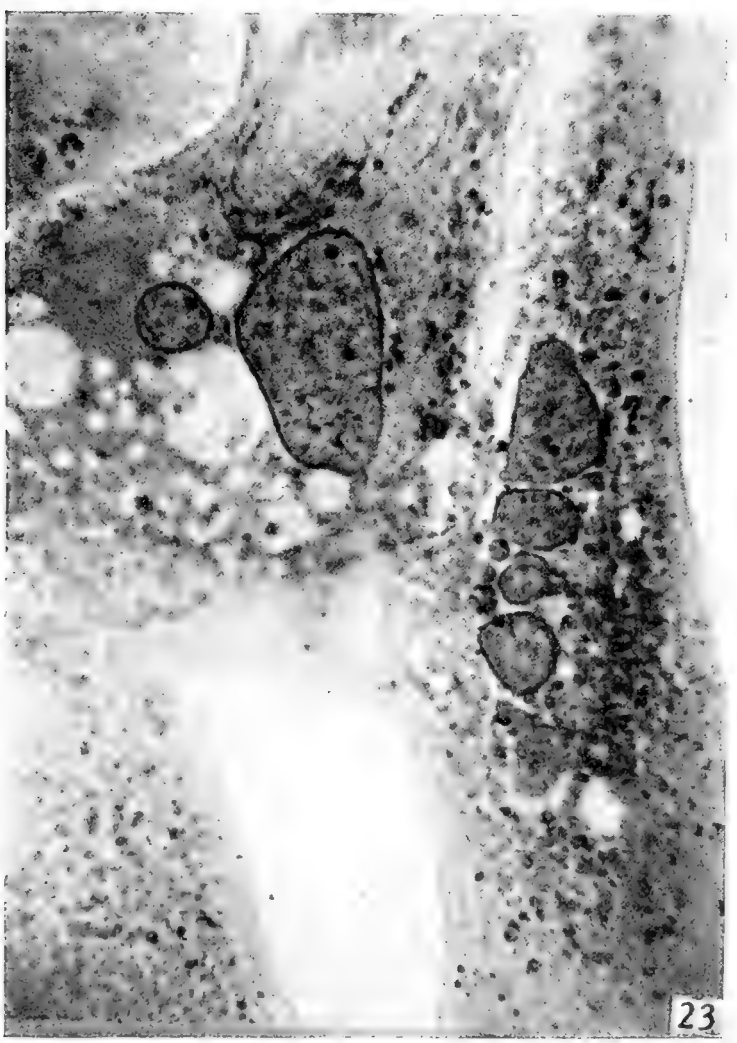
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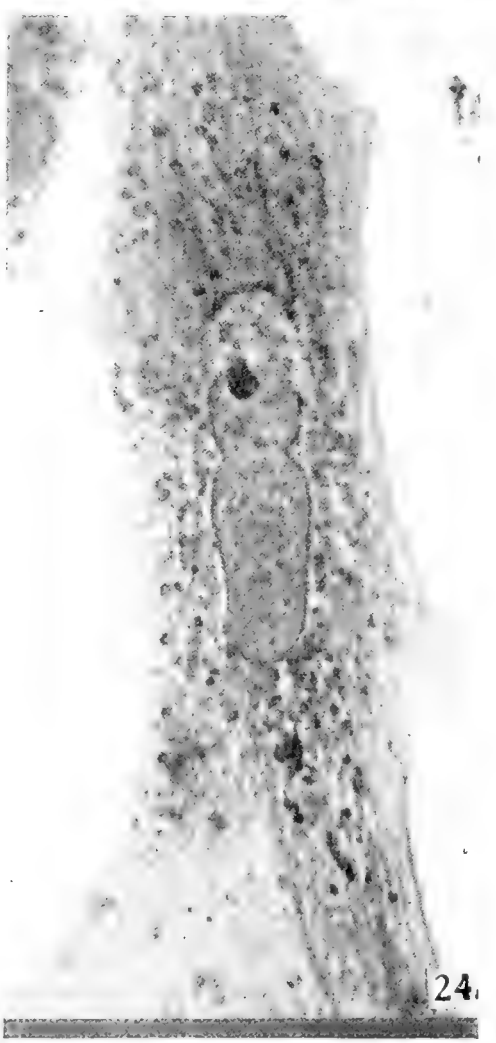
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Resumen por el autor, Otto F. Kampmeier

El desarrollo de los linfáticos anteriores y corazones linfáticos de los embriones de los anuros

El orígen y desarrollo del seno linfático maxilar primario, lifáticos yugulares y corazones linfáticos anteriores de embriones de sapo es objeto de descripción en el presente trabajo. El seno se origina a expensas de pequeños esbozos discontínuos que aparecen como engrosamientos del endotelio de las yugulares externas en vías de desarrollo o como islotes en el mesenquima que rodea a las anteriores. Al principio macizos, estos esbozos adquieren una cavidad y mediante proliferación se alargan, se funden, ramifican y forman de este modo una red complicada. Esta red se transforman en un reservorio espacioso mediante expansión de los canales interanastomóticos y reducción de los cordones mesenquimóticos que los separan. El linfático yugular se desarrolla a expensas de un plexo venolinfático que deriva de las tres primeras venas intersegmentarias (tributarios dorsales del seno venoso pronéfrico), cuando estas se separan del sistema venoso. Después el linfatico yugular establece continuidad con el seno linfático maxilar primario y con el corazón linfático anterior.

El corazón linfatico anterior se origina en una porción circunscrita del plexo veno-linfático, mencionado anteriormente, al nivel de la tercera vena intersegmentaria original. El esbozo plexiforme se desarrolla en la cámara cardiaca por distensión y fusión de sus canales reunidos. Temporalmente se aisla del plexo veno-linfático circunyacente, pero persiste unido con las venas en la boca de la tercera vena intersegmental, de la cual deriva la vena vertebral anterior. Las comunicaciones entre el corazón linfátido y los linfáticos aferentes se reestablecen ulteriomente. La formación de las válvulas y la histogénesis de las paredes del corazón son también objecto de descripción.

Translation by José F. Nonidez
Cornell Medical College, New York

AUTHOR'S ABSTRACT OF THIS PAPER ISSUED
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THE DEVELOPMENT OF THE ANTERIOR LYMPHATICS AND LYMPH HEARTS IN ANURAN EMBRYOS[1]

OTTO F. KAMPMEIER

Department of Anatomy, College of Medicine, University of Illinois, Chicago

THIRTY-FIVE FIGURES INCLUDING EIGHT COLORED PLATES[2]

THE LYMPHATIC GROUND-PLAN OF THE TADPOLE

As is well known, the vessels which collect the lymphatic fluid and convey it to the lymph hearts and thus to the veins in the fully developed anuran Amphibia are in the form of extensive subcutaneous sacs and deep sinuses. This condition, however, is a relatively late acquisition in development, appearing during the metamorphosis of the individual. Before this period, the lymphatic conduit system consists of narrower ducts and capillary networks, similar to those found in the higher vertebrates. In fact, we can recongnize three periods in the development of the lymph channels in Anura: firstly, the initial formative period, secondly, a phase of specific ducts and plexuses, and, thirdly, the final condition, characterized by broad lymph sacs and sinuses—periods which in a general way coincide with the three into which we arbitrarily divide the embryogeny of frog and toad, namely, early embryonic, larval or tadpole, and metamorphic phases. To follow intelligently the nature of events which occur during the formation of the anuran lymphatic system from its inception to its final configuration, as well as to emphasize particular components and to propose a terminology which will facilitate comparison with other vertebrates, it seems expedient to

[1] The present communication represents a portion of a monograph intended for publication in 1918. Other papers which will follow complete the subject-matter of this monograph. The reason why it was broken into a number of separate parts is explained in a footnote of the first installment which appeared in The Anatomical Record, vol. 19, July, 1920.

[2] The cost of illustrations in part borne by the Anatomical Laboratories.

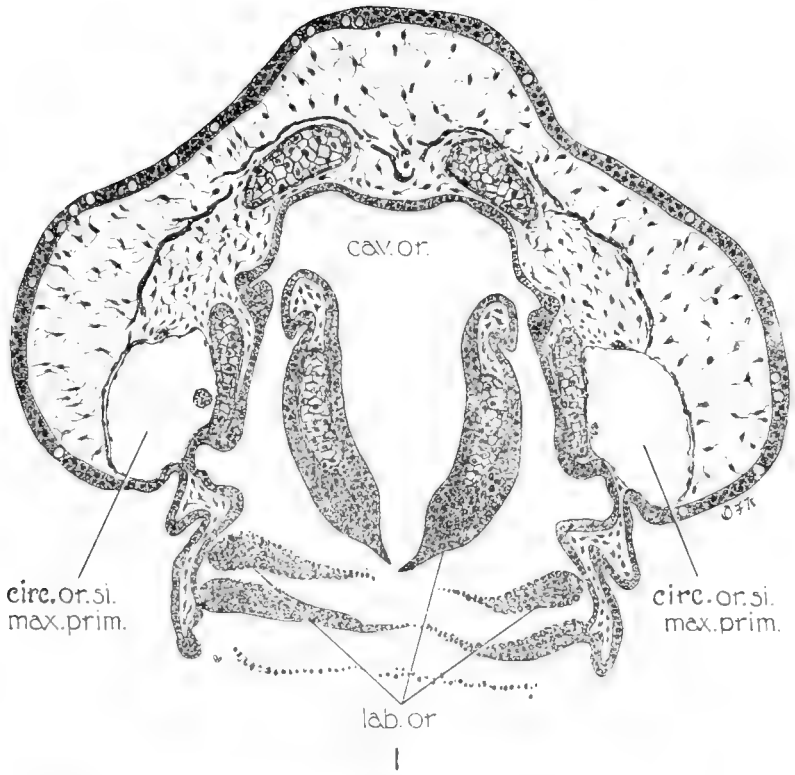
delineate the topography of the lymphatics functional in an intermediate stage.

In Bufo tadpoles,[3] 12 to 15 mm. long, the chief lymph vessels are already laid down, so that we may say the second phase of lymphatic organization, suggested above, begins at this time.

[3] I spent the spring and summer of 1913 with great profit and pleasure at the Anatomical Institute of the University of Munich, where, through the kindness of Professor Rückert, I was able to enjoy all the facilities of those delightful laboratories. I wish also to mention Dr. H. Marcus, who placed his series of larval Gymnophiona at my disposal. Further, I express my gratitude to Mr. Otto Balbach, of the University of Pittsburgh, who prepared the later series of my numerous sectioned anuran embryos.

Toad embryos constitute by far the bulk of the material used in the investigation. These specimens are of two species, the American common toad, Bufo lentiginosus, and the European, Bufo vulgaris (?), and are respectively from New Jersey and Wisconsin, and from the marshes along the Isar River near Munich. The writer neglected to determine with absolute certainty the specific name of the latter form before leaving Munich. There are but two species which can be considered, Bufo vulgaris and B. viridis, but after comparing them as to distribution, breeding habits, etc., as described in the standard works of Zoology, he is confident that it is Bufo vulgaris. The descriptions and figures are based mainly on the embryos of this European form which were gathered later in the course of the investigation when the writer had attained greater success in the preparation of tissues so profusely filled with yolk as are amphibian embryos. The ova of these Anura were collected shortly after laying and developed in the laboratory aquaria. To procure a closely graded ontogenetic series, active individuals were fixed and preserved at intervals of three to four hours.

The ordinary methods of technique were employed in the preparation of the serial sections. Before the embryos exhibited movement, they were fixed directly in Zenker's fluid, but later embryos were first anesthetized in a weak chloretone solution to prevent the distortion or tearing of the delicate tissues which might result from the writhing or twitching of the body when placed in the irritating fixative. The difficulties at first encountered in making satisfactory serial sections, apparently due to the brittleness of the yolk-laden tissues when xylol was used as the clearing reagent, were overcome by using cedar oil instead and by diminishing the time of paraffin infiltration to a minimum. Both the graphic method and the modified Born's wax-plate process were enlisted in the execution of the reconstructions. In every case, the outline drawings of the sections were made with the Edinger projection apparatus. The writer also attempted to inject the vascular channels of a number of embryos, but on the whole he met with little success. The continuous layer of brown pigment in the skin of the toad larvae hides the underlying structures, and the cannula needle can therefore not be directed with the same degree of certainty as when transparent fish embryos or the much larger pig or chick embryos are injected under the binocular microscope.



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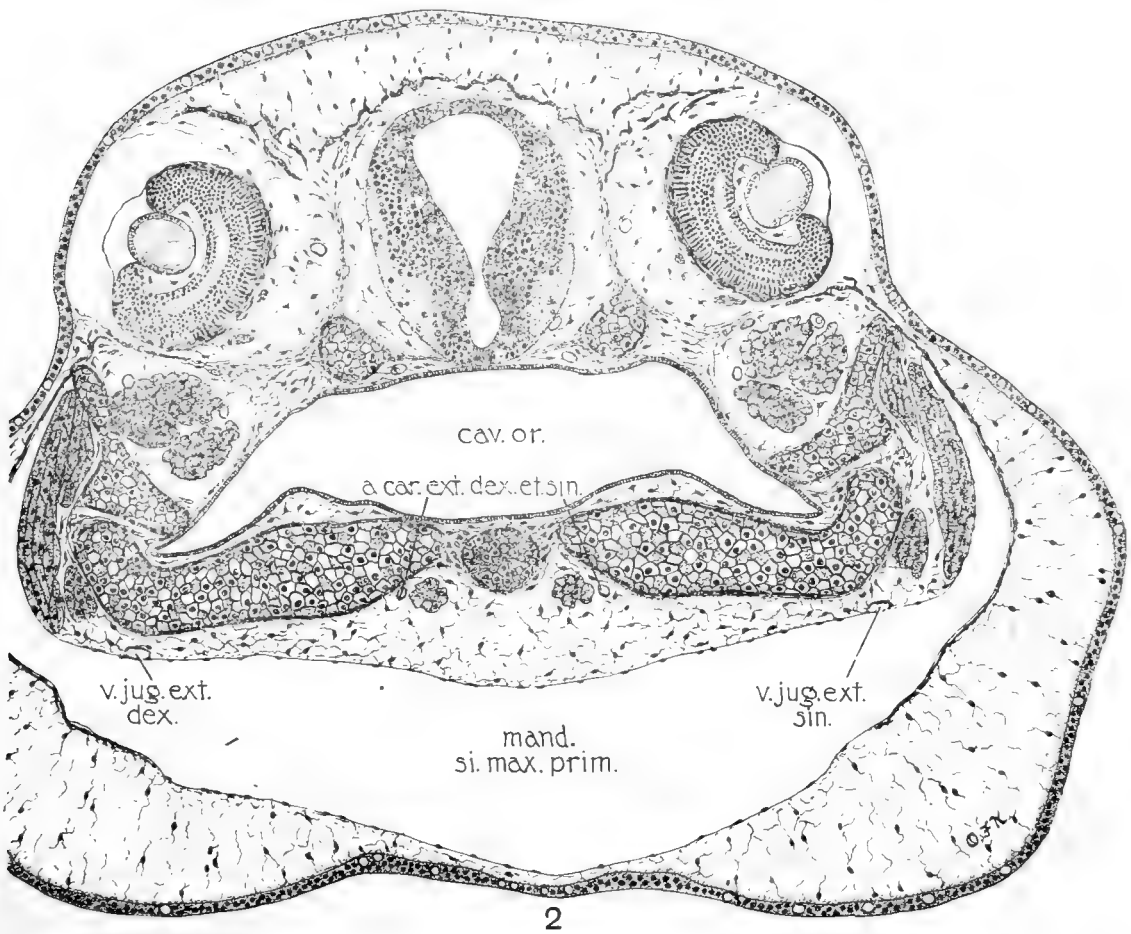

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Fig. 1 Transverse section of a 13-mm. embryo of Bufo vulgaris at the level of the mouth. × 50. *circor. si. max. prim.*, circumoral division of the primary maxillary sinus; *cav. or.*, cavum oris; *lab. or.*, labial structures of the larval sucking mouth.

Fig. 2 Same, at the level of the eyes. × 50. *mand. si. max. prim.*, mandibular division of sinus lymphaticus maxillaris primigenius; *v. jug. ext. dex.* and *sin.*, vena jugularis externa dextra and sinistra; *a. car. ext. dex. et sin.*, arteria carotis externa dextra and sinistra; *cav. or.*, cavum oris.

The most marked feature of the disposition of the lymphatics in the head is the relatively enormous expanse of a lymph sinus situated in the ventral and lateral cephalic territory. Its limits in a 26-mm. frog larva are shown in Hoyer's sketches, illustrated in Wiederscheim's 'Vergleichende Anatomie der Wirbeltiere' (7th et al. editions), and in the wax reconstruction of the vascular channels in the head of a toad embryo in figure 28. This lymph reservoir, which the writer designates the primary maxillary sinus (sinus lymphaticus primigenius maxillaris)[4] because eventually it is resolved into the secondary lymph sinuses in the region of the jaws, is developed very early and, in general form, extent and proportions, is virtually complete in 9- or 10-mm. toad embryos, hence, at a period when most of the other lymphatics are still in the formative state. Figure 28 shows that this sinus does not possess a simple contour, but is composed of several interconnecting chambers of diverse shape and size. For convenience and clearness, we may refer to the several subdivisions by different names. The broad, roughly rectangular division (figs. 2, and 28, *mand. si. max. prim.*) on the ventral side of the head may be regarded as the mandibular one; it is the largest, the first to develop, and the other portions of the sinus arise from it by outgrowth and extension. In continuity with it anteriorly is the circumoral division (figs. 1 and 28, *circ. or si. max. prim.*), which encircles the mouth opening. The third division, a pair of temporal chambers (figs. 3 and 28, *temp. si. max. prim.*), appears in the wax model as two lateral wing-like expansions of the mandibular sac; these extend as far as the pronephroi, where each contracts into a narrow duct which leads to the anterior lymph heart of the respective side. The fourth division of the primary maxillary sinus may be termed the pericardial (figs. 3 and 28, *pericard. si. max. prim.*); it constitutes a second path of communication between the mandibular and temporal sacs, but at a deeper level. It is paired and branches, as a more slender and somewhat plexiform channel, from the mandibular sac near

[4] Hoyer calls this the 'Kehlsack' and Jourdain 'sac gulaire,' and in my paper on the origin of the lymphatics in Bufo ('15) it is spoken of as the ventral cephalic sinus, but these terms are too general.

its posterior margin, but in its course it curves dorsally, that is, centrally or inwardly, and, closely associated in position with the external jugular vein, passes back along the heart towards

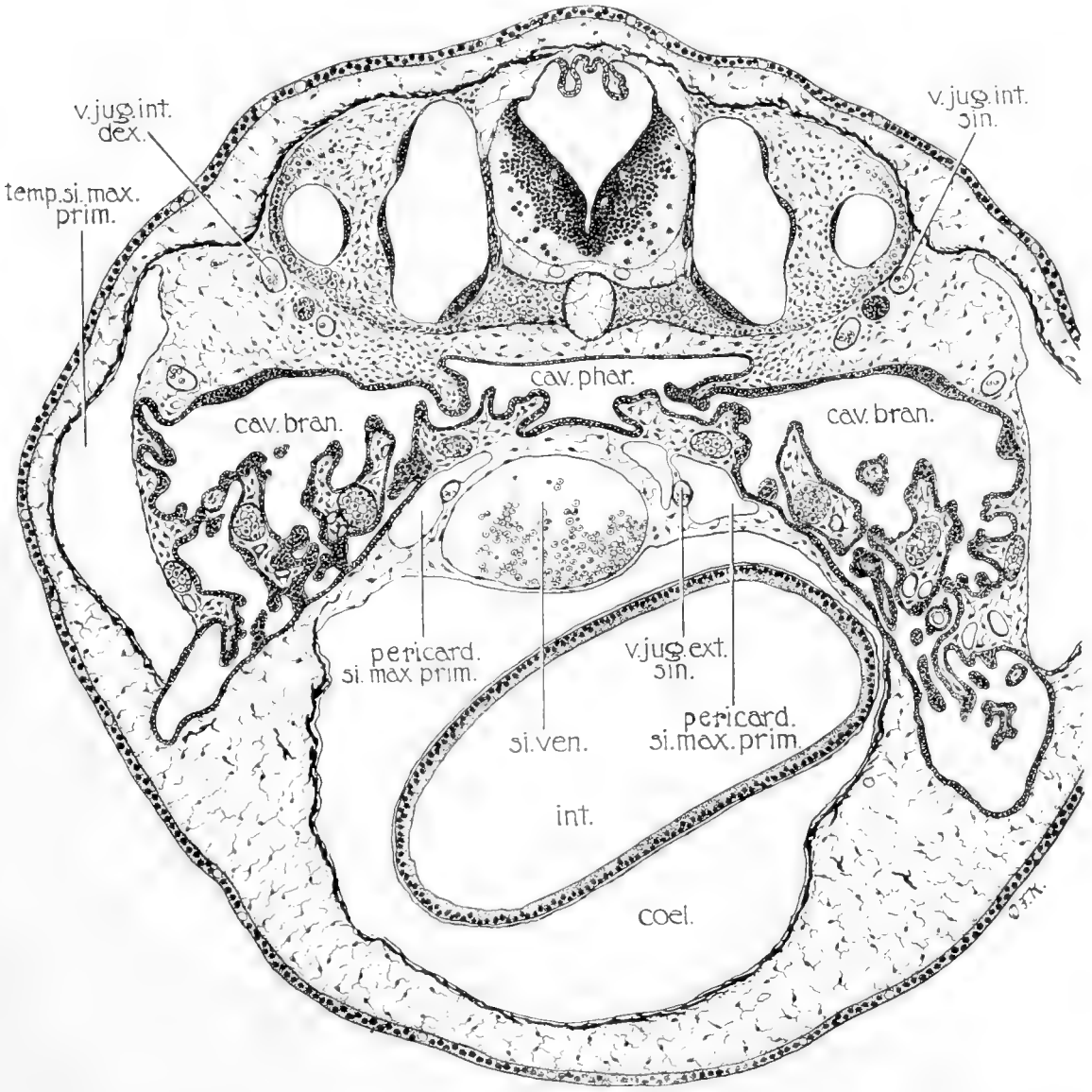
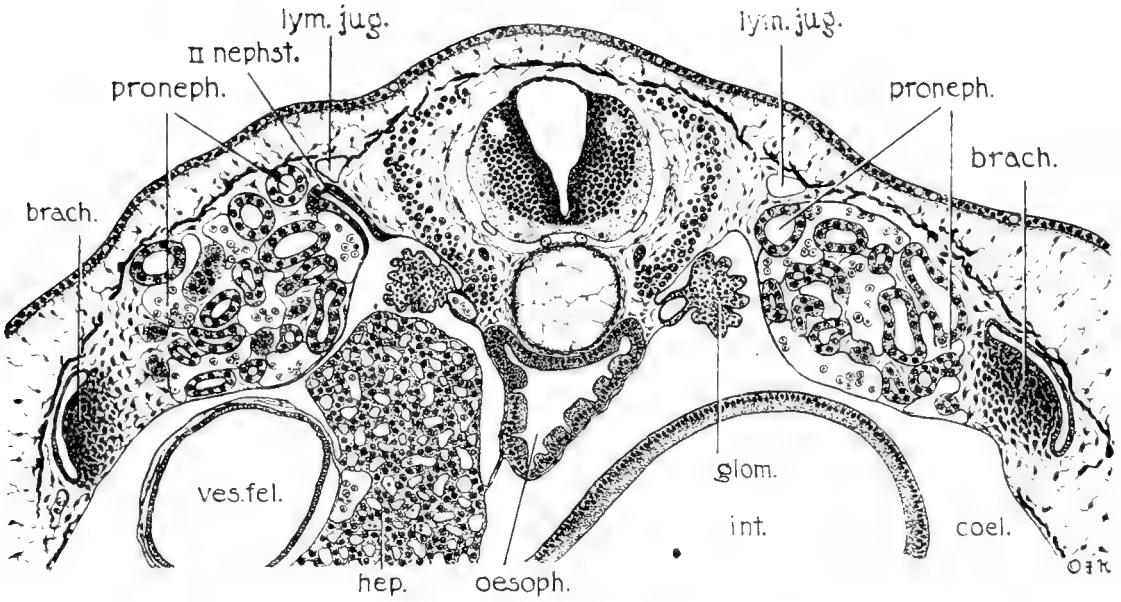


Fig. 3 Same, at the level of the auditory capsule. × 50. *temp.* and *pericard. si. max. prim.*, temporal and pericardial divisions of the primary maxillary sinus; *v. jug. int.* and *ext. dex.* and *sin.*, venae jugulares internae and externae dextrae and sinistrae; *si. ven.*, sinus venosus; *cav. phar.*, cavum pharyngeus; *cav. bran.*, cavum branchialis; *int.*, intestinum; *coel.*, coelom.

the sinus venosus and thence makes a broad sweep outward along the duct of Cuvier to join the hinder end of the temporal sac.



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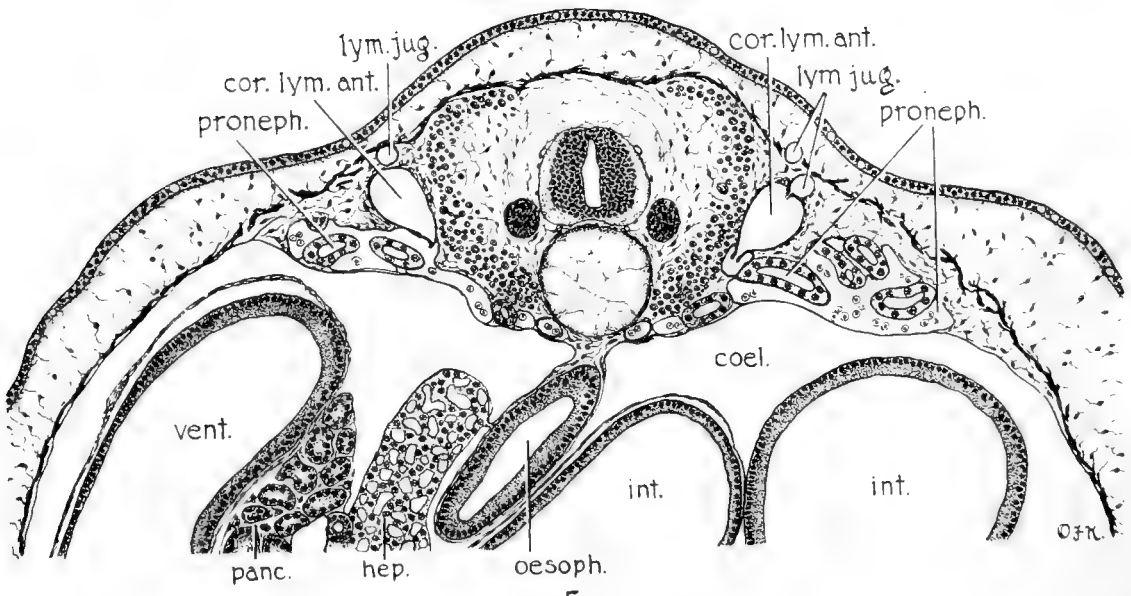

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Fig. 4 Same, at the level of the anterior limb bud (*brach.*). × 50. *lym. jug.*, lymphatica jugularis; *proneph.*, pronephric tubules and sinusoids; *II nephst.*, 2nd nephrostome; *glom.*, pronephric glomerulus; *int.*, intestinum; *oesoph.*, oesophagus; *ves. fel.*, vesica fellea; *hep.*, hepar; *coel.*, coelom.

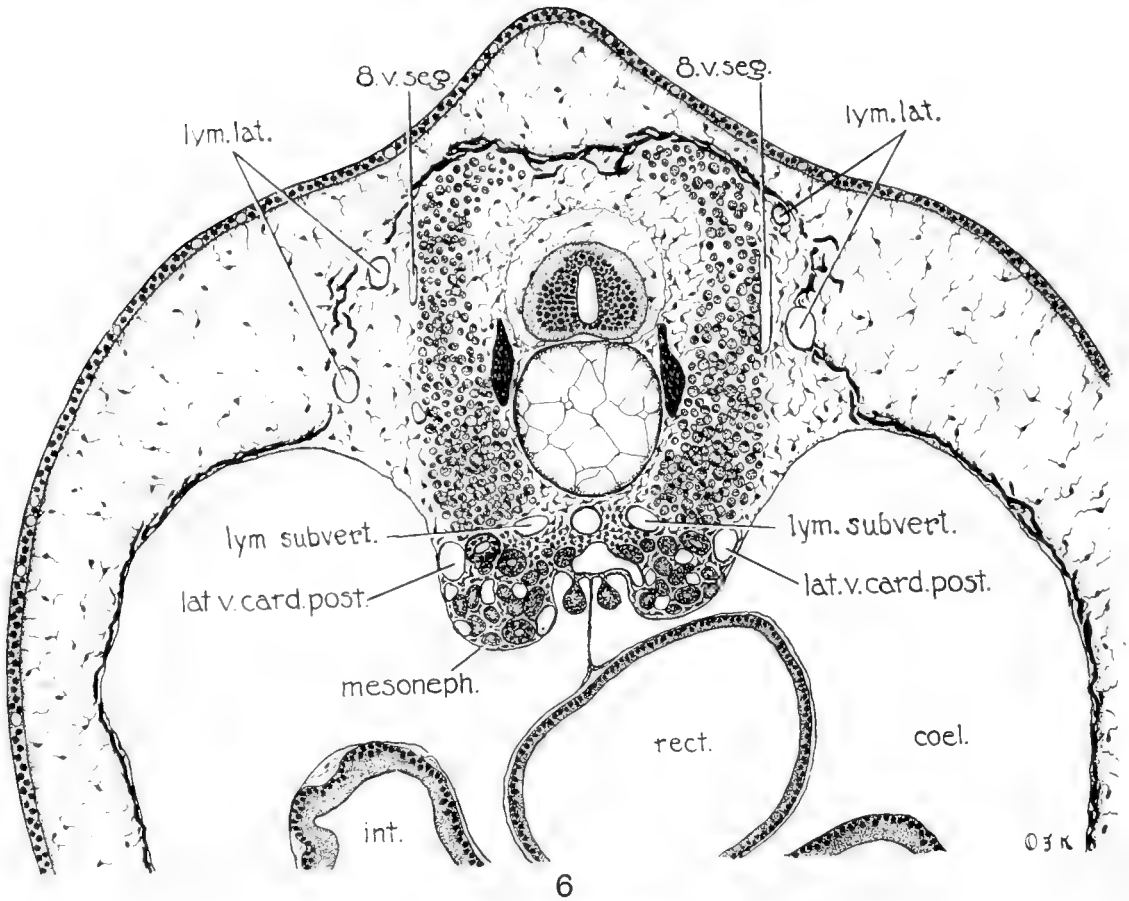
Fig. 5 Same, at the level of the 3rd spinal ganglia. × 50. *cor. lym. ant.*, cor lymphaticum anterius; *vent.*, venter. Other references as in the preceding figure. Lying medial to the pronephric tubules and sinusoids (*proneph.*) are, in the order named, the primary excretory duct, the subcardinal vein and the aorta.

The primary maxillary sinus receives the lymphatic drainage of the head, as indicated in Hoyer's sketches. The lymph then flows posteriorly towards the anterior lymph heart of the same side through the channel (fig. 28, *lym. jug.*), which has been mentioned as a caudal prolongation of the temporal portion of the sinus, though genetically it has an independent origin. Hoyer has called this vessel the 'cephalic duct' or 'Kopfgefäss,' but the term jugular lymphatic (lymphatica jugularis)[5] seems more appropriate on account of its probable homology with a similar vessel in all other vertebrates. It lies immediately dorsal to the pronephros (fig. 4) and only a short distance below the skin. Near the lymph heart a tributary is given off which extends to the anlage of the forelimb, at this time a knob-like condensation of mesenchyme beneath the operculum, and, as it is the parent of the future lymph vessels of the arm, this tributary may be called the brachial lymphatic (lymphatica brachialis).

The relative size, shape, position, and connections of the pair of anterior lymph hearts during the second embryonic phase are exhibited in the wax reconstruction (fig. 28) and in the section of a 13-mm. embryo (fig. 5). Each heart is globular in form, placed superiorly at the posterior limit of the pronephros and is in continuity with both vein and lymphatic duct. It is located in the triangular area, bounded by skin, myotome, and the roof of the coelom at the level of the third spinal ganglion. On its ventral side it opens at the junction of the pronephric sinus and a short dorsal venous extension, the rudiment of the anterior vertebral vein of the adult. In the opposite wall of the heart the afferent lymphatic vessel has its entrance.

Not only the lymphatic drainage of the head is poured into the anterior lymph hearts, but also the greater quantity of the lymph from the trunk is conveyed to them by two pairs of important ducts, the subvertebral lymphatics (lymphaticae subvertebrales), lying deep, and the lateral lymphatics (lymphaticae laterales) of the trunk situated superficially, one on each side. The latter

[5] The usage of the word 'lymphatic' and its Latin form, 'lymphatica,' has been made clear in the author's paper in The Anatomical Record, vol. 16, no. 6, August, 1919.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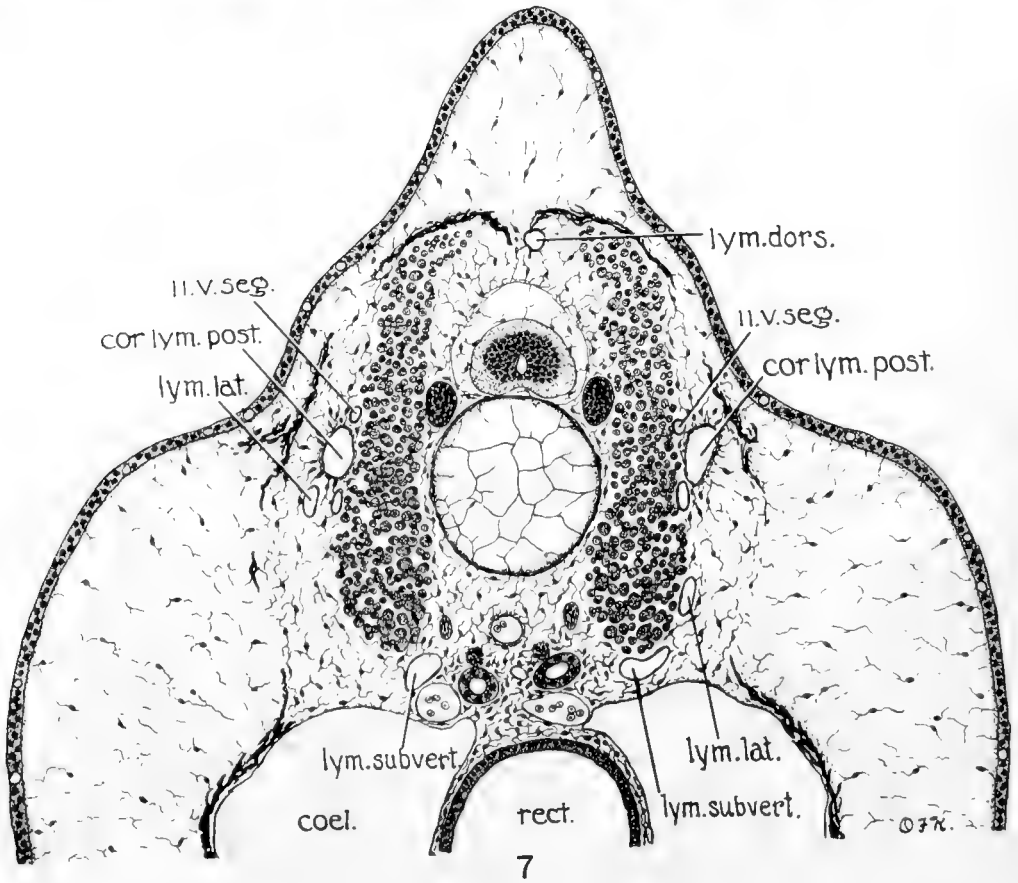
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coel.
rect.

7

are a direct continuation backwards of the jugular lymphatics, as illustrated in figure 28, and together they possess a common opening into the lymph hearts. In its course caudalward, each lateral lymph duct runs between the myotomes and the epidermis (fig. 6, *lym. lat.*), in the lateral-line region. About halfway towards the posterior lymph heart, it sends off a branch which passes over the upper edge of the myotome to fuse with its fellow of the opposite side and assuming a median position (fig. 7, *lym. dors.*), proceeds distally into the tail as the dorsal lymphatic (lymphatica dorsalis).

The paired subvertebral lymphatic, corresponding to the thoracic ducts of the higher vertebrates, has a position along the aorta and dorsal to the postcardinal veins. Anteriorly, it curves outward under the lower margins of the myotomes to join the anterior end of the lateral lymph ducts in the immediate neighborhood of the anterior lymph hearts. It retains the axial location (fig. 6, *lym. subvert.*) throughout almost its entire extent and later becomes connected with its companion by occasional anastomoses. In the vicinity of the posterior lymph heart, the duct again bends outward to reunite with the lateral lymph vessel of the same side (fig. 7). The common duct so formed later combines with the opposite one in the ventral midline and is prolonged caudally into the tail as the ventral caudal lymphatic (lymphatica ventralis) in the base of the ventral tail fin. In this region, too, the iliac lymphatic (lymphatica iliaca) is given off to the hind limb bud.

Fig. 6 Transverse section of a 15-mm. embryo of Bufo vulgaris through the trunk at the level of the 9th spinal ganglia. *lym. lat.*, lymphatica lateralis; this duct is plexiform in character; *lym. subvert.*, lymphatica subvertebralis (thoracic duct); *8 v. seg.*, 8th intersegmental vein; *lat. v. card. post.*, lateral division of the postcardinal vein; the medial divisions (subcardinals) have fused to form the postcava which lies ventral to the aorta; *mesoneph.*, mesonephric tubules and sinusoids; *rect.*, rectum; *int.*, intestinum; *coel.*, coelom.

Fig. 7 Same, at the level of the 11th spinal ganglia. × 50. *cor lym. post.*, cor lymphaticum posterius; *lym. dors.*, lymphatic dorsalis; *11 v. seg.*, 11th intersegmental vein. Other references as in the preceding figures. Medial to the subvertebral lymphatics are the aorta, primary excretory ducts and the postcardinal veins.

The paired posterior lymph heart is similar in shape to the anterior, though somewhat smaller in size at this period (15-mm. embryo), and lies lateral to the myotomes in the intersegment of the 11th and 12th (fig. 7, *cor. lym. post.*). It joins the 11th intersegmental vein (*11 v. seg.*) which becomes, as shown previously,[6] the proximal portion of the posterior vertebral vein. The heart receives the lymph stream from the hinder regions of the trunk and the tail through the lateral lymph duct.

All of the main lymphatic conduits described possess subsidiaries and capillary plexuses, the ramifications of which in frog larvae are admirably shown in injected specimens, as illustrated by Hoyer.

In the present paper, the origin and development of the primary lymph sinus, the jugular lymphatics, and the anterior lymph hearts will be considered. The formation of the lymphatics of the trunk and tail, including the posterior lymph hearts, will be taken up in a succeeding article.

THE DEVELOPMENT OF THE PRIMARY MAXILLARY LYMPH SINUS[7]

In 5-mm. embryos (Bufo vulgaris) a crude vascular plexus exists ventral to the oropharyngeal cavity and has its greatest concentration in the vicinity of the thyroid diverticulum. From this plexus the external jugulars[8] and external carotids and their tributaries subsequently differentiate. But at this time veins and arteries are still broadly confluent; all channels are alike in histological appearance, and merely the definite and constant position of certain ones enables us to pick out the future arterial and venous components. Farther back towards the heart, however, a division has already occurred between them, and the external jugulars and carotids are independent, the former curving laterally around the ventricle to join the common cardinal veins

[6] Anatomical Record, vol. 9, July, 1920.

[7] A short description of the genesis of the primary lymph sinus in the head of Bufo embryos was published by the writer in The American Journal of Anatomy, vol. 17, 1915.

[8] In using the term 'external jugular vein' the author is following Gruby and Ecker; Goette and many other authors refer to this vein as the 'inferior jugular.'

and the latter being in continuity with the aortic arches. In the reconstruction reproduced in figure 29 their topographical relations are clearly indicated, though this deals with a later stage, a 6-mm. embryo, in which the demarcation between jugular (*v. jug. ext.*) and carotid (*a. car. ext.*) is complete except anteriorly, where they are still in broad plexiform connection.

The inception of the primary maxillary sinus takes place in 5-mm. embryos during the period of the indifferent jugulocarotid plexus, just described. Its initial anlagen arise along those channels which are to become the external jugular veins, and at first many of them are in the form of short knot-like cellular thickenings adhering to their lining. Such a lymphatic anlage is shown in the photomicrograph, figure 8, as a compact protuberance (*lym.*) of the intima of the blood vessl (*v. jug. ext. dex.*). A transverse section of another sinus anlage of the same specimen, but from the opposite side, is pictured in figure 9, *B*. In longitudinal extent, it passes through seven sections (each 6 μ thick). It is a solid cell cord or column attached to the wall of the vein (*v. jug. ext. sin.*) by its anterior end, while throughout the remainder of its course it lies free in the mesenchyme ventral and parallel to this vessel.

Besides the adherent lymphatic anlagen, there are at this stage other anlagen, which, though they be similar to them in size, shape, and location, are not in immediate contact with the lining of the blood channel, and the question naturally arises: Were such anlagen formerly connected with the haemal endothelium, or did they arise independently? Observations on the succeeding genetic stage, as well as the investigation of the developing lymphatics of the trunk region, furnish evidence that points to the independent origin of such anlagen and besides reduces the significance which we would attach to the adhesion of some of the earliest lymphatic anlagen to the primitive blood channels. The theoretical aspects of this problem will be discussed after the steps in the development of the primary maxillary lymph sinus have been described.

The lymphatic anlagen, like the endothelium of all blood channels, especially in the head region during early development,

are stuffed with large yolk globules from tip to tip—a fact that clearly distinguishes them from the surrounding mesenchymal cells which have for the most part lost their yolk content. The nuclei of incipient endothelium, regardless of whether haemal or lymphatic, show no difference, except possibly in chromatic density when compared with those of mesenchyme; indeed, the endothelium presents a very unspecialized appearance. The fact of the longer retention of yolk spherules by the cells of vascular anlagen and channels was reported by the author ('15) and emphasized as a diagnostic trait of considerable value in discriminating between these tissues during the earlier embryonic period. Their distinctions were accurately expressed in the colored figures of that paper, to which the reader is referred.

The next older stage, a 6-mm. embryo, is characterized by the numerical increase of sinus anlagen along the external jugular veins, by their growth in length and budding of branches, their detachment from the venous intima at the original point of contact, and by their acquisition of lumina. The reconstruction in figure 29 displays the number, size, form, affinities, and distribution of these lymphatic anlagen. It furnishes a convincing picture to show that the sinus does not originate by centrifugal sprouting from any specific foci, but that it has a multiple origin in proximate relation with vessels of the primitive vascular network, and that the initial anlagen are discontinuous. Further, it shows the bilateral origin of the sinus and also that its principal or mandibular division is the first component to be formed, the other divisions, such as the circumoral, temporal and pericardial appearing somewhat later.

The time at which the individual lymphatic anlagen that are adherent to the venous wall retract from it varies greatly, neither the time of their beginning nor their length entirely conditioning it. In the reconstruction (fig. 29) some of them still cling to the blood channel, while others, even smaller ones, lie independently in the surrounding tissue. Nor is the size of the anlage a criterion of the possession of a lumen; one may acquire such very early, even in its incipient stage, while another may remain solid for a longer period of time. When a lumen does appear, it is at

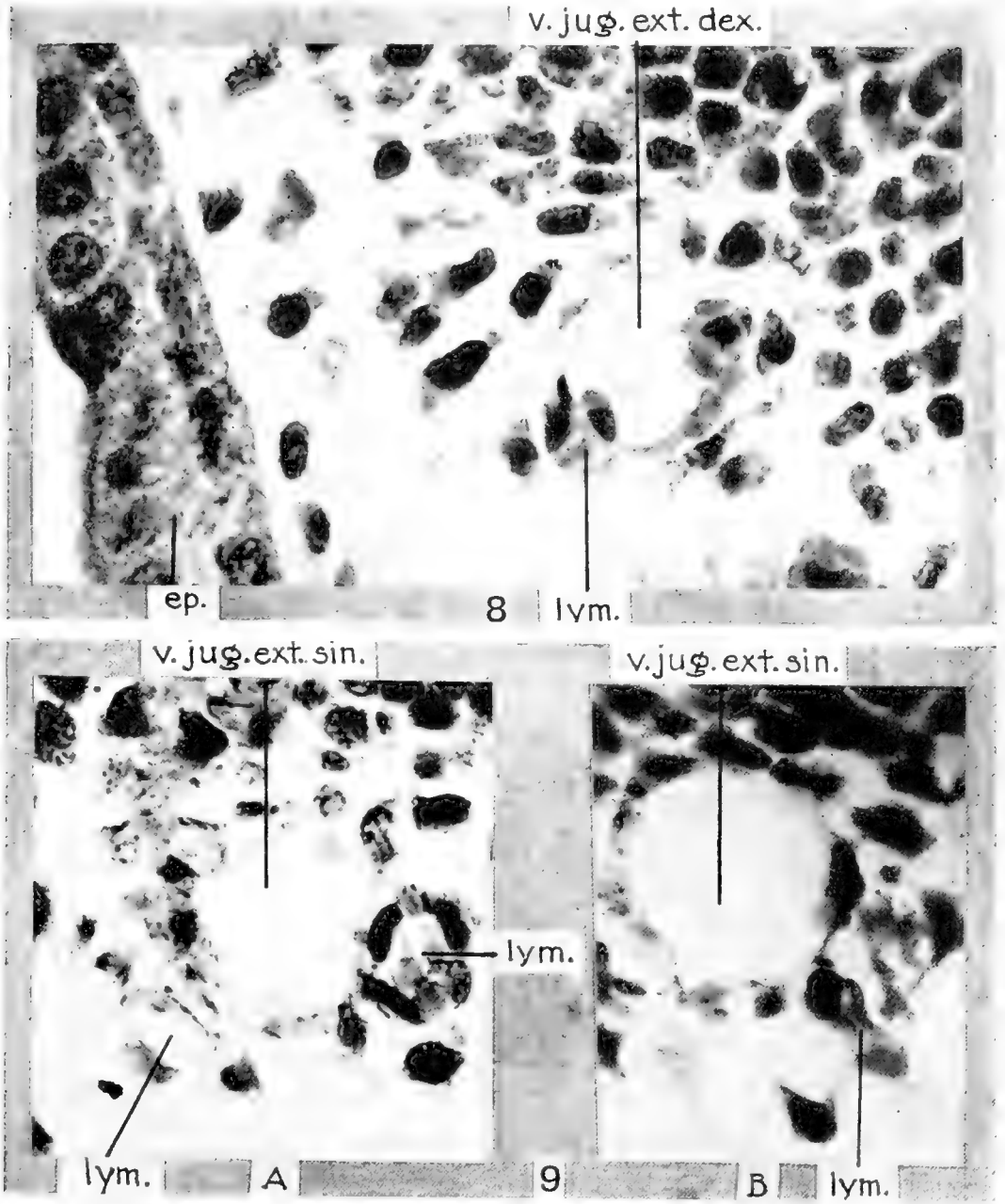

Fig. 8 Photomicrograph of a transverse section through the right ventral cephalic region in a 5-mm. embryo of Bufo vulgaris (Kampmeier Embryological Collection, series B 25, slide 1, section 71). × 690. (Zeiss Apochromat. Obj. 4 and Compensat. Project. Oc. 4,). *ep.* epidermis; *v. jug. ext. dex.*, vena jugularis externa dextra; *lym.*, and initial lymphatic anlage of the primary maxillary sinus; the structure lying within the upper right-hand portion of the lumen of the vein is a yolk-filled blood cell. In this and the following photographs, the yolk globules can be easily distinguished from the dense cell nuclei by their smaller oval shape and their uniform gray color.

Fig. 9 Photomicrograph of transverse sections through the left ventral cephalic region in a 6-mm. embryo of Bufo vulgaris (K. E. C., series B 54, slide 1, sections 75 (*A*) and 80 (*B*). × 690. *v. jug. ext. sin.*, vena jugularis externa sinistra; *lym.*, initial anlagen of the primary maxillary sinus.

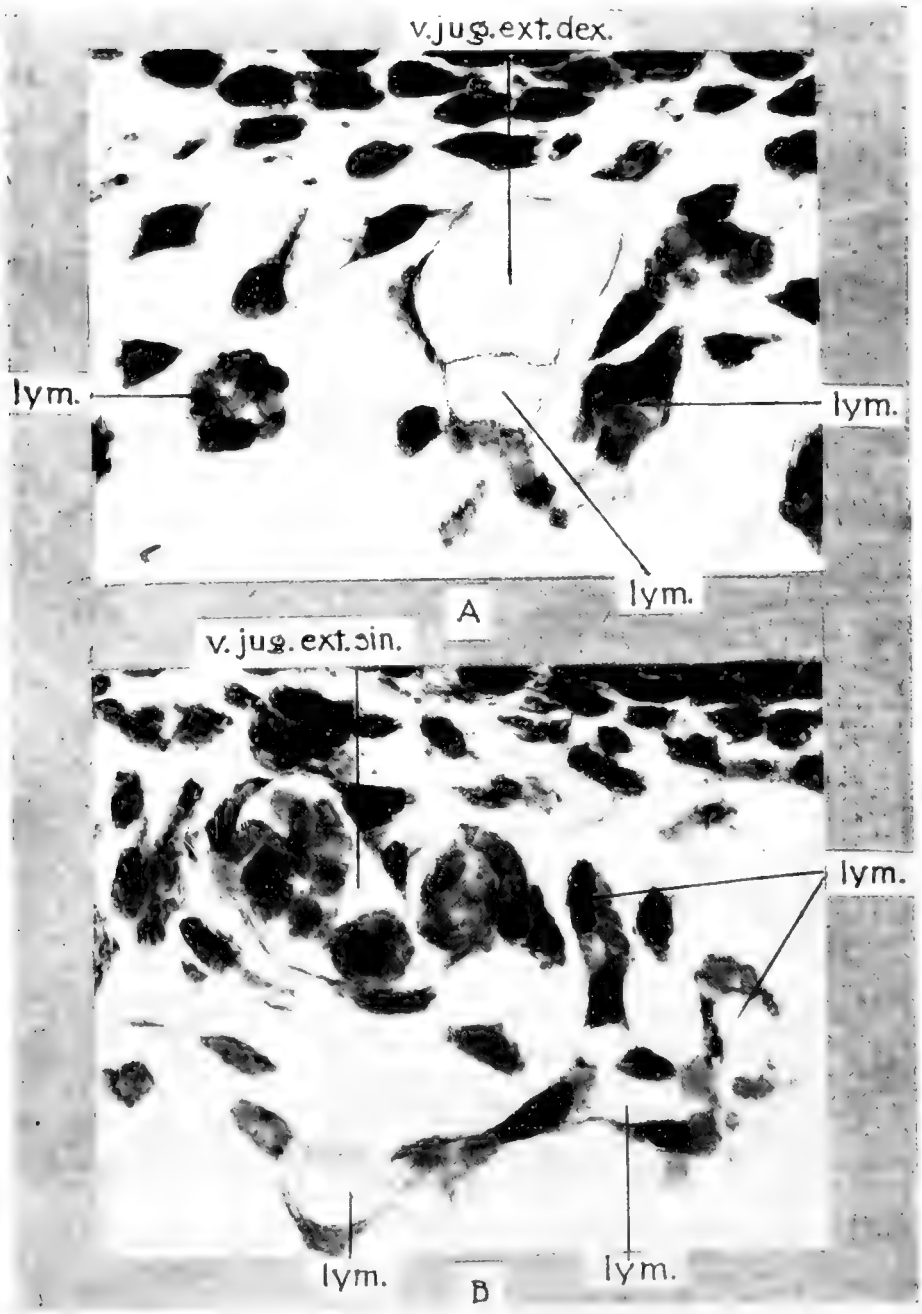

Fig. 10 (*A*) Photomicrograph of a transverse section through the right ventral cephalic region of a 6-mm. embryo of Bufo vulgaris (K. E. C., series B 53, slide 1, section 90). × 690. (*B*) Section through the left ventral cephalic region of a 7-mm. embryo (series B 52, slide 1, section 84). × 690. *v. jug. ext. dex.* and *sin.*, vena jugularis externa dextra and sinistra; *lym.*, anlagen of the primary maxillary sinus.

first a cleft or vacuole in the cytoplasm[9] between the large prominent yolk globules (fig. 10, *A*), or if the anlage be larger, it may consist of a number of crevices which soon coalesce to produce a more conspicuous cavity, (figs. 9. *A*, and 10). The lumen naturally expands with the growth of the anlage, but during several successive stages the confines of these lymphatics, like those of the haemal vessels, remain irregular and of varying thickness and appear gnarled, particularly in section (fig. 10), owing to the groups of large ovoid yolk globules which they contain and which do not entirely vanish until a relatively late period of sinus formation.

From now on the development of the sinus makes rapid progress. The discrete lymphatic anlagen of the same side establish continuity with one another by end-to-end fusion and begin to send out endothelial extensions in a ventromedial direction. These sprouts actively proliferate, branch and rebranch, and freely anastomose with one another in such a way as to produce a plexus, the meshes of which all lie in the same plane. As the identical condition prevails on the opposite side of the head, the two plexuses approach each other, meet and combine in the midline and so create a broad intricate network (fig. 11, from *lym.* to *lym.*) extending in a curved plane from the vicinity of one external jugular to that of the other through the loose mesenchyme between the thyroid and the epidermis on the ventral surface of the head. This network is the anlage of the principal or mandibular division of the primary maxillary sinus and is shown in the reconstruction in figure 30 (*si. mand.*). In the drawing, the vascular channels are pictured in a flat plane, though in reality the most distal structures bend dorsolaterally. It may

[9] The formation of the lumen, as indicated, raises the question, whether it is of intracellular or intercellular origin. The answer rests partly on our definitions of 'cell' and 'syncytium.' Are the spaces of mesenchyme to be considered as 'intercellular' or 'intracellular'? The originally solid lymphatic anlagen, described above, are probably, like other mesenchymal tissue, syncytial in nature, and accordingly I would look upon the vacuole-like beginnings of their lumen as being intracellular in situation. Subsequently, with the expansion of the lymphatic anlage into a definite vessel and the appearance of distinct cell boundaries in its endothelium, the lumen acquires its intercellular character.

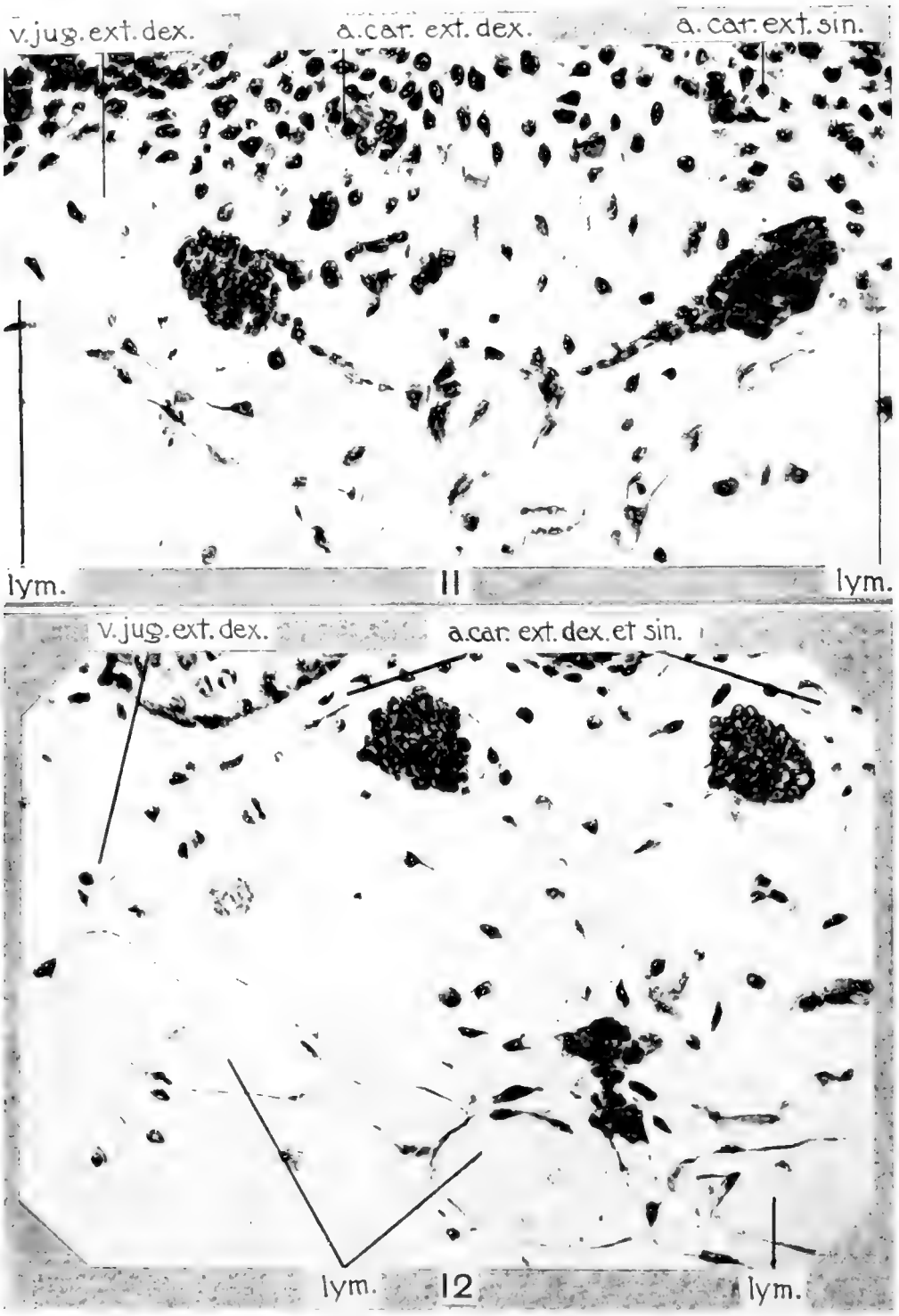

Fig. 11 Photomicrograph of a transverse section through the ventral cephalic region in a 7-mm. embryo of Bufo vulgaris (K. E. C., series B 27, slide 1, section 100). × 290 (Leitz 4-mm. Obj. and Zeiss Compensat. Project. Oc. 4). *a. car. ext. dex.* and *sin.*, arteria carotis externa dextra and sinistra; *v. jug. ext. dex.*,

be noted that the outer limit of the principal or mandibular plexus is sharply defined by a pair of broader, longitudinal vessels which genetically represent the oldest portion. In this stage (a 7-mm. embryo) also the anlagen of the other divisions have made their appearance as outgrowths from the principal one. Anteriorly, the circumoral division (*si. circor.*) is an extension, on either side, growing forward along a ring-like vessel which is a branch of the external jugulars and encircles the mouth opening. Eventually the two halves of this division, by further elongation, meet and unite in front of it. Posteriorly, the pericardial division (*si. pericard.*) of the sinus consists of a pair of caudally directed extensions, closely accompanying the external jugular veins towards the sinus venosus, where in subsequent stages they become prolonged outward to join the terminal portion of the temporal division of the same side. In the reconstruction under consideration each temporal division (*si. temp.*) is shown as a derivative of a slender lymph vessel which extends laterally around the oropharyngeal cavity in association with a tributary of the external jugular vein. At this time the temporal division has already made considerable advance in plexus formation, but further tips continue to proliferate and to anastomose. One of these offshoots, passing back in the broad expanse of loose tissue lateral to the aortic arches, is highly distended locally (fig. 30), a condition manifestly produced by the pressure of the lymph collected within its lumen. A similar saccular enlargement of the temporal plexus exists also on the opposite side. In several sections on the right side, the writer was unable to follow with certainty its connection with the remaining part of the plexus, and on the reconstruction this point has been indicated by an

vena juglaris externa dextra; the heavy masses in the center of the figure are muscle anlagen; in a curved line from *lym.* to *lym.*, sections of the interanastomosing channels of the primary maxillary lymph sinus during its plexiform stage (cf. fig. 30).

Fig. 12 Photomicrograph of a transverse section through the ventral cephalic region in an 8-mm. embryo of Bufo vulgaris (K. E. C., series B 49, slide 1, section 88). × 290. *lym.*, the channels of the plexiform primary maxillary sinus are beginning to coalesce with one another by their expansion. Other references as in figure 11.

interrogation mark. It is probable that during the fixation of the embryo, the very slender connecting channel had collapsed or contracted into such a delicate strand that it became impossible to distinguish it from the surrounding mesenchymal reticulum.

The reconstruction (fig. 30) shows that the primary outgrowths of the circumoral, temporal, and pericardial divisions from the principal plexus keep close to haemal vessels, potential veins. In fact, frequently, and particularly in the case of the pericardial division, the lymphatic extension adheres to the wall of the blood vessel. The writer has been unable to decide whether or not, in the elongation of such lymph channels, the endothelium of the blood vessel contributes cells to the growing tip. It is conceivable that the latter might simply advance along a path of least resistance or in accordance with certain stresses or currents that may closely parallel the blood vessel. As yet we are entirely ignorant of the presence or absence of any such pronounced currents in the tissue interstices before the advent of the haemal and lymphatic capillary systems, and the suggestion that the paths invariably taken by these primary lymphatic extensions may be predestined by the existence of definite antecedent streams, acting as a stimulus or directive force to the proliferating endothelium, is pure conjecture. A cross-section of the pericardial division illustrating the adhesion of the lymph vessel to the haemal one is shown in figure 14 (*lym.* and *v. jug. ext. sin.*).

During the formation of the plexus phase of the primary maxillary sinus, the sprouts and the most recently established anastomoses are usually solid, the acquisition of lumina, however, occurring very soon. During this period, too, the number of yolk spherules in the lining cells are still very abundant.

The next phase in the development of the lymph sinus is the transformation of the plexus into a spacious and uninterrupted chamber. This process is a rapid one, being practically finished in the embryonic period between 8- and 10-mm. stages (B. vulgaris). The genetic changes consist in the progressive expansion of all the anastomosing channels, so that the gaps in the network are reduced and the mesenchyme filling them is compressed

into trabeculae, which become more and more attenuated, and finally break and disappear as the sinus becomes more greatly distended in its vertical, that is, dorsoventral, diameter. These successive steps are clearly exhibited in the inserted photomicrographs, figures 11, 12, and 13, the last two illustrating how the mesenchymal strands are drawn out and tear and how their remnants persist for a time as longer or shorter spurs which project into the sinus cavity.

During the further growth and enlargement of the sinus, I was unable to find the addition of separate mesenchymal spaces by concrescence, such as I described in the development of the thoracic duct in the pig ('12) or those of McClure ('15) in the formation of the subocular lymph sac in the trout, or those of Huntington ('11) on the growth of the periaortic lymphatics in Chelydra.

I can but believe that the coalescence of the originally discontinuous lymphatic anlagen, the formation of the intricate lymphatic plexus and its conversion into the relatively enormous sinus is largely, perhaps wholly, due to the accumulation within their lumen of lymph, which, as it increases in quantity, increases the internal pressure on its walls and achieves the extension and distention of the developing sinus, for *during this important genetic period the sinus possesses no outlet; it is not confluent with the veins.* The saccular and expanded posterior prolongations of the temporal plexus shown in the reconstruction (fig. 30) certainly point to such an interpretation. A similar view was expressed by McClure ('15) in his preliminary paper on the development of the anterior lymphatics in teleost embryos.

Coincident with the expansion of the lymph sinus, its lining cells assume all of the attributes of typical endothelia. The cells become much flattened, and their nuclei, which in earlier stages resembled those of mesenchyme in their spherical shape and their coarse chromatic texture, become more and more compact and dense like the intimal nuclei of older vascular channels. The yolk corpuscles in the cytoplasm of the endothelium also gradually disappear, although in 9- and 10-mm. embryos a few are still to be found.

In embryos, approximately 10 or 11 mm. long, the primary maxillary sinus acquires an outlet. The posterior extremity of the temporal division by further backward prolongation (figs. 34 and 35) becomes confluent with the jugular lymphatic, which in turn gains access to the anterior lymph heart and conveys thither the lymph collected by the sinus.

During the later larval and metamorphic periods, the primary maxillary sinus, as well as the other lymph channels laid down in the embryo, are converted into the superficial and deep lymph sacs found in the adult.[10] Such changes will be reserved for a later paper.

From the foregoing account of the appearance and relations of the early adherent anlagen of the primary maxillary lymph sinus, reinforced by the evidence of the photomicrographs illustrating it, the conclusion is forced upon one that they are probably derivatives by proliferation from the walls of the external jugular components of the early unspecialized jugulocarotid vascular plexus. Formerly ('15) I believed that these observations afforded fairly decisive evidence in favor of the origin of lymphatics from venous epithelium, and I suggested tentatively that certain discontinuous mesenchymal spaces of Amniotes, which had been described previously as incipient lymphatics, might have been derived early from neighboring blood channels in a manner hardly perceptible on account of the absence of any special differential characteristic in either the vascular intima or the mesenchyme. But after investigating more thoroughly other lymphatic channels in anuran embryos, as well as considering the evidence contained in the mass of literature which has accumulated in recent years on the problem of vasculogenesis, that opin-

[10] In The Anatomical Record, vol. 16, 1919, the writer stated that topographical relations and genetic data show the primary maxillary lymph sinus of anuran tadpoles to correspond to the subocular lymph sinus of fishes. Since then I have carried on a comparative study of the lymphatic system in the different classes of vertebrates, and the available data force me to modify that statement. Possibly only the dorsal lateral extensions of the principal portion of the primary maxillary sinus are concerned in the homology. Further observations bearing on this question will be considered in the comparative anatomy of the lymphatic system which is in process of preparation.

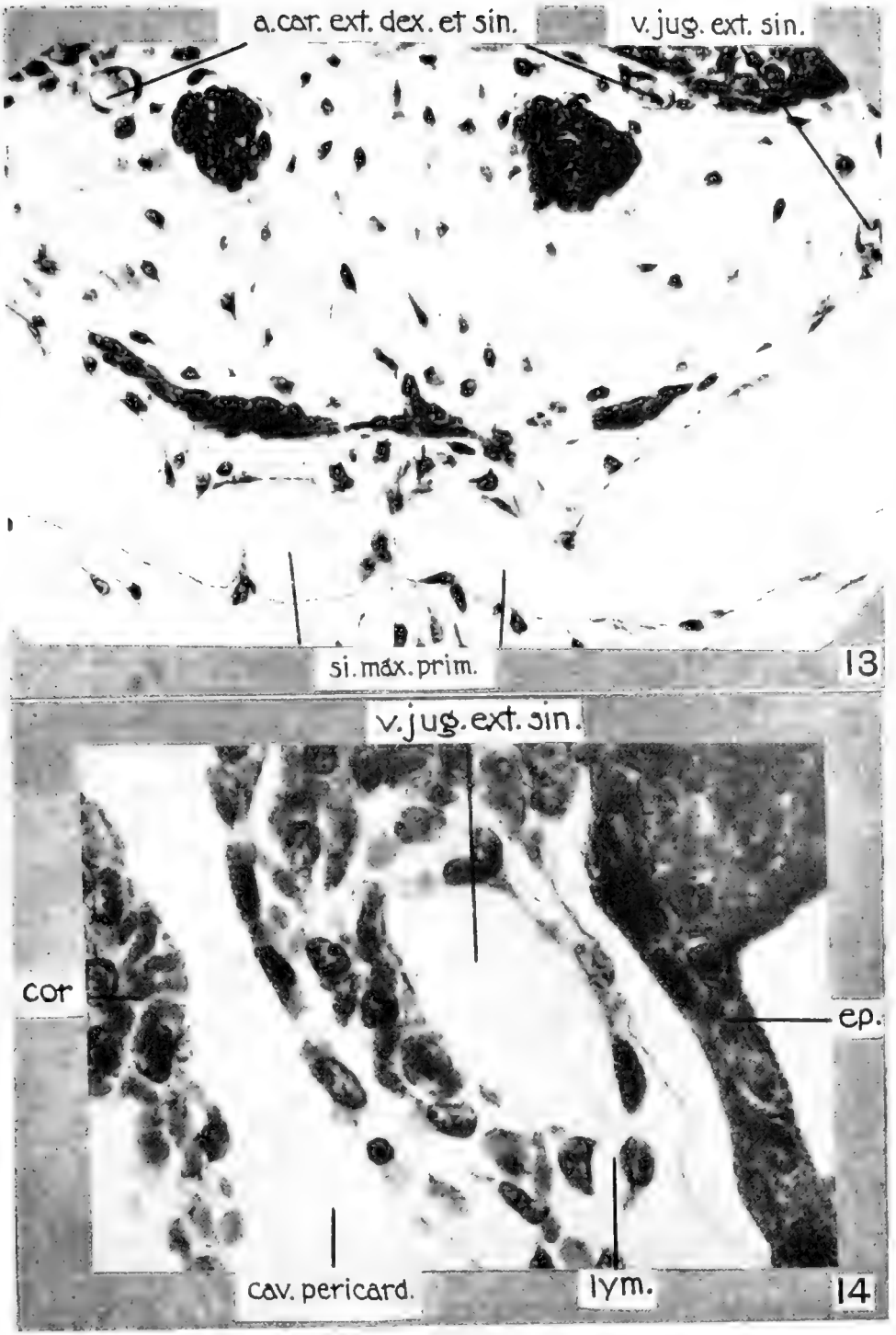

Fig. 13 Photomicrograph of a transverse setion through the ventral cephalic region in a 9-mm. embryo of Bufo vulgaris (K. E. C., series B 2, slide 1, section 115). × 290. *si. max. prim.*, primary maxillary lymph sinus; the spurs of tissue projecting into its lumen are the vestiges of the former bands of mesenchyme between the lymph channels during the plexus stage of the sinus. Other references as in previous figures.

Fig. 14 Photomicrograph of a transverse section through the left ventral region of the body at the level of the heart in a 7-mm. embryo of Bufo vulgaris (K. E. C., series B 27, slide 1, section 178). × 690. *cor*, wall of the heart; *cav. pericard.*, cavum pericardium; *ep.*, epidermis; *v. jug. ext. sin.*, vena jugularis externa sinistra; *lym.*, anlage of the pericardial division of the primary maxillary sinus.

ion loses weight—a source of gratification to the writer in so far as he is not compelled to regard his earliest work ('12) as fundamentally wrong in its deductions. The recent investigations have brushed away many difficulties, and the interpretation of the observations rests on firmer ground; views which were thought conflicting only a few years ago can now be reconciled.[11] The origin of the earliest vascular anlagen in the embryo is the basic problem of vasculogenesis, not that of vessels, regardless of their vascular function, which develop later. If it be true that the earliest anlagen arise from the mesenchyme, as most of the modern research on vasculogenesis would indicate, then the two views of lymphatic development, the venous origin and the mesenchymal origin of lymph vessels, are not in diametrical opposition as was formerly vehemently asserted. Indeed, there may be a number of variations in the genesis of such channels, but the differences can now be judged superficial, presupposing, of course, that the haemal and lymphatic systems are not primordially and phyletically distinct, as some investigators tacitly hold. Lymphatic anlagen may proliferate from components of the early indifferent embryonic vascular plexus, or certain channels may separate from it (just as arteries and veins are differentiated from it) and assume a lymphatic function; and, again, they may be formed directly from mesenchyme independently of vascular channels already existing. What determines the several variations of lymphatic development is still obscure, although the time and the site at which they first appear, as conditioned by physiological needs, may be the causative factors. The first method is illustrated by the origin of the primary maxillary sinus which, with the exception of the anterior lymph hearts, is the first lymphatic to appear in Bufo embryos, and perhaps most of the anlagen of which originate, as has just been described, in connection with the endothelium of potential haemal vessels before that endo-

[11] Anyone wishing to follow the controversy regarding the origin of lymphatics is referred to the numerous papers which have appeared during the last decade in America on the problem of lymphatic development. The larger papers of Sabin, Huntington, and McClure on the development of the mammalian lymphatic system contain a comprehensive list of the literature.

thelium has become specialized, that is, has acquired the attributes of the typical flattened lining cells. The second method is seen in the formation of the jugular lymph sac of mammals, and in that of the anterior lymph hearts and the jugular lymph ducts of the toad. The development of the latter structures will be described in following sections of this paper, but it may be stated here that they arise from vessels which, at first, are freely confluent with the embryonic blood vessels and function as such, but later separate and become an integral part of the lymphatic channel system. The third method, the formation of a lymph vessel by the fusion of mesenchymal spaces, somewhat like the origin of the earliest vascular channels, is illustrated by the development of a considerable portion, at least, of the thoracic duct and other large lymph vessels in mammals, birds, reptiles, and fishes, as portrayed in numerous papers that have appeared within the last decade. It was therefore not surprising to discover this method active also, perhaps solely, in the formation of the large lymph ducts in Anura which arise later than the anterior lymph hearts and primary maxillary sinus, at a time when the blood circulatory system of the embryo had become better organized and its components more specialized.

THE DEVELOPMENT OF THE JUGULAR LYMPHATIC

Hoyer describes the development of the jugular lymphatic (cephalic duct) in frog embryos as a centrifugal outgrowth of the anterior lymph heart, but the writer's observations show that in toad embryos, at least, its origin is not so simple.

The jugular lymphatics (fig. 28, *lym. jug.*), one on each side, develop at the same time as the anterior lymph hearts and in the same general region so that they might be discussed together, though for systematic reasons they will be treated separately. Figures 31 to 35, inclusive, which illustrate reconstructions of the important structures in the territory of the left pronephros in several consecutive stages, furnish a clear idea of the salient and progressive events that occur. Besides the vascular channels which are directly and indirectly concerned in the formation of the lymphatics, other organs, such as the pronephros, spinal

ganglia[12] and a segment of the neural tube, were introduced in the reconstructions for the purpose of orientation.

I ('20) described the series of intersegmental vessels that appear in the development of the venous system as dorsal tributaries of the pre- and postcardinal trunks and found them situated at the intersegments of successive myotomes. The first two reconstructions (figs. 31 to 32), besides illustrating the beginning of the anterior lymph heart as a circumscribed plexus of the proximal portion of the third intersegmental, shows the development of a more open-meshed network of vessels formed by anastomoses between the first, second, and third intersegmentals. The jugular lymph duct is derived from the latter plexus. Passing from the 6- to the 7-mm. stage, the genetic changes consist in the separation of this intersegmental plexus (which in view of its former and its future functions may temporarily be called a venolymphatic) from the precardinal vein and the pronephric venous sinus. The channels of connection contract in caliber, like any other small redundant vessel, and finally are cut off entirely. This is indicated in the reconstruction in figure 33, where the points marked by a star still show minute and slender connections, the last traces of the originally freely confluent condition of the intersegmental veins and their parent trunk. Farther forward in the figure are two other vestiges in the form of venous spurs extending towards the lymphatic duct. As the veno-lymphatic plexus (potentially lymphatic) is severed from the veins, its channels distend, evidently due to the accumulation of the lymph within their lumen.

While the foregoing is taking place, a notable event, the transient isolation of the lymph heart anlage from the surrounding lymphatic plexus, occurs, a process which will be considered more fully in the following section. Such a phase is illustrated in the reconstruction in figure 34. The secondary junction between heart and afferent lymphatic is brought about a little later. The

[12] The first pair of spinal ganglia are evanescent structures in the anourous Amphibia, being present in toad embryos (B. vulgaris) only during the 6-mm. stage and vanishing completely very soon after. The second pair become the first of the adult.

reconstruction exhibits a number of other features. The jugular lymphatic (*lym. jug.*) by growth cephalad and the temporal division of the primary maxillary lymph sinus (*temp. si. max. prim.*) by growth caudad (figs. 30, 34, and 35) have met and become continuous. Further, as shown in figure 34, the jugular and the lateral-line (*lym. lat.*) lymphatics, united from the beginning, develop prominent ventral branches lateral to the pronephros. The other tributaries, extending dorsally and showing a metameric tendency, unquestionably represent the distal portions of the intersegmental vessels from which the jugular lymphatic was derived. Finally, the minute connection (starred) between this plexiform duct and the pronephric sinusoids may be noted, which has managed to persist until this time. In a later stage (10-mm. embryo) the jugular, in common with the lateral-line lymphatic, has reunited with the lymph heart, and farther forward the junction with the temporal division of the primary maxillary sinus has expanded (fig. 35).

A few words respecting the venous circulation of the region under consideration will explain certain difficulties. Since the anterior intersegmental veins function as haemal conduits before their transformation into the plexus of the jugular lymphatic, as soon as their complete separation from the cardinal venous trunk is accomplished, the region of the myotomes which they drained would be left without a blood vascular return, but for the development of secondary channels from the cardinal veins. Accordingly, such tributaries are laid down at this time, but they are situated chiefly on the inner suface of the myotomes and accompany the spinal nerves and ganglia; here, besides receiving branches from the myotomes, they communicate broadly with similar channels from the aorta. In order not to complicate the reconstruction more than was necessary, the entire medial blood vascular plexus, except the main cardinal tributaries, was omitted. Besides these medial segmental tributaries, two or three lateral ones develop (figs. 34 and 35) in proximity to the lymph heart and are closely pressed against the outer side of the myotomes. At a later period, these venules anastomose, become larger, and combine to form the definitive anterior vertebral vein and its branches (fig. 35).

THE DEVELOPMENT OF THE ANTERIOR LYMPH HEARTS[13]

Jourdain ('83) probably was the first to make a statement respecting the development of the lymph hearts in Anura. But his paper is chiefly concerned with the formation of several lymph sinuses in the frog, and his allusion to the hearts is very cursory, these being dismissed in a few sentences. He pointed out that a small pulsating vesicle, the posterior lymph heart, is visible, one on each side, at the base of the tail in tadpoles on which the hind limbs are budding, and that it conducts the lymph into a branch of the postcardinal vein. On the other hand, the lymph from the anterior regions of the larva flows directly, according to him, into the precardinal vein, as in fishes. The anterior pair of lymph hearts are considered independent (?) structures, which do not appear until the pectoral girdle has been formed.

[13] It would seem superfluous again to draw the distinction between 'lymph heart' and 'lymph sac' or 'sinus', were it not for the confusion of terms and ideas that is evident in several recent papers on the lymphatic system. In Mrs. Eleanor L. Clark's paper ('15) on the early lymphatics of the chick, the difference between lymph sacs and lymph hearts is disregarded, as may be instanced by the following quotation: "According to Baranski and Fedorowicz, the lymph hearts are formed from two or three lymphatics instead of from a luxuriant plexus, as in birds and mammals. However, Knower and Kampmeier state that in frog and toad embryos, the anterior lymph heart is formed from numerous lymphatic capillaries." In criticism, I wish to state that the researches of Baranski and Fedorowicz, here mentioned, deal only with the genesis of the posterior lymph hearts in Anura, and genetic peculiarities distinguish them from their fellows in the anterior region of the body. Further, Knower is mistated, and my paper, to which reference was made, published in 1915, is absolutely not concerned with the development of the anterior pair of lymph hearts, but describes the origin of a few lymphatic ducts and especially that of the large lymph sinus of the head which is not the same thing as the lymph heart. My studies of the heart were briefly reported for the first time before the American Anatomists during the Christmas holidays of 1916, a year after Mrs. Clark's article appeared. In the Amphibia, lymph heart and lymph sac or sinus are distinct structures, one possesses muscular walls and pulsates, the other is a modified lymph duct or a transformed lymph capillary plexus.

Miss Sabin also uses lymph heart and lymph sac indiscriminately. Thus ('13, p. 56), she has the following sentence: "Weliky, Jossifov, and Favaro thought that the posterior lymph heart arose from the dilatation of the caudal lymph trunks which grow from the anterior lymph hearts, and Jourdain describes them as being formed by a rapid destruction of connective tissue." Jourdain's account, here mentioned (Comptes Rendues, 96, 1883), does not pertain to the formation of lymph hearts, but refers to that of the lymph sacs.

In 1891 Field published his excellent work on the development of the pronephros in the frog, and in a footnote ('91, p. 240) described briefly, though correctly, a 'peculiar sac' which he found in 8-mm. embryos lateral to the myotomes at the niveau of the third nephrostome and joined to the postcardinal vein. "Respecting the fate and the significance of this singular structure," he says, "I have no suggestions to offer." Reference to this observation is made by Gaupp ('99, pt. 2, p. 380) who calls it a 'Blutbläschen' of unknown function. We now know that this enigmatical organ is the anterior lymph heart.

Hoyer ('05), in his work on the formation of the lymphatic system in frog larvae, states that the anterior lymph heart makes its appearance during the stage when the external gills begin to vanish, as a small spindle-shaped evagination from the short anterior vertebral vein anlage at the point where this vein branches dorsally from the pronephric venous plexus. At that time the walls of the fusiform heart are composed of an inner endothelium and an outer layer of stellate cells. In 6-mm. embryos the heart has become larger, but it is still in broad, open communication with the vein and contains numerous blood corpuscles in its cavity. Its walls become thicker and a few cross-striated muscle fibers are visible in the outer coat. During this stage in the living specimen the heart occasionally quivers, but distinct rhythmic contractions do not become evident until later, when the embryo has reached the length of 12 mm. and the muscle elements have increased in number and in configuration. In the meantime valves have appeared, one at the junction of heart and vein and another at the opening of the lymph vessel into the heart. After the formation of these structures, blood cells are only exceptionally found in the heart chamber. The fundamental changes in the development of the heart have now occurred, and in subsequent stages it merely grows larger and acquires its definitive character. But it retains its original position lateral to the second myotome throughout the entire period of genesis and growth up to metamorphosis.

Knower, in a short paper ('08), remarks that the anterior lymph heart is the first lymphatic to be formed in the frog and

agrees with Hoyer that it makes its appearance in approximately 6-mm. embryos (Rana palustris, R. virescens, R. sylvatica).[14] He observed that during this period the heart is situated dorsal to the posterior end of the pronephros and arises from the 4th intersegmental vein, thus differing from Hoyer. Knower does not state explicitly how it originates, but I assume that he regards it as a local expansion of the vein. According to him, the heart opens directly into the plexiform venous sinus of the pronephros just back of the last nephrostome. Striated muscle fibers appear early in its walls, and in 8-mm. embryos already are arranged in bundles which branch freely; he believes that these fibers are derived from the adjacent myotomes, the fourth and the fifth, since the heart is developed in the intersegment in proximity to the ventrolateral portions of these muscle segments. Finally, he notes the development of valves at both the afferent and efferent portal of the heart.

A very brief preliminary account of the development of the anterior lymph hearts in Bufo was presented by the writer before the American Anatomists in 1916.

Morphogenesis

Except that they state definitely the time of appearance and the location of the anterior lymph heart in frog embryos, neither Hoyer nor Knower offers a detailed description of its formation; their accounts are brief and rather general. After more extensive study, in which numerous graphic reconstructions and some wax models were made, the writer is able to demonstrate with greater preciseness, perhaps, its origin and progressive changes. Such an exposition will show that, in Bufo embryos at least, its genetic history and the nature of its changes are not so simple as Hoyer's and Knower's descriptions would lead us to suppose. The first indefinite rudiments are already suggested in approximately 4-mm. embryos (Bufo vulgaris), thus appreciably earlier than

[14] The early origin of the anterior lymph heart in the frog was indicated by Knower five years earlier (before the American Society of Zoologists, 1903), two years before Hoyer's first paper on the development of the lymphatics in the frog appeared.

was specified for the frog; yet, I am inclined to believe that even in frog larvae the first hint of a lymph heart may be demonstrated earlier by means of reconstructions, which, if accurately executed,

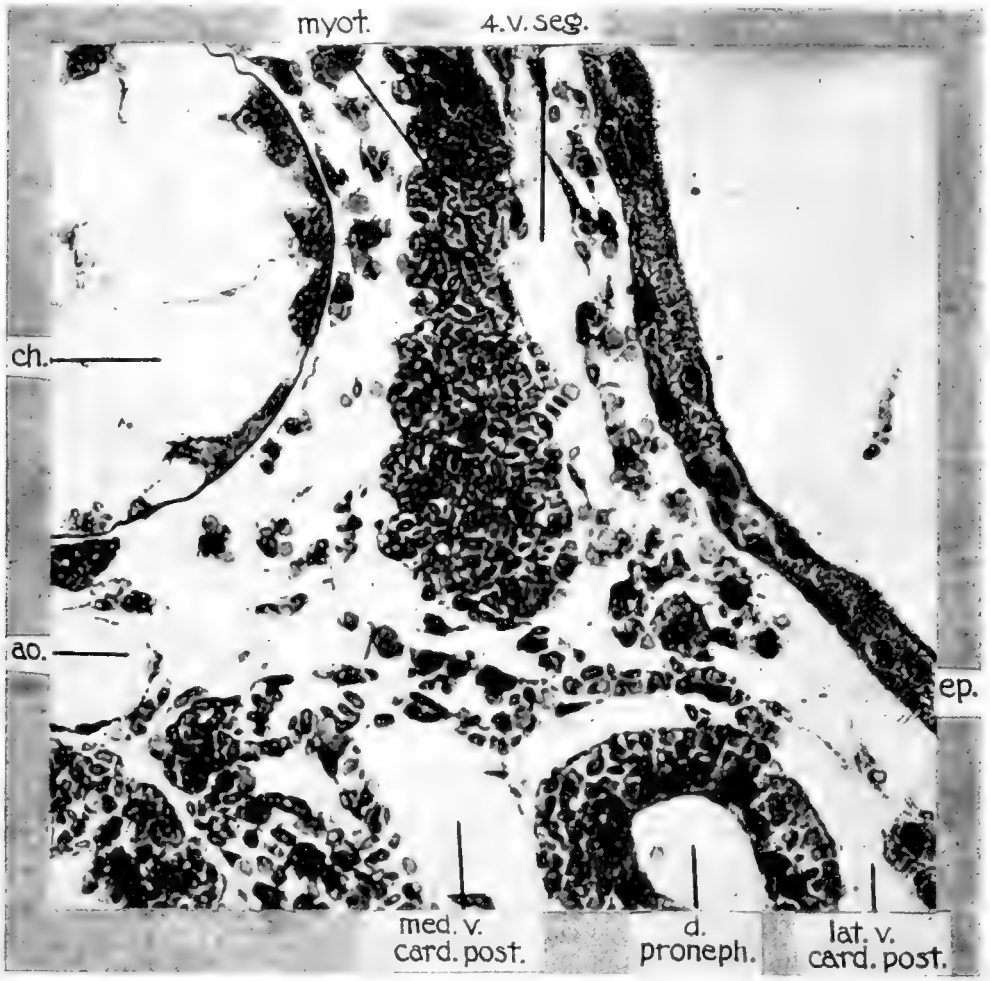

Fig. 15 Photomicrograph of a transverse section through the left lateral and anterior trunk region in a 5-mm. embryo of Bufo vulgaris (K. E. C., series B 44, slide 2, section 142). × 340. (Zeiss Apochromat. Obj. 8 and Compensat. Project. Oc. 4). *4 v. seg.*, 4th intersegmental vein; *med.* and *lat. v. card. post.*, medial (subcardinal) and lateral divisions of the postcardinal vein; *d. proneph.*, pronephric or primary excretory duct; *ao.*, aorta; *ch.*, chorda dorsalis; *myot.*, myotome; *ep.*, epidermis.

bring to view significant twists and turns and other topographical details that frequently escape the strictest scrutiny of serial sections. However, the relative time at which the lymph heart arises is a matter of little importance; we are interested more in

the manner in which it originates; but here, too, my observations are not in agreement with the views expressed by the above-named investigators. In Bufo embryos at least, it arises neither

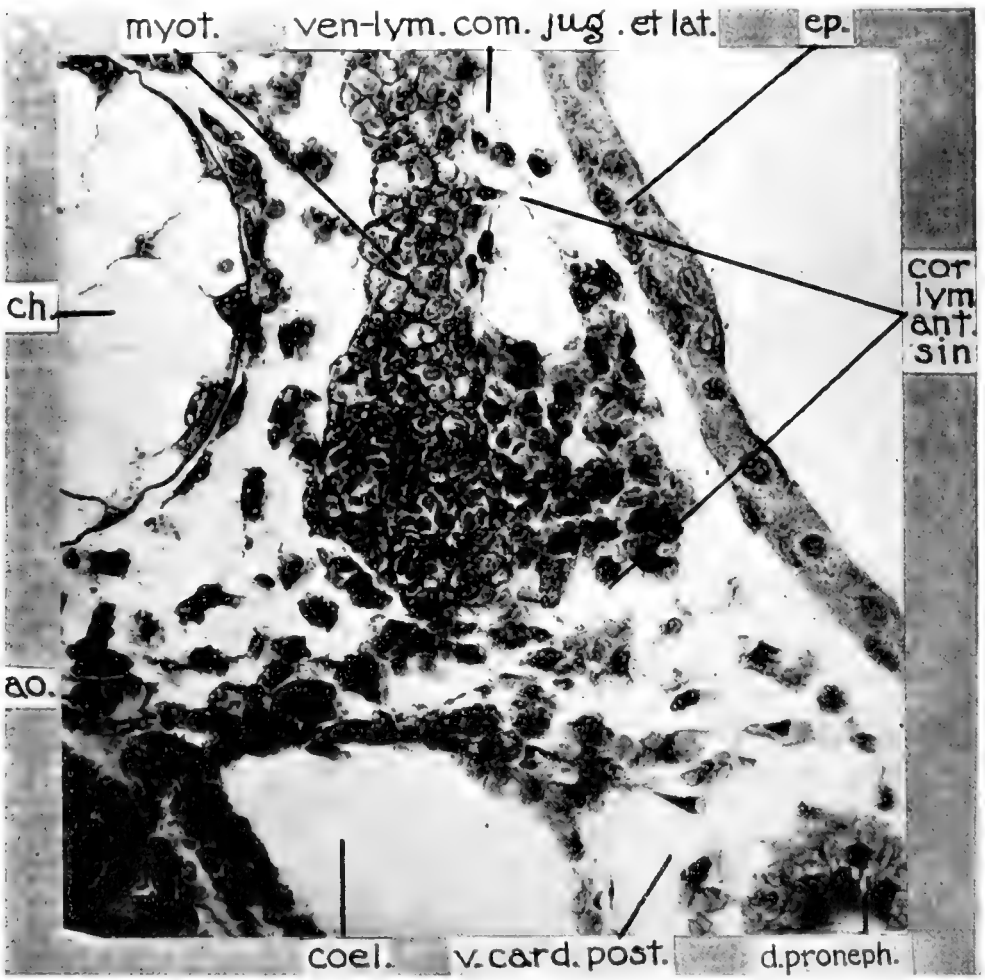

Fig. 16 Photomicrograph of a transverse section through the left anterior lymph heart region in a 5-mm. embryo of Bufo vulgaris (K. E. C., series B 44, slide 2, section 126). × 340. The line of demarcation and the difference of appearance between the cells of the myotome (*myot.*) and those of the lymph heart anlage (*cor. lym. ant. sin.*) is clearly expressed; *ven-lym. com. jug.* et *lat.*, common segment of the jugular and the lateral line venolymphatics. Other references as in figure 15.

as an evagination of the anterior vertebral vein nor as a direct expansion of a particular intersegmental vein, although it is conceivable how its appearance in certain developmental stages might lead to such suppositions. The readiest way of obtaining

a lucid idea of the morphogenesis of the anterior lymph heart is to examine a consecutive series of reconstructions representing different genetic stages. Such a series is pictured in figures 31 to 35, inclusive, attention to which has already been directed in the preceding section on the development of the jugular lymph duct.

The reconstruction in figure 31, reproducing the conditions in a 4-mm. embryo, shows the vague beginnings of the anterior lymph heart as an incipient vascular plexus between the second and the fourth intersegmental vessels and in connection with the proximal portion of the third. It is so inconspicuous and ill-defined that the observer would overlook it but for the striking changes that occur in the same locality soon after.

In 5-mm. embryos, the above venous, or better, venolymphatic plexus, the anlage of the anterior lymph heart, has become more sharply outlined. In comparison with the preceding stage, the plexus not only has joined the second and fourth intersegmental vessels by longitudinal anastomosis, but also has increased the number of its connections with the pronephric sinus from one, the original mouth of the third intersegmental, to several.

By the distention of the interjoined channels of the lymph-heart plexus, these coalesce, resulting in a single cavity. In figure 32 the loop-hole in the anterior part of the anlage is still indicative of its previous plexiform state. Viewed from the side, as pictured the contour of the anlage already suggests its future globular form. In reality, however, its shape at this time is lenticular, for its lateromedial diameter is not much greater than the third intersegmental vessel from which it sprang, and accordingly in transverse section through its center (fig. 17, *cor. lym. ant. sin.*) it would appear as a spindle-shaped expansion of this vessel. The connection of the heart with the pronephric venous sinus and with the surrounding intersegmental network, which, as already shown, is involved in the formation of lymphatic ducts, vary little in position and in number, as a comparison of several specimens of the same age has shown. At the lower margin of the lymph-heart anlage (fig. 32) the delta-like confluence with the pronephric sinusoids is to be regarded as a complication of the

mouth of the former third intersegmental vein, and the extensions at the upper margin as the distal portion of this vessel. The anterior and posterior junctions of the lymph heart with the

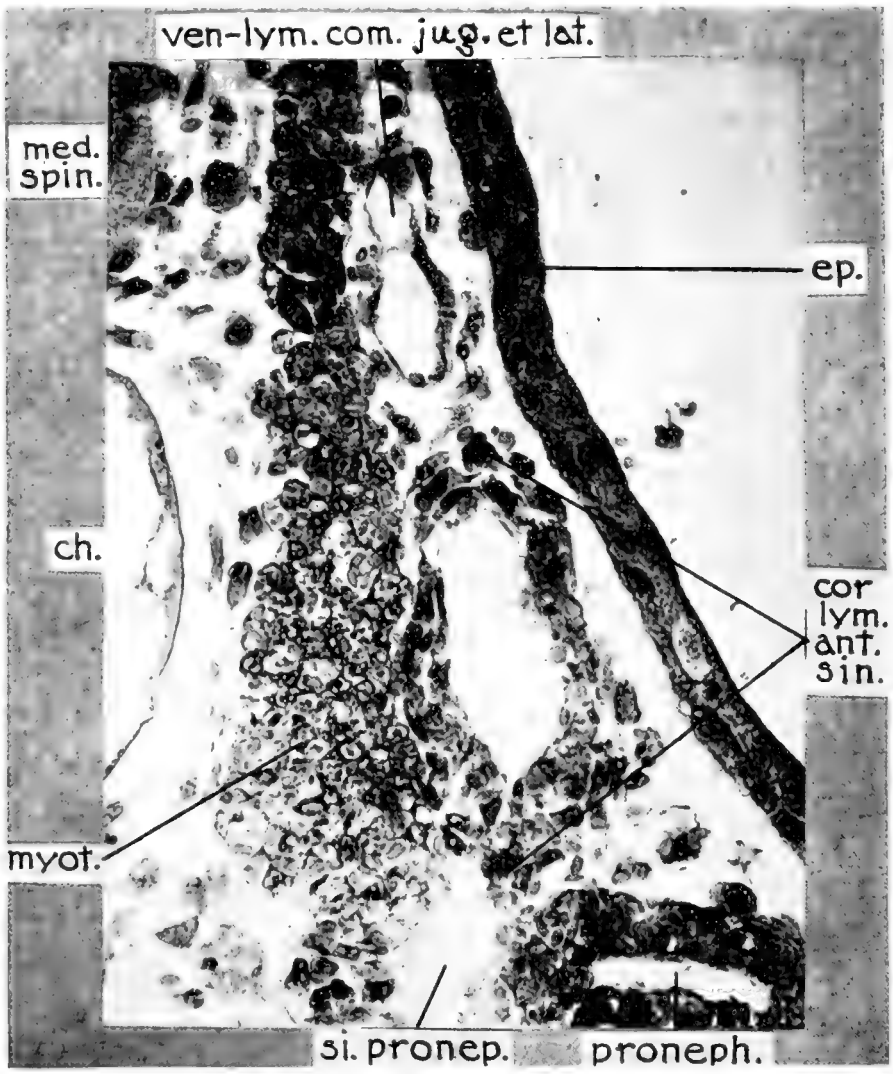

Fig. 17 Photomicrograph of a transverse section through the left anterior lymph heart region in a 6-mm. embryo of Bufo vulgaris (K. E. C., series B 54, slide 2, section 130). × 340. *si. proneph.*, the mouth of the original 3rd intersegmental vein, branch of the pronephric sinus; *proneph.*, pronephric tubule; *med. spin.*, medulla spinalis. Other references as in figure 15.

second and fourth intersegmentals, respectively, may also be resolved into plexiform channels. Thus, four fairly constant groups of connections may be recognized, a ventral, a dorsal, an anterior, and a posterior group.

In 7-mm. embryos, the connections between the lymph heart and the circumjacent venolymphatic plexus begin to break away. An early stage in this process is shown in figure 33. The anterior connection is still broad; on the dorsal surface of the heart one has already severed relations and another is very much constricted; the one on the posterior surface, too, shows signs of contraction when compared with its homologue in figure 32. On the ventral aspect of the heart one channel of confluence is just being pinched off, but the more anterior connections are fusing into one and so constitute the anlage of the anterior vertebral vein and the lymphaticovenous tap. During these progressive events, the heart becomes more spheroidal as the area between the myotomes and the epidermis widens, associated with the rounding out of the back and sides of the embryo (fig. 18).

In 8-mm. embryos, mere vestiges, in the form of small projections, remain of the former union between the lymph heart and the neighboring lymph vessels, as delineated in the reconstruction in figure 35, but in every case their coincidence with the points of union of earlier stages can be made out readily. At this period, then, there seems to be no open passage whatsoever between the cavity of the lymph heart and the remainder of the lymphatic conduit system. It is a blind globular chamber attached to the anlage of the vertebral vein at its anterior and ventromedial surface and is confluent with it.

During the period between 8- and 10-mm. stages the secondary or permanent communication is established between the lymph heart and the afferent lymph duct. A comparison of figures 34 and 35 and the photomicrographs, figures 19 to 23, shows plainly how this is accomplished. By uniform growth and dilatation of the lymph heart as well as of the circumjacent lymph vessels, the common segment of the jugular and lateral ducts (*lym. com. jug. et. lat.*) and the dorsal aspect of the heart are gradually brought together, this approximation continuing until the duct comes to lie in a shallow groove-like indentation or depression of the heart wall. Along this line of contact the first afferent ostium appears. In later stages, as more tributaries of the aforesaid lymph channels are formed, some of them, situated nearest the heart, cross over its surface and come to lie against it, and

eventually break through at certain points, so increasing the number of portals of entry for the lymph stream, as shown in the drawing (fig. 24) of the lymph heart in a young toad. In figure 35

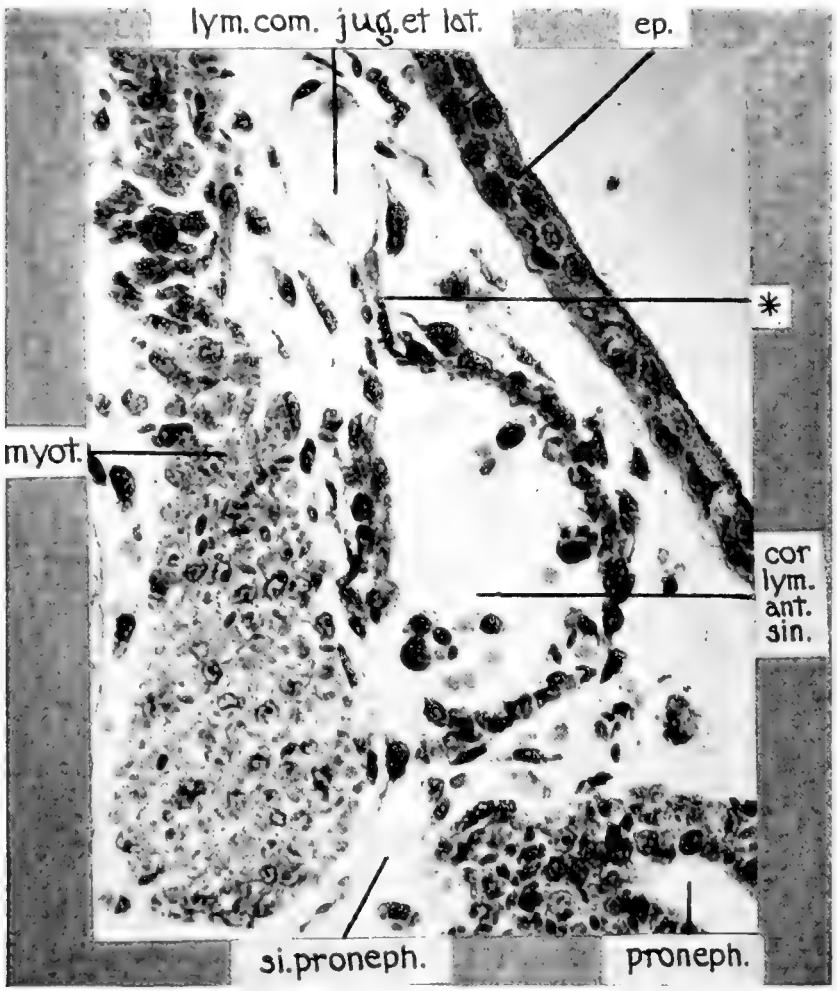

Fig. 18 Photomicrograph of a transverse section through the left anterior lymph heart region in a 7-mm. embryo of Bufo vulgaris (K. E. C., series B 27, slide. 2, section 83). × 340. *lym. com. jug.* et *lat.*, common segment of the lymphatica jugularis and lymphatica lateralis; *, temporary breaking away of the lymphatics just mentioned from the lymph heart (*cor. lym. ant. sin.*). Other references as in the preceding figure.

such a condition is already intimated by the lymph vessel which branches off from the jugular duct and passes diagonally over the outer surface of the lymph heart.[15]

[15] During the period of metamorphosis, the plexus of lymphatic vessels in the vicinity of the anterior lymph heart develop into the sub-scapular lymph sinus. The description of the transformation of the lymphatic system in the tadpole

One detail still remains to be considered in the morphogenesis of the anterior lymph heart, namely, the shifting of the efferent portal, or ostium venosum. In the earlier stages, this connection is with

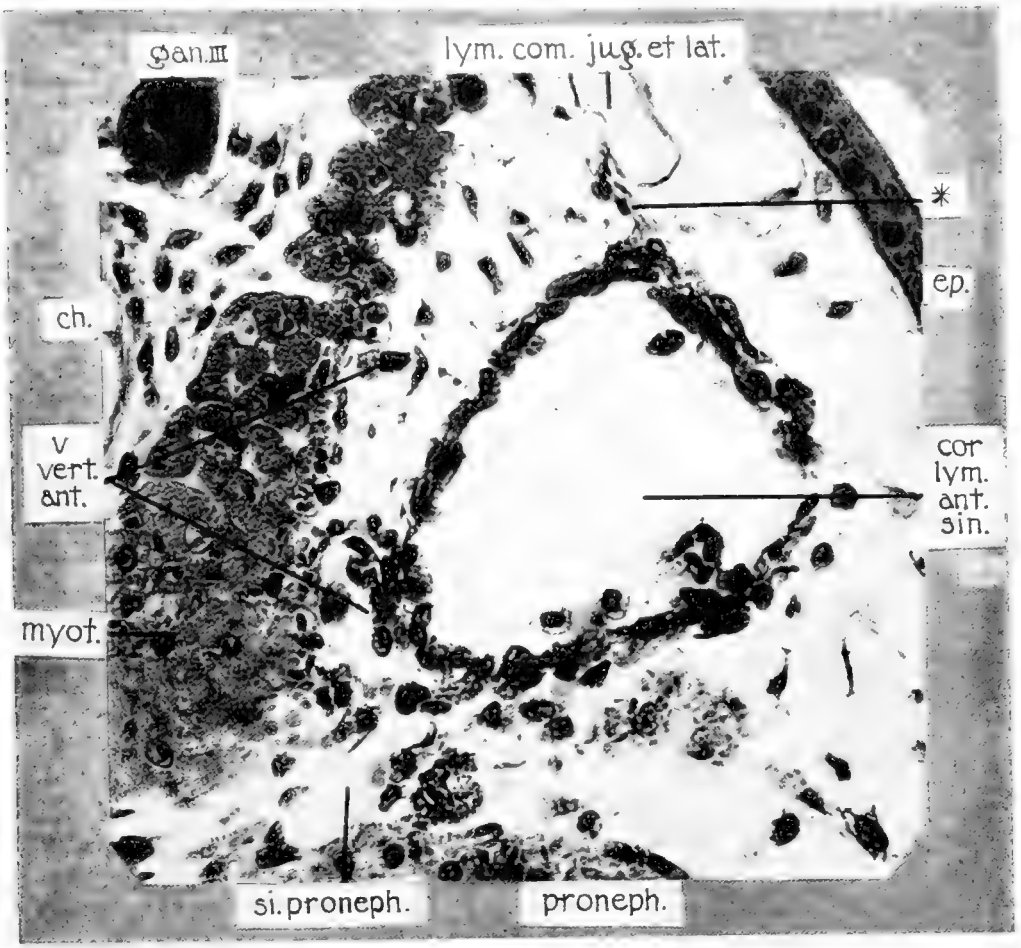

Fig. 19 Photomicrograph of a transverse section through the left anterior lymph heart region in an 8-mm. embryo of Bufo vulgaris (K. E. C., series B 49, slide 2, section 112). × 340. *v. vert. ant.*, vena vertebralis anterior, branch of the pronephric sinus (*si. proneph.*), its mouth being that of the original third intersegmental vein; *, point of former connection, in the form of a small spur, between the common segment of the jugular and lateral lymphatics and the lymph heart; *gan. III*, third spinal ganglion. Other references as before.

the pronephric venous sinus and is found squarely on the ventral side of the heart (fig. 32). This condition is changed by three factors: first, the breaking away of the posterior channel of the

into that of the fully formed animal represents another section of the original monograph and will appear later as a separate paper.

multiple junction and the amalgamation of the other channels into a larger one: secondly, the outgrowth of the anterior vertebral vein (*v. vert. ant.*) just internal to the latter, and, thirdly, the distention of the lymph-heart cavity, the bulging of which is more pronounced laterally than medially where the myotomes resist its expansion (cf. photomicrographs figs. 18 to 22). As a result of the interaction of these factors, the lymphaticovenous tap is shifted forward and medially. The ultimate condition has not yet been attained, however, for in the young toad the junction is at the anterior, more conical, end of the heart (fig. 24). Between the stage figured in figure 35 and the final one, it is evident, therefore, that considerable displacement still occurs, but to specify all of the underlying causes is impossible and is of little importance. Unquestionably, it is correlated with the stresses and strains due to other bodily changes that take place in the neighborhood of the lymph heart during development, such as the atrophy of the pronephros and the proximal segment of the postcardinal,[16] the absorption of the pronephric sinus by the internal jugular, the consequent shifting of the mouth of the anterior vertebral vein, the differentiation of the myotomes, and the rearrangement of the resulting muscles, to mention only a few of the most evident modifications.

Histogenesis

a. The lymph heart wall. Having described the conformation of the lymph heart and its venous relations, the development of its walls and valves remains to be discussed.

In early stages (4- and 5-mm. embryos) when the anterior intersegmental vessels have just been established, the area between the epidermis and the myotomes is very narrow, not much wider than is sufficient to accommodate these vessels (fig. 15). The mesenchyme, too, is very scanty here except in the region of the 3rd intersegmental vein, where its yolk-laden cells soon become more numerous and are locally massed against

[16] The medial division of the postcardinal vein has been shown by the writer (Anat. Rec., vol. 19, 1920) to correspond to the sub-cardinal vein of higher vertebrates.

the confines of that channel. This is especially true during the stage when the lymph-heart anlage is plexiform, a section of which is shown in the photomicrograph, figure 16 (*cor. lym. ant. sin.*). As suggested in the figure, the masses of mesenchymal cells are not uniformly arranged around the outlines of the lymph-heart plexus, but are irregularly distributed, at one level being crowded against its lateral side, at another, against its medial, and more frequently between the meshes of its interanastomosing channels. Incidentally, it is evident that the reconstruction, which simply represents an enlarged cast of the lumen of the channels, does not exhibit all of the essential features of the developing structure, and to acquire a correct conception of the genetic processes, the sections as portrayed in the photomicrographs must be examined together with the reconstructions. The mesenchymal cells are so closely packed together and so filled with yolk globules that it is impossible to determine their individual boundaries. At several points, too, such aggregations seem to bound the cavity of the lymph heart anlage directly, at least no distinct intima lining it can be recognized. Otherwise the lining is quite sharply defined, though only part of the endothelial cells tend to the flattened shape, while others still retain the unspecialized form in which the nuclei are in general either oval or spherical and resemble those of ordinary mesenchymal cells. The nuclei of these mesenchymal masses stain deeply and are coarsely chromatic, and many of them have an indented circumference which conforms to the large yolk bodies in the cytoplasm.

At another level of the lymph-heart plexus, just back of the section shown in figure 16, primitive spherical blood cells, also stuffed with yolk globules, are crowded together and block the lumen of a connecting channel. Generally speaking, there is already a marked difference between the nuclei of circulating blood cells and those of mesenchymal cells; the former are dense and opaque and take almost a black color when stained with haemotoxylin, while the latter possess lighter staining areas between the large chromatic granules. A few exceptions, however, were observed; several of the nuclei of the mesenchymal

cell aggregations approach the haemal nuclei in density, though the writer is unable to demonstrate decisively the transition and conversion of one into the other. This observation immediately calls to mind the researches of Miller ('13) and Allen ('13) on the development of the thoracic duct in the chick and the caudal lymph heart in Polistotrema stouti, respectively, where it was discovered that some mesenchymal cells were converted into blood cells during the early genetic stages of these lymphatics. Consequently the question arises: Does the incipient anterior lymph heart in Anura also function transiently as a haemopoietic organ? Do some of the cells of the mesenchymal masses contiguous to and between the channels of the plexiform anlage become differentiated into blood corpuscles? All my efforts to demonstrate this proved futile in the face of that prime obstacle, the abundance of yolk, which obscures and erases the more delicate tissue distinctions.

In 6-mm. embryos not only has the periphery of the lymph-heart lumen become more definite than in the previous stage, but the surrounding masses of mesenchymal cells are becoming more evenly spread out over its outer surface (fig. 17).

In the next older stage (7 mm.) the rearrangement of the cells composing the walls of the lymph heart is such that in general two layers may be distinguished (fig. 18), a lining or internal layer and a covering layer, but which, as yet, are not sharply delimited. This indistinctness is further emphasized by the fact that the intimal cells are not all flattened, as we should expect in this relatively advanced stage, but still retain their generalized character. Indeed, one is not able to discern any striking difference between them and the other mesenchymal cells; as regards size, form, and appearance, the nuclei seem identical. The yolk globules have decreased in number in both layers, and for the first time one can get a glimpse of the shape of the cell body. Some of the cells of the outer or covering layer are becoming definitely fusiform, with their long axis directed parallel to the circumference of the heart cavity. A considerable number of blood cells are present in the latter (figs. 18 to 22), a fact of no special significance, however, for the heart is in broad

open communication with the pronephric venous sinus and no valve has yet been established at this lymphaticovenous junction.

In the subsequent period of development the wall of the lymph heart changes very slowly in character. Instead of increasing in thickness, it becomes relatively thinner during the time between 9- and 13- or 14-mm. embryos, due, in the first place, to the progressive flattening of the cells of the intima layer; secondly, to the attenuation of the covering cells, and, thirdly, to the loss of the large yolk corpuscles. In fact, during these stages, the second or covering layer does not form a complete investment of the endothelium, for there are bare spots (figs. 20 and 21) where endothelium constitutes the only line of demarcation between the cavity of the lymph heart and the surrounding mesenchymal reticulum. The scantiness of the covering layer at this time is probably explained by the slow specialization of its cells and the more rapid expansion of the heart lumen with the resultant stretching of its lining cells. In 16-mm. embryos it again forms a continuous single cell-sheet (fig. 23), though it is still quite as thin as the endothelial layer, and is composed of slender spindle-shaped cells which show delicate striations. These cells, differentiated, as we have seen, from the mesenchymal cell aggregations so conspicuous during the initial stages of the lymph heart, compose the anlage of its muscle coat.

Knower claims there is evidence that the cells of the muscle coat are derived from the adjacent myotomes, but the writer is unable to furnish proof for the contention. In 5-mm. embryos a radical difference already obtains between the cells of the myotomes and those which surround the lymph-heart anlage—a distinction strikingly revealed in figure 16. Nevertheless, this fact does not discount the possibility that in much earlier stages the potential muscle cells of the lymph heart may proliferate from the myotomal elements; but, if this is found to be true, then it will be equally true that other mesenchymal cells of the same region have a similar source, provided the absence of any visible difference whatsoever between the cells of the mesenchyme and those of the lymph-heart anlage is any criterion of the similarity of origin.

The further development and thickening of the muscle coat is not consummated until some time after metamorphosis, for even in the young toad it is not conspicuous and is composed of only three or four cell layers.

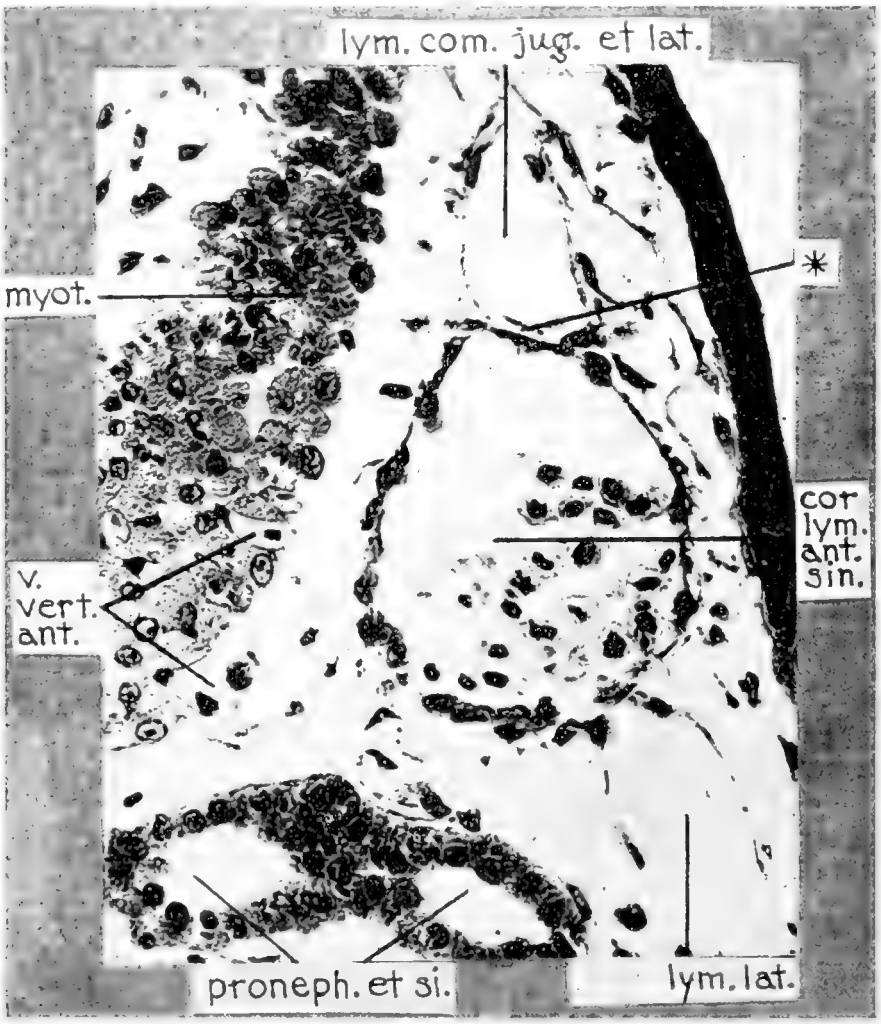

Fig. 20 Same, in a 9-mm. embryo of Bufo vulgaris (K. E. C., series B 13, slide 3, section 14). × 340. *, the common segment of the jugular and lateral lymphatics by expansion have again come in contact with the lymph heart; *lym. lat.*, a ventral branch of the lateral lymphatic (lymphatica lateralis) and continuous with the dorsal one at a further level. Other references as before.

The intima, too, of the lymph heart is slow in acquiring its definitive character, which is not attained until approximately in 15- or 16-mm. stages. Even in 10- or 11-mm. embryos the endothelial cells show little advance over those present in the heart of somewhat younger individuals. Some do have the finished

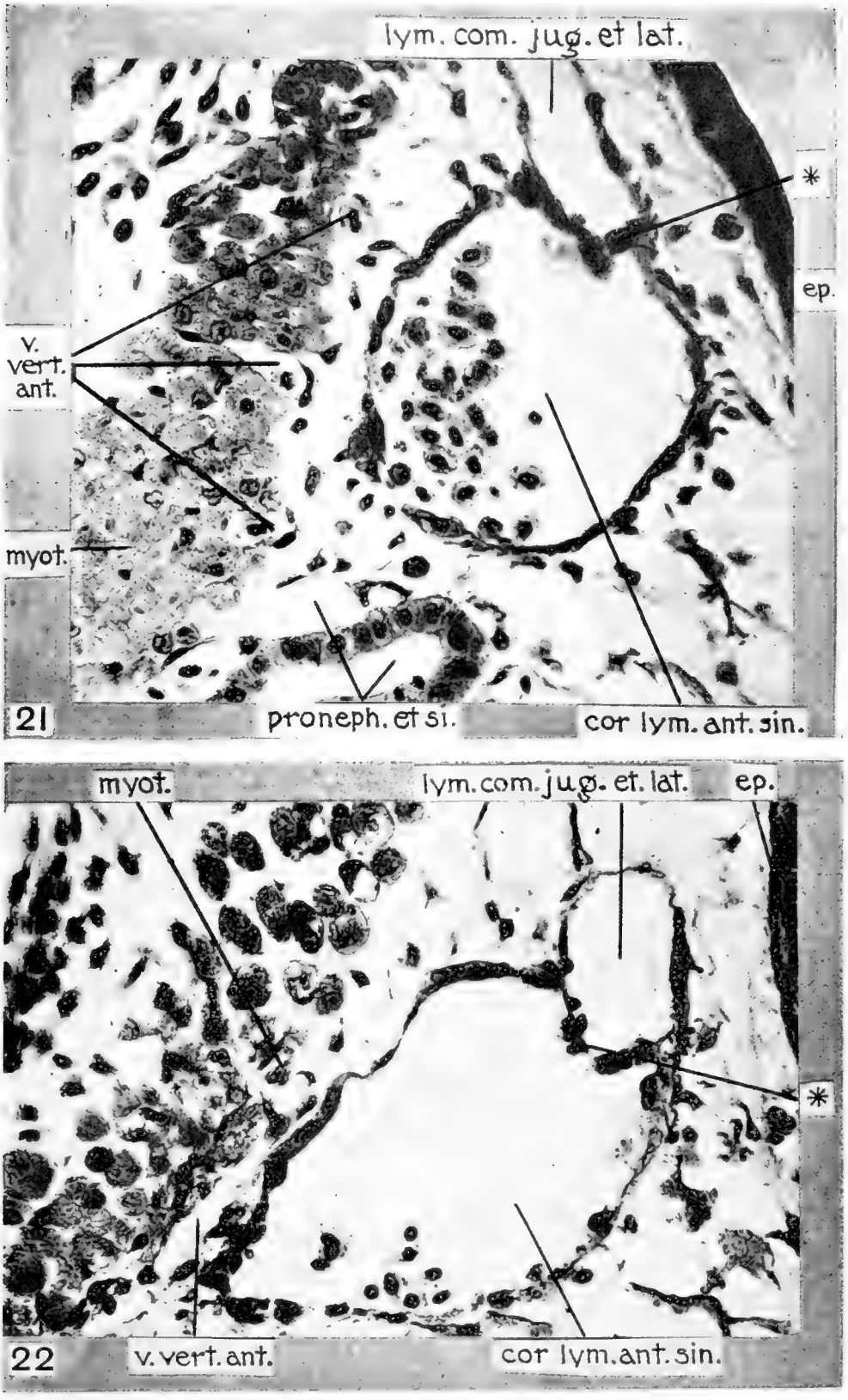

Fig. 21 Photomicrograph of a transverse section through the left anterior lymph heart region in a 10-mm. embryo of Bufo vulgaris (K. E. C., series B 34, slide 2, section 83). × 340. *, the common segment of the jugular and lateral lymphatics has indented the dorsal wall of the lymph heart, which has become thickened at this point. Other references as before.

Fig. 22 Same, in a 12-mm. embryo of Bufo vulgaris (K. E. C., series B 11, slide 3, section 34). × 340. *, the wall between the lymph duct and the lymph heart has broken through in the middle and the two flaps so formed represent the valves of the afferent portal. Other references as before.

form, in so far as their nuclei appear compressed and uniformly dense, but others again contain large, spherical, and coarsely chromatic nuclei and protrude into the heart cavity like little humps or hillocks, which call to mind the observations of certain investigators on haemopoiesis where endothelium germinated blood cells, but the author was unable to discover an undoubted case where one became constricted off.

The further differentiation and thickening of the heart wall occurs during the period of growth after metamorphosis, and histological examination of a section through the lymph heart of the adult anuran reveals three well-defined coats or layers: a tunica interna or intima, a tunica media, and a tunica externa or adventitia. The first is composed of the layer of highly flattened lining cells and a very thin stratum of connective tissue, probably elastic in nature, immediately external to them. As we should expect from the great energy displayed by the lymph hearts during life, the muscular tunica media, the second coat, is the broadest layer of the heart. Its muscle cells or fibers are of varying length and thickness and group themselves into small bundles which branch and interlace in a complex manner. Hoyer ('04) claims that the individual muscle fibers themselves branch and anastomose and possess numerous cross bands, which call to mind the intercalated discs of human cardiac muscle. A large number of elastic strands are also contained in the media. No sharp boundaries exist between media and adventitia. The latter is made up of fibrillar connective tissue in which are scattered pigment cells. The nerve fibers to the anterior pair of lymph hearts are apparently supplied by the III spinal nerve. According to Waldeyer ('64), both medullated and non-medullated nerve fibers are found in the walls of the fully developed lymph hearts.

Before discussing the formation of the valves, a variable feature may be mentioned in connection with the development of the walls. In about half of the lymph hearts examined between 8- and 16-mm. stages, a strand or trabecula, sometimes delicate and sometimes fairly thick, bridged the cavity. Occasionally they were imperfect, simply projecting as slender filaments

(fig. 26). It is possible that these trabeculae correspond to the incomplete partition which, according to Radwanska ('06), is of constant occurrence in the anterior lymph hearts of adult frogs.

b. The afferent portals. As was indicated earlier, during the first part of its functional life the lymph heart of the anuran embryo possesses but two valvular openings, a lymphaticovenous or efferent one and the entrance of the afferent lymph vessel. It is only in later embryonic and postmetamorphic periods that the number of afferent gateways is increased from one to about twelve. The development of this type will be considered first.

In the discussion of the morphogenesis of the lymph heart the writer has described how the developing lymphatic plexus surrounding it temporarily detaches and recedes from it and how the longitudinal channel of the plexus, the common segment of the jugular and lateral-line ducts lying dorsal to the heart, again comes into juxtaposition with it by the dilatation of both structures, whereupon the permanent communication is established. Figure 20 is a section of the lymph-heart region during the phase of simple apposition. Here the heart wall, having the same appearance and thickness as elsewhere along its periphery, separates the cavity of the heart (*cor. lym. ant. sin.*) from that of the lymph duct (*lym. com. jug. et lat.*), and there is as yet no indication of the future opening between the two. In the next older stage (10-mm.), the heart and vessel are more intimately applied to each other by the partial invagination of the latter into the heart, as shown in figure 21 (*); in the reconstruction (fig. 35) the vessel lies in a shallow furrow of its heart wall at *af.* It is along this surface of contact that the partition dividing the two cavities thickens considerably (fig. 21) by the proliferation of its cells. Somewhat later, a cleft develops in the center of the thickened area (fig. 22, *) by the separation of its cells, evidently the effect of the increasing pressure within the afferent lymph duct. The margins of the simple rupture now serve as the valve. These, by further proliferation, may become longer, and as they converge and project into the lumen of the lymph heart they produce the typical teat-like form in section (fig. 23, *). The other valvular afferent portals which arise later (fig. 24, *af.*) are developed in

a similar way. In a young toad (Bufo lentiginosus), shortly after the period of metamorphosis, the author observed five such points of entry in the anterior lymph heart, but doubtlessly their

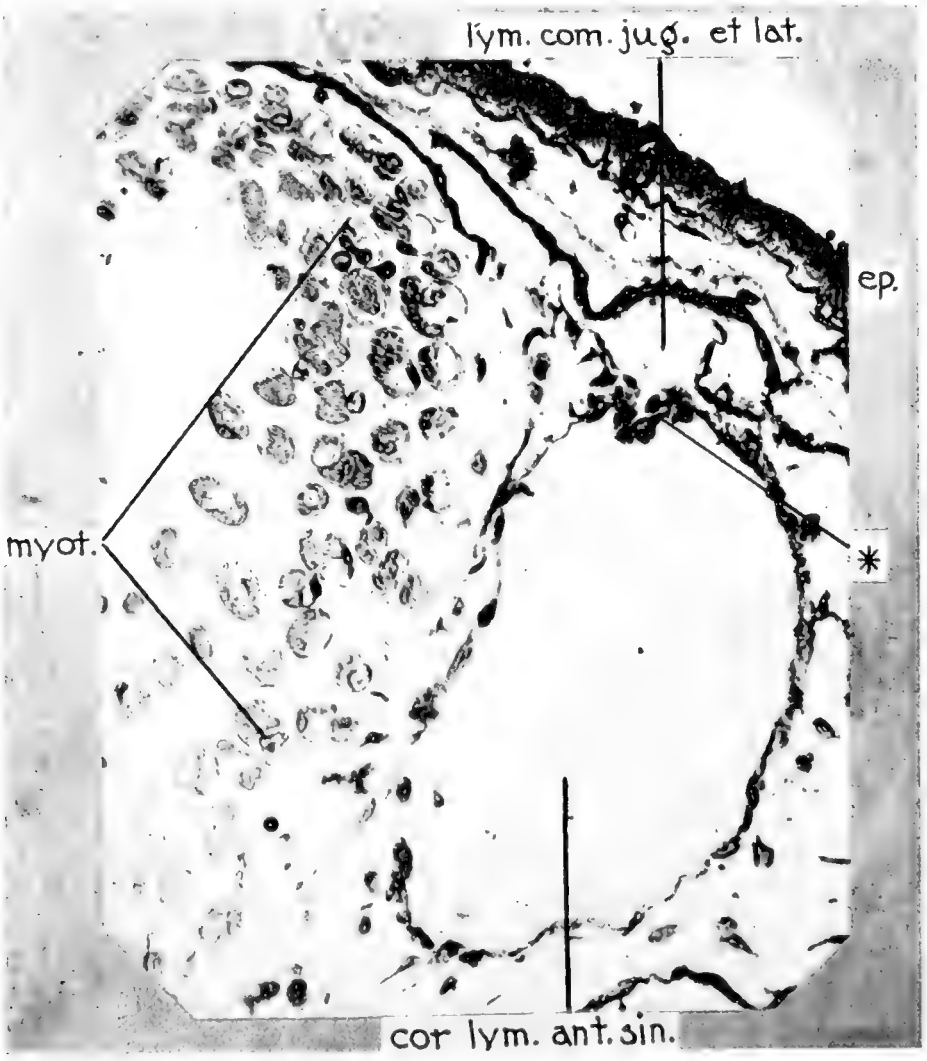

Fig. 23 Photomicrograph of a transverse section through the left anterior lymph heart region in a 16-mm. embryo of Bufo vulgaris (K. E. C., series B 39, slide 5, section 51). × 340. *, valve at the afferent portal. Other references as before.

number is increased with the growth of the toad towards maturity, for Radwanska ('06) counted more than a dozen on the same organs in adult frogs.

c. The efferent portal, or ostium venosum. The formation of the valve at the lymphaticovenous junction is perhaps not so

diagrammatic. It develops, however, at the same time as the other valve. In 7-mm. embryos the lymph heart is still in broad open communication with the anlage of the vertebral vein (fig. 18). In the next few succeeding stages (8-, 9-, and 10-mm. embryos) the junction becomes progressively constricted by the local thickening of its surrounding wall. In fact, in some cases it was observed that the cell proliferation was so considerable as to block almost entirely the channel of connection (figs. 19 and 22).

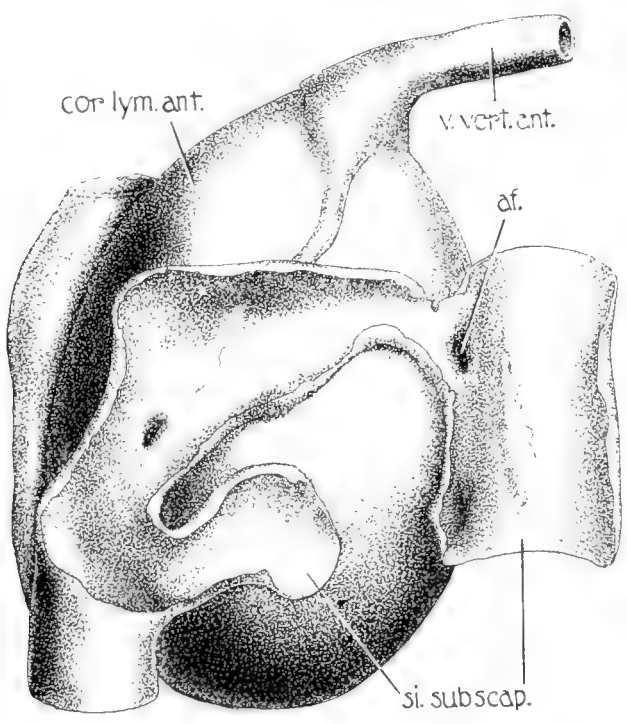

Fig. 24 Quasischematic reconstruction of the left anterior lymph heart of the young toad immediately after metamorphosis. × 150. Ventromedial view. *cor lym. ant.*, cor lymphaticum anterius; *v. vert. ant.*, vena vertebralis anterior; *si. subscap.*, subscapular sinus; *af.*, one of the afferent portals.

Then, by the elongation of its thickened sides (fig. 25,*) associated with the expansion of the venous lumen up and around it towards the lymph-heart wall, the connection becomes telescoped, as it were, into the cavity of the vein, so that the thickened cell masses project as the lips of the valve (fig. 26,*). This process is completed in 10- to 12-mm. toad embryos (B. vulgaris). In the outline sketches in figure 27, the formation of both the afferent and the efferent portal is expressed graphically.

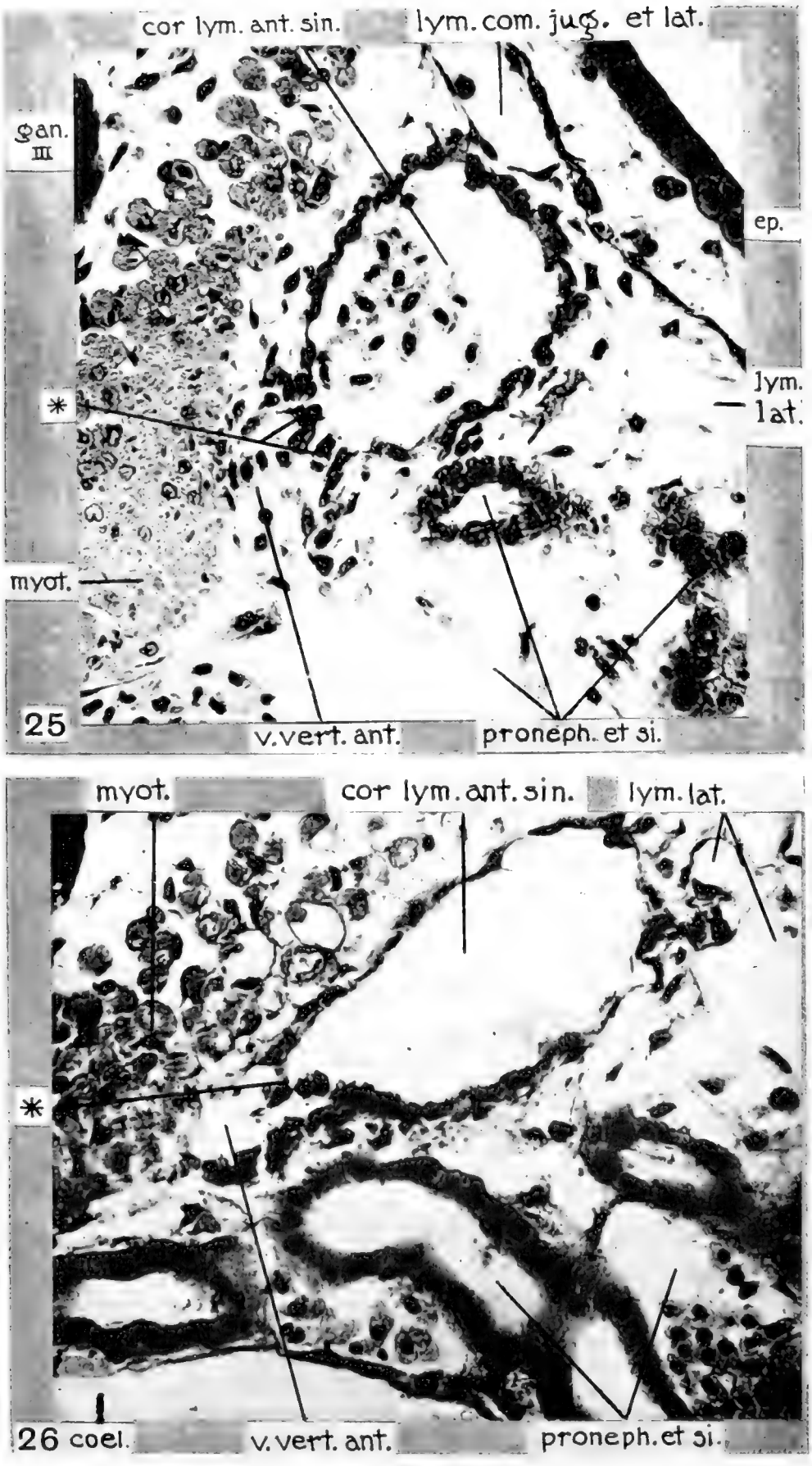

Fig. 25 Photomicrograph of a transverse section through the left anterior lymph heart region in a 10-mm. embryo of Bufo vulgaris (K. E. C., series B 34, slide 2, section 68). × 340. *, formation of the valve at the efferent portal. Other references as before.

Fig. 26 Same, in a 12-mm. embryo of Bufo vulgaris (K. E. C., series B 11, slide 3, section 41). × 340. *, valve of the efferent portal. Other references as before.

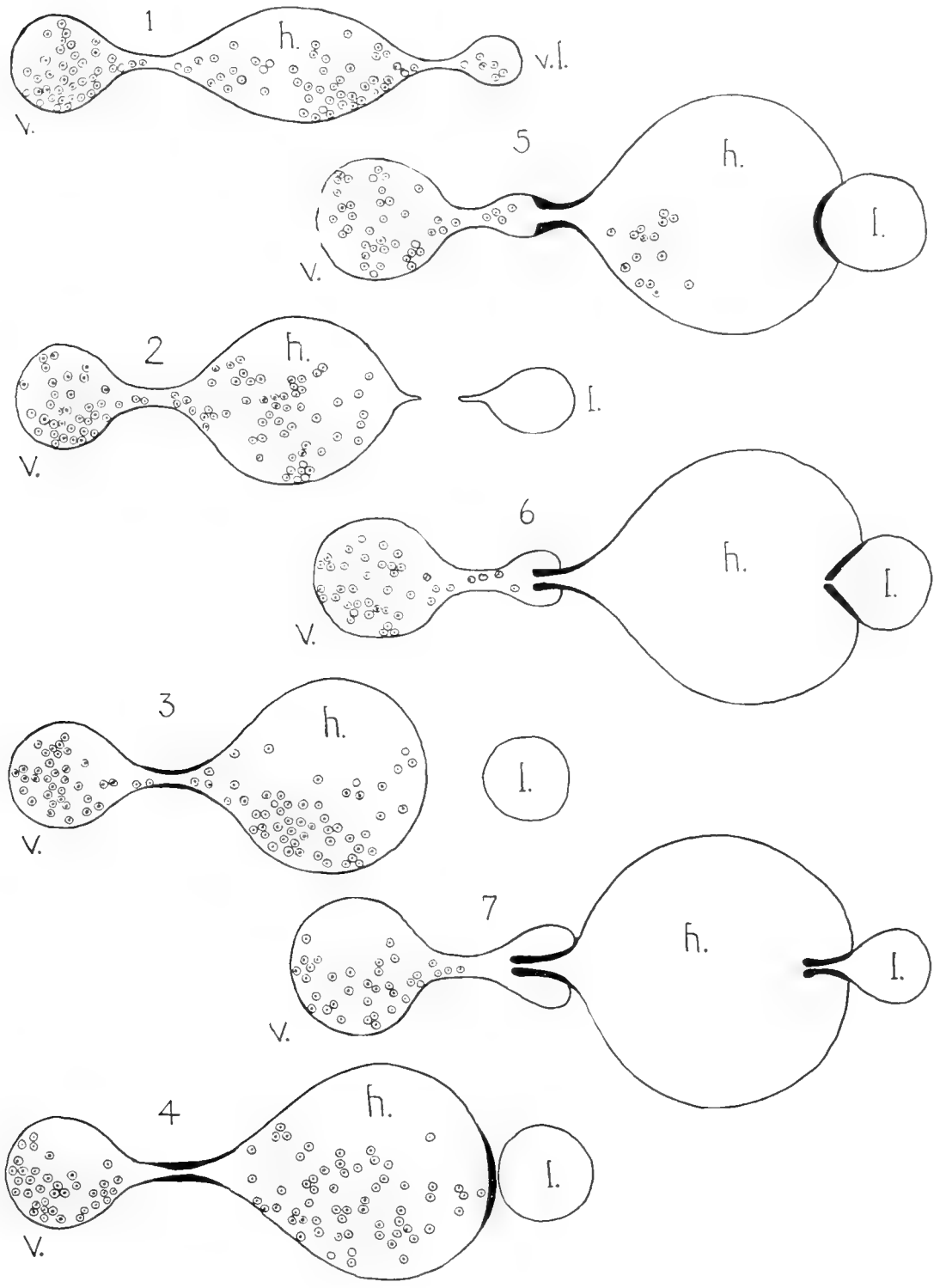

Fig. 27 Diagrams (1 to 7) illustrating the formation of the afferent and efferent ostia of the lymph heart (based on transverse sections). *v.*, vein; *h.*, lymph heart; *v. l.*, venolymphatic, a channel of the intersegmental vein plexus, and converted into the afferent lymph vessel, *l.*

Since blood cells have free access to the cavity of the lymph heart before the appearance of the valve at the lymphatico-venous junction, in stages up to and including 10-mm. embryos, they are abundant in it (figs. 18 to 22 and 25). In 12-mm. embryos and later (figs. 23 and 26), they are rarely present, and we may conclude from this and the fact that the valves are now functionally complete and efficient that the pulsations of the lymph heart commence at this time, for the first few contractions would certainly cause the evacuation of all haemal elements.

On account of the abundance of pigment in the integument of Bufo embryos, it was impossible to determine accurately by direct observation on the living specimen at which time the pulsations of the anterior lymph heart commenced, but, according to Hoyer ('05), they first become evident as irregular quiverings in the more transparent frog embryos (R. temporaria) when they are 12 to 13 mm. long. Later the pulsations of the lymphatic heart become more rhythmic, but the beats coincide neither with those of the haemal heart nor with those of its companion on the opposite side. In the mature animal it throbs as often as sixty to seventy times every minute, and since its capacity is about 0.5 cu. mm. (Radwanska, '06), the quantity of lymph pumped into the anterior vertebral vein during this period is 30 cu. mm., and in one hour reaches the relatively considerable amount of 180 cu. mm. During systole of the lymph heart, the efferent valve, projecting into the vein, opens for the discharge of the lymph, but closes and prevents the backflow of blood into the heart chamber during diastole. Similarly, the afferent gateways permit the entrance of the lymphatic current from the circumjacent lymph sinuses, yet avert its reflux during systole.

SUMMARY

1. On the development of the primary maxillary lymph sinus

The sinus begins in approximately 5-mm. embryos of Bufo vulgaris in the form of small discontinuous anlagen, which appear either as cellular thickenings of the endothelium of the developing jugular veins or as islands lying in the mesenchyma in the immediate vicinity of these vessels.

During development all vascular anlagen of the head region, both haemal and lymphatic, can be distinguished from the surrounding mesenchyma by the greater number of yolk globules present in their endothelium.

The originally solid lymphatic anlagen acquire lumina, which have their inception as small crevice-like spaces in the cytoplasm between the large yolk globules.

By continued proliferation and growth, the individual anlagen increase in length, bud collateral branches, coalesce with one another, and in time form a complex tubular network extending in a curved plane from the region of one external jugular vein to that of the opposite side; this network represents the principal or mandibular division of the primary maxillary lymph sinus.

The other divisions, the circumoral, temporal, and pericardial, arise from the mandibular division by outgrowth and extension.

The lymphatic network becomes transformed into a spacious and uninterrupted sinus by the progressive expansion of all the anastomosing channels and by the reduction and tearing of the intervening mesenchymal strands and trabeculae.

During the preceding genetic stages, the sinus possesses no outlet; it is not confluent with the veins. The sinus receives an outlet in approximately 10-mm. embryos as the posterior prolongations of its temporal divisions join the jugular lymphatics and thereby are placed in communication with the anterior lymph hearts and through them with the venous system.

The extension and distention of the developing sinus are probably achieved by the increasing internal pressure on its walls of the accumulating lymph before an exit is established. During the expansion of the sinus, the lining cells become progressively flattened and assume typical endothelial qualities.

2. On the development of the jugular lymphatic

In 5- to 6-mm. embryos, the first three intersegmental veins, which are dorsal vertical tributaries of the pronephric venous sinus (common segment of pre- and postcardinal veins), become joined longitudinally by interanastomoses and consequently take on a plexiform character.

The aforesaid intersegmental vein plexus, which in view of its original relations and its future function may be called a venolymphatic one, gives rise to the jugular lymphatic, the important change consisting in its gradual separation from the veins (pronephric venous sinus).

By the expansion, approximation, and fusion of the longitudinal components of the plexus, the main channel of the jugular lymphatic is definitely established, and it eventually makes connection anteriorly with the temporal division of the primary maxillary lymph sinus and at its posterior end, in common with the lateral line lymphatic, joins the anterior lymph heart.

3. On the development of the anterior lymph heart

The anterior lymph heart, on either side, arises from a circumscribed portion of the venolymphatic plexus, mentioned in the preceding section, at the level and in the axis of the original 3rd intersegmental vein.

The plexiform anlage of the lymph heart becomes transformed into the uninterrupted heart chamber by the expansion and fusion of its interjoined channels.

The developing lymph heart in approximately 7- or 8-mm. embryos severs connection with the circumjacent venolymphatic plexus, but remains in continuity with the venous system via the mouth of the former 3rd intersegmental vein, now the mouth of the anterior vertebral vein.

A communication is reestablished between lymph heart and afferent lymphatic, the common segment of the jugular and lateral-line lymphatics, in approximately 10-mm. embryos; this is accomplished by the gradual approximation of the two structures, due to their growth and dilatation, and by the perforation

of the intervening wall at the line of contact to form a teat-like valve. Other permanent afferent portals are formed later in a similar manner, there being five of these in Bufo lentiginosus immediately after the period of metamorphosis.

The valve at the efferent or lymphaticovenous tap is developed from a circular endothelial cushion projecting into the lumen of the anterior vertebral vein, followed by the telescoping of this valvular portion of the heart deeper into the lumen of the vein.

During the later development of the heart the efferent tap is shifted forward from a ventral position on the heart to an anterior one.

While the heart is expanding, the lining cells become progressively flattened; the mesenchymal cells external to these become spindle shaped and ultimately develop into muscle cells.

Before the efferent valve has become differentiated, numerous blood corpuscles are found in the heart cavity. The evacuation of these elements doubtlessly occurs at the first vigorous contractions of the heart.

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APPENDIX

At the time when the above paper had already appeared in proof, I found a reference in the literature to an article by Bles on "The life-history of Xenopus laevis" (Trans. Roy. Soc. Edinb., vol. xli, 1905) in which he described and pictured the anterior lymph hearts in the larvae of this anuran. Reference to this paper will be made in my work on the comparative morphology of the systemic lymphatics which is in preparation.

PLATES

PLATE 1

EXPLANATION OF FIGURE

28 Reconstruction of the larger haemal and lymphatic vessels in the head and anterior trunk region of a 7.5-mm. embryo of Bufo lentiginosus (K. E. C., series B 31), dorsal view. × 50.

Structures not colored: lymphatics, anterior end of spinal cord and brain, olfactory, optic, and auditory vesicles, and pronephros and its duct; the latter structures omitted on the right side.

Lymphatics: *si. circ. or.*, circumoral division of the sinus maxillaris primigenius; *si. mand.*, mandibular division; *si. temp.*, temporal division; *si. pericard.*, pericardial division; *lym. jug.*, lymphatica jugularis; *cor. lym. ant. dex.* and *sin.*, cor lymphaticum anterius dextrum and sinistrum; *lym. lat.*, lymphatica lateralis.

Veins (blue): A portion of the sinus venosus is shown ventral to the myelencephalon joined by the hepatic sinusoids, the external jugular veins and cuvierian ducts. The external jugular accompanies the pericardial lymphatic and anteriorly receives two branches, a medial (hidden by the mesencephalon), probably the anlage of the vena lingualis, and a lateral, lying closely against the inner side of the principal and circumoral divisions of the primary maxillary sinus and representing the future vena mandibularis and branches. In the region of the pronephric sinusoids (a large section omitted on the right side) the cuvierian duct is joined by the precardinal or internal jugular, which passes laterally around the auditory vesicle and possesses three large tributaries, the vena orbitonasalis, the vena ophthalmica, and a large intracranial vein. The lateral and medial (subcardinal) divisions of the postcardinal, situated along the pronephric duct, and anteriorly, near the pronephric sinus, receive the anterior vertebral vein into which the anterior lymph heart opens.

Arteries (red): The heart, external carotids and ventral roots of the aortic arches are not shown in the drawing. The radices aortae are broadly divergent in the region of the auditory vesicles, where they connect with the dorsal roots of the aortic arches which, as they curve ventrad, lie closely against the inner side of the temporal lymphatics. Anteriorly the radices aortae are continued forward as the internal carotids which give off in the order named the following important branches: arteria palatina, a. ophthalmica, and a. carotis cerebralis. The pronephric glomeruli branch from the radices aortae immediately anterior to their convergence and fusion to form one trunk (ventral to the spinal cord).

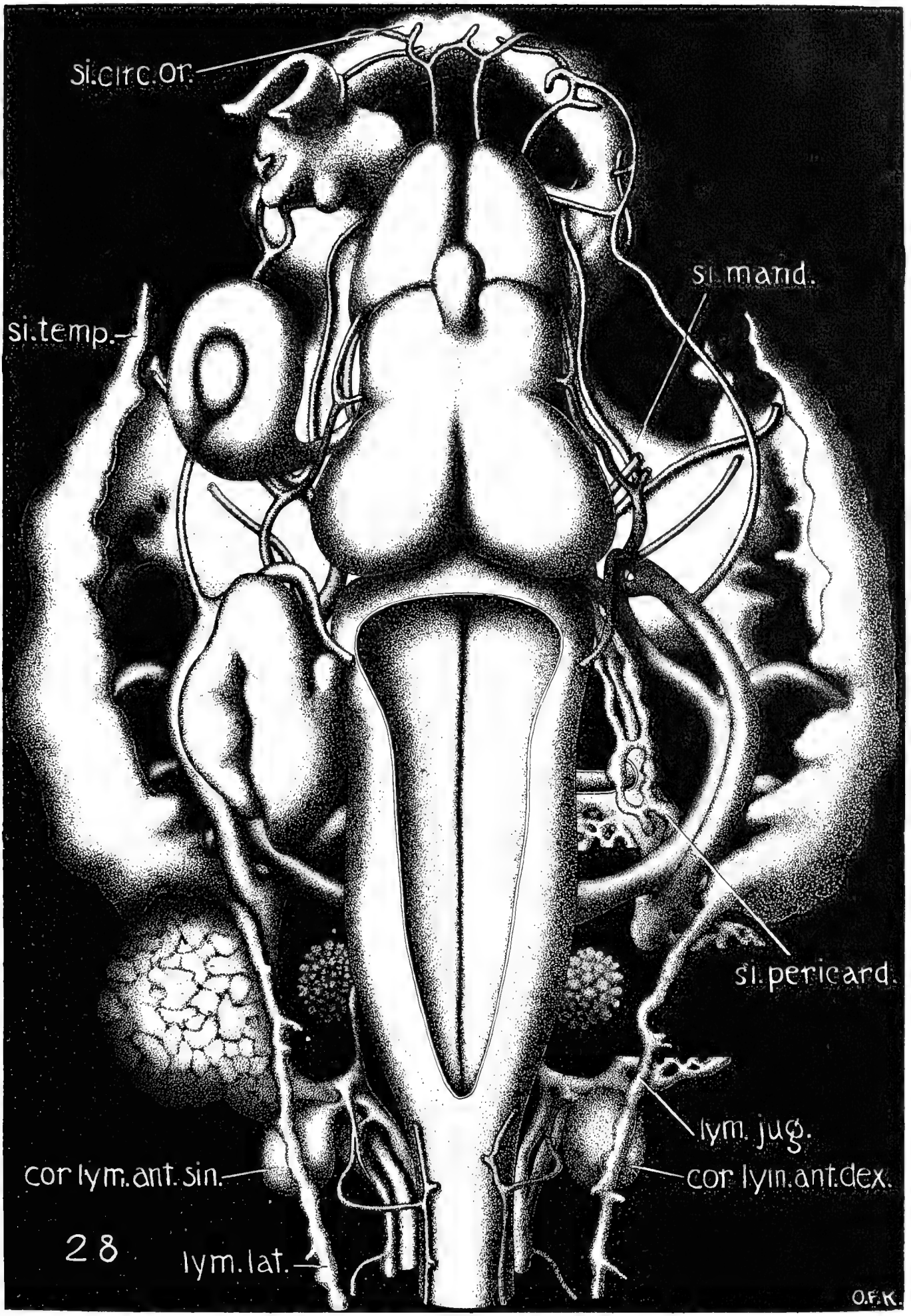

si.circ.or.
si.mand.
si.temp.
si.pericard.
lym. jug.
cor lym.ant.sin.
cor lym.ant.dex.
28
lym.lat.
O.F.K.

PLATE 2

EXPLANATION OF FIGURE

29 Reconstruction of the vascular channels of the ventral cephalic region in a 6-mm. embryo of Bufo vulgaris (K. E. C., series B 53), ventrai veiw. × 125.

v. jug. ext., vena jugularis externa.

a. car. ext., arteria carotis externa.

g. thyr., glandula thyroidea.

ao., aortic arches.

vent., ventriculus of the heart.

d. Cuv., ductus Cuvieri.

sin. ven., sinus venosus; its cut edge shows its attachment to the liver, for at this period the hepatic sinusoids open directly into it.

The lymphatics, the anlagen of the sinus maxillaris primigenius arise independently of each other along the venous components of the jugulocarotid plexus; some of them have severed contact with the blood vessel wall, while others still adhere to it.

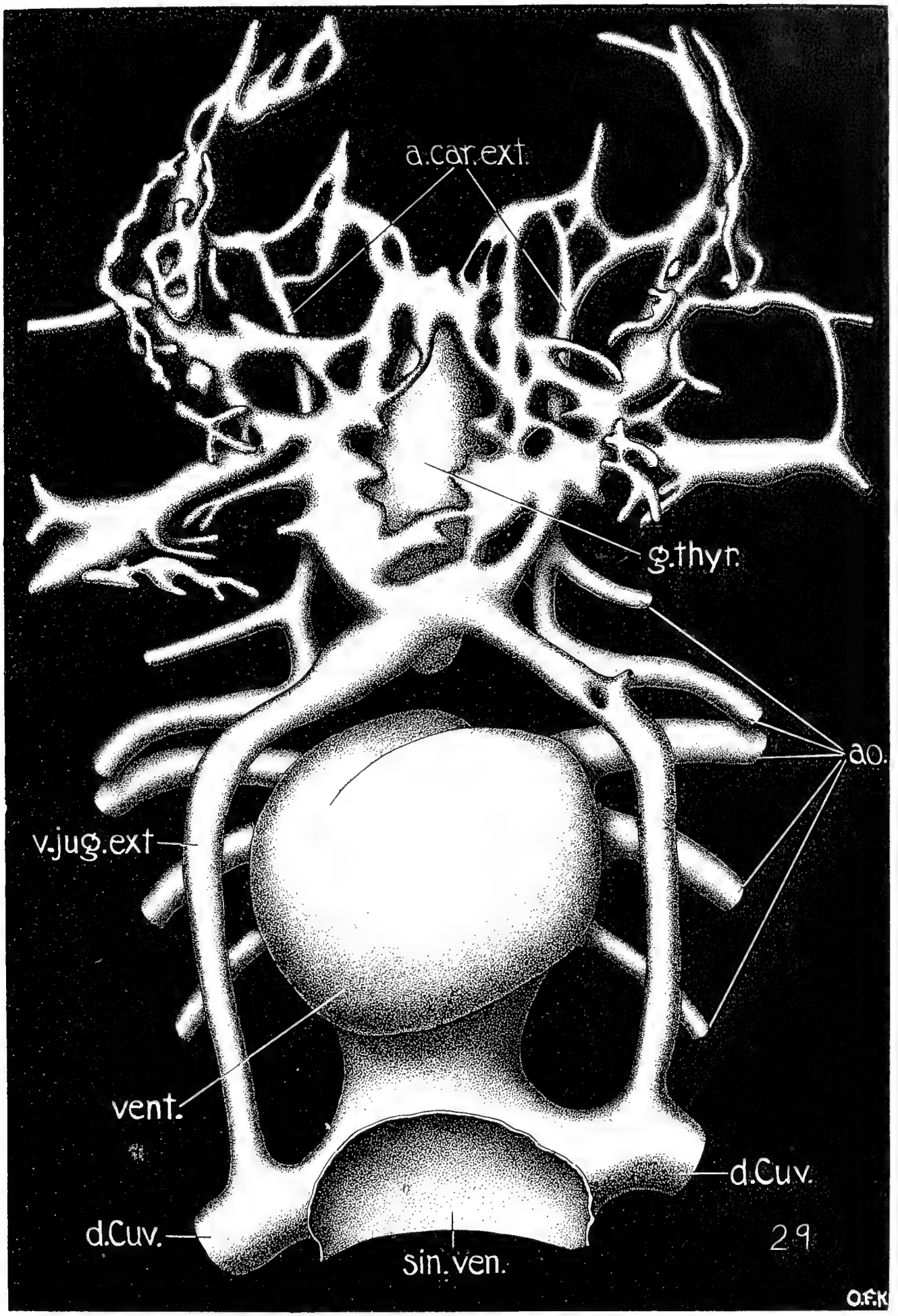

a.car.ext.
g.thyr.
ao.
v.jug.ext
vent.
d.Cuv.
d.Cuv.
sin. ven.
29
O.F.K

PLATE 3

EXPLANATION OF FIGURE

30 Reconstruction of the vascular channels of the ventral cephalic region in a 7-mm. embryo of Bufo vulgaris (K. E. C., series B 27), ventral view. × 125.

si. circ. or., circumoral division of the sinus maxillaris primigenius.
si. mand., mandibular division of the sinus maxillaris primigenius.
si. temp., temporal division of the sinus maxillaris primigenius.
si. pericard., pericardial division of the sinus maxillaris primigenius.
g. thyr., glandula thyroidea.
a. car. ext., arteria carotis externa.
ao., aortic arches.
v. jug. ext., vena jugularis externa.
d. Cuv., ductus Cuvieri.
v. card. post., vena cardinalis posterior.
sin. ven., sinus venosus.
v. jug. int., vena jugularis interna.
vent., ventriculus of the heart.

At the point marked *?*, the author is unable to decide whether a direct connection exists or not.

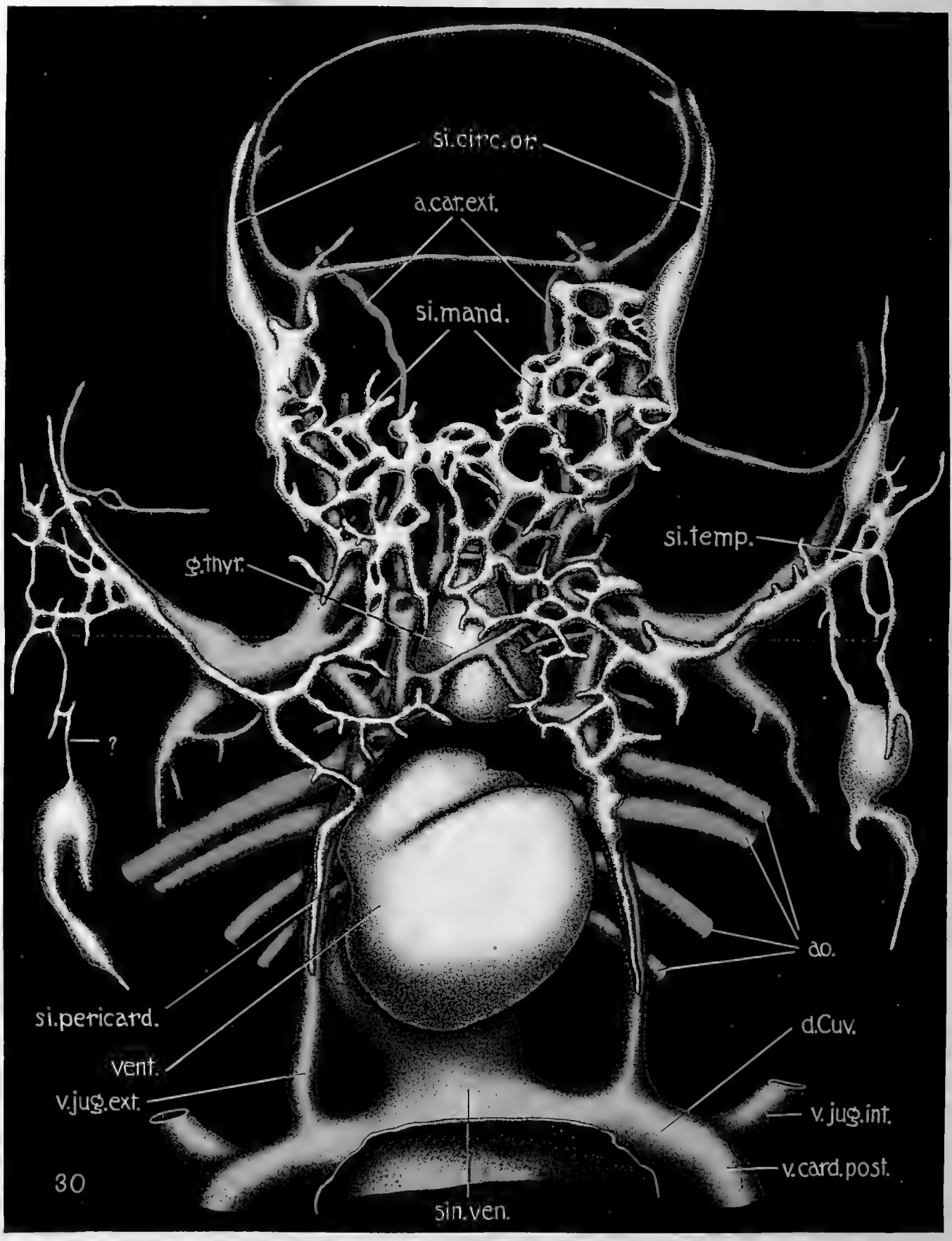

si.circ.or.
a.car.ext.
si.mand.
si.temp.
g.thyr.
?
ao.
si.pericard.
vent.
v.jug.ext.
d.Cuv.
v. jug.int.
v.card.post.
sin.ven.
30

PLATE 4

EXPLANATION OF FIGURE

31 Reconstruction of the vascular channels and other structures in the region of the left pronephros in a 4-mm. embryo of Bufo vulgaris (K. E. C., series B 45), lateral view. × 166.

1–4 v. seg., 1st to the 4th intersegmental veins; the beginning of the formation of the venous plexus in the development of the anterior lymph heart is already indicated by the short and irregular branches of the 3rd intersegmental vein.

v. jug. int., vena jugularis interna (precardinal)

v. jug. ext. vena jugularis externa

v. card. post., vena cardinalis externa

d. Cuv., ductus Cuvieri

nephst., *I*, *II*, et *III*, nephrostomes of the pronephros

d. proneph., pronephric or primary excretory duct

med. spin., medulla spinalis

II, *III*, *IV*, 2nd, 3rd and 4th spinal ganglia

vag., a ganglionic prolongation from the vagus group back along the medulla oblongata; it may be a vestige associated with the lateral-line organs and later disappears.

A reconstruction of the above structures in a 5-mm. embryo, representing an intermediate stage between that pictured on the opposite plate and that on the following one, was omitted with several other illustrations to reduce the cost of publication, although it revealed very strikingly the lymph heart plexus before its coalescence into a uniform cavity, a process almost completed in plate 5.

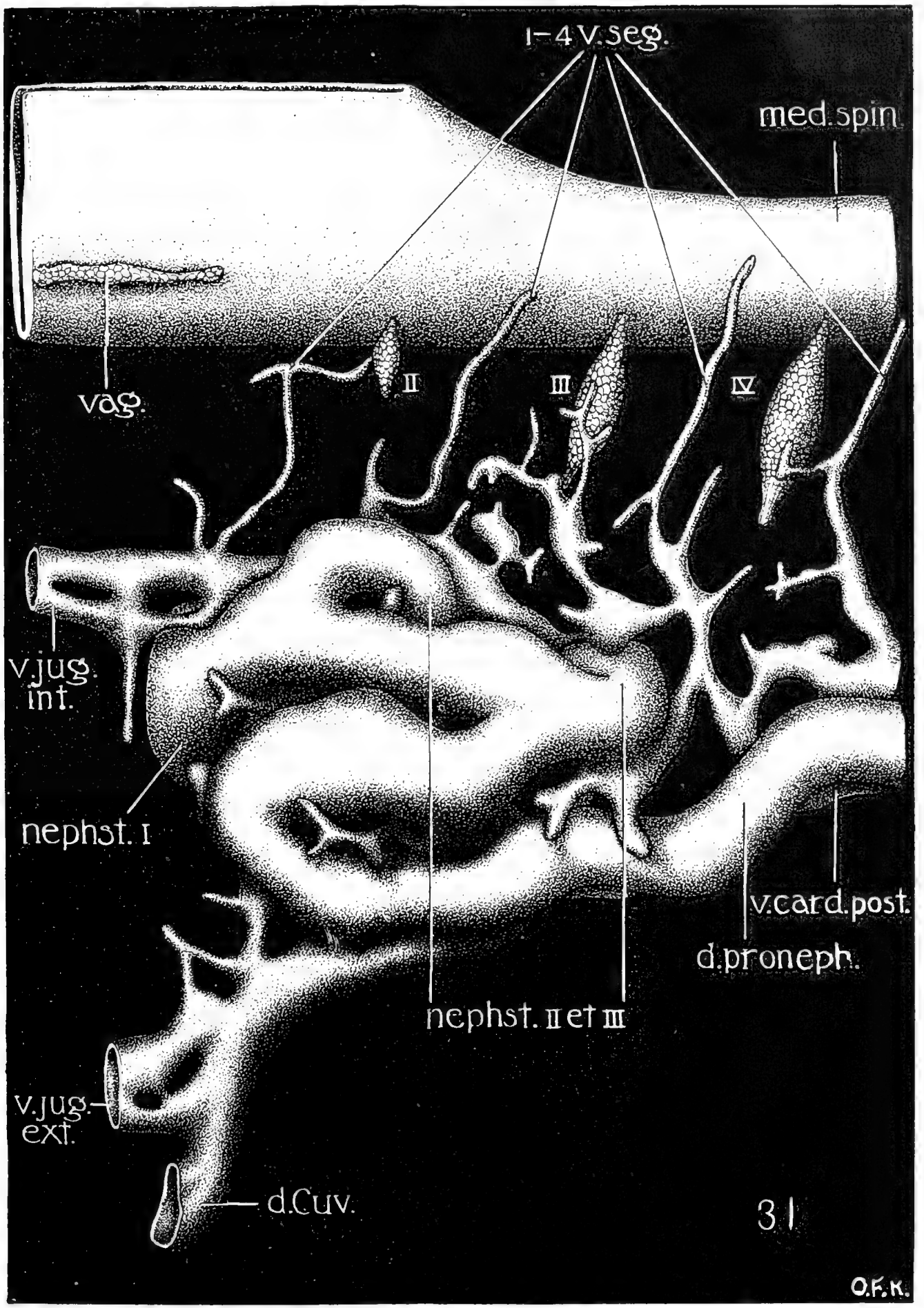

1–4 V. seg.
med. spin.
vag.
II
III
IV
V. jug. int.
nephst. I
nephst. II et III
V. card. post.
d. proneph.
V. jug. ext.
d. Cuv.
31
O.F.K.

PLATE 5

EXPLANATION OF FIGURE

32 Reconstruction of the vascular channels and other structures in the region of the left pronephros in a 6-mm. embryo of Bufo vulgaris (K. E. C., series B 54), lateral view. × 166.

1–4 v. seg., 1st to the 4th intersegmental veins; by the formation of interanastomosis between them a plexus results, which may be called a venolymphatic one, in view of the fact that it subsequently gives rise to the lymph vessels in this region.

cor. lym. ant., anlage of the anterior lymph heart.

v. jug. int., vena jugularis interna.

v. jug. ext., vena jugularis externa.

v. card. post., vena cardinalis posterior.

d. Cuv., ductus Cuvieri.

d. proneph., pronephric duct.

nephst. I, II, et *III*, pronephric nephrostomes.

med. spin., medulla spinalis.

I. II, III and *IV*, spinal ganglia; the 1st spinal ganglion is a very transitory and vestigial structure appearing for the first time in 6-mm. embryos and disappearing very soon after; the 2nd spinal ganglion becomes the 1st of the adult.

vag., ganglionic prolongation of the vagus group (cf. fig. 31).

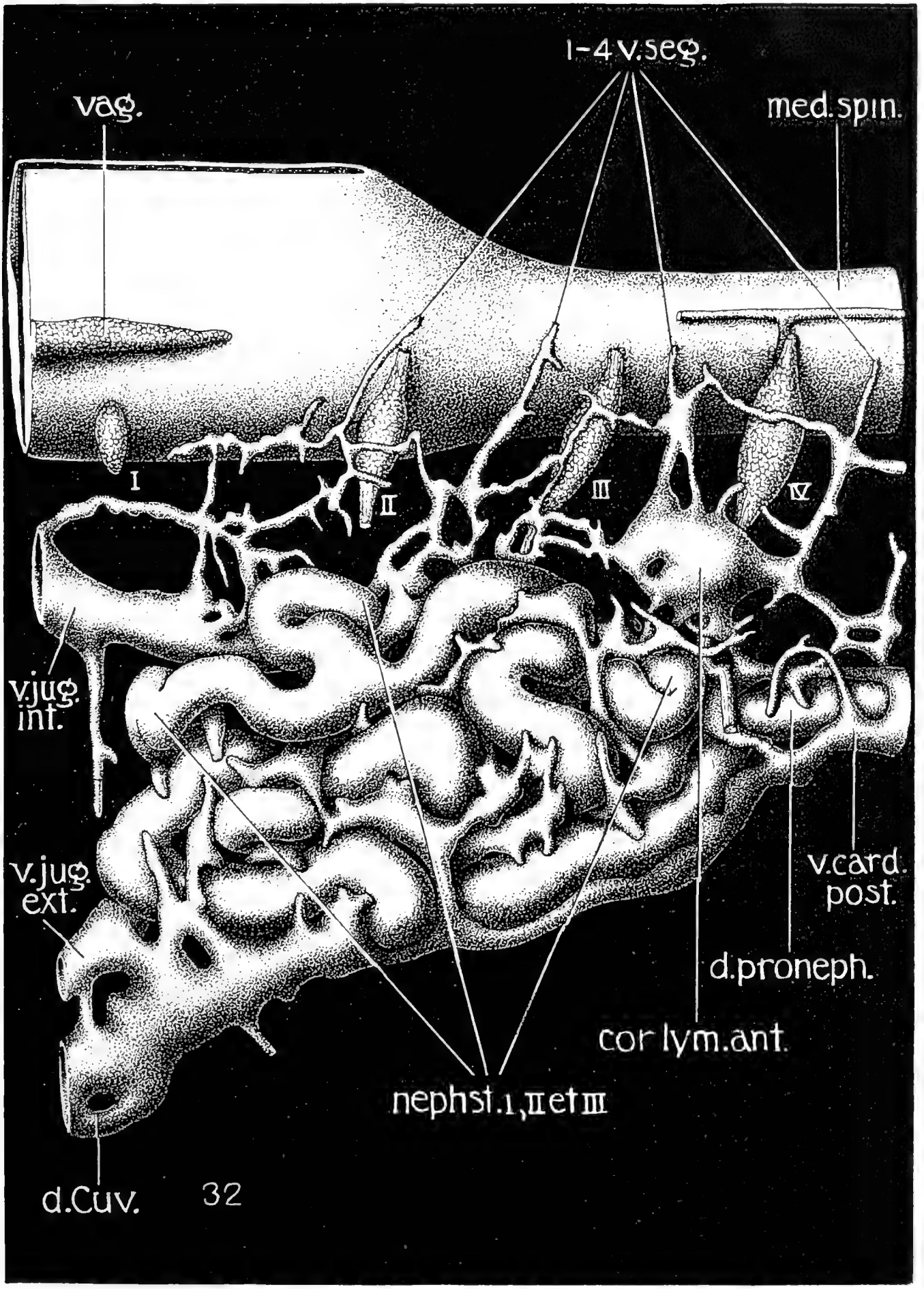

1-4 V. seg.
vag.
med. spin.
I
II
III
IV
v. jug. int.
v. jug. ext.
v. card. post.
d. proneph.
cor lym. ant.
nephst. I, II et III
d. Cuv.
32

PLATE 6

EXPLANATION OF FIGURE

33 Reconstruction of the vascular channels and other structures in the region of the left pronephros in a 7-mm. embryo of Bufo vulgaris (K. E. C., series B 27), lateral view. × 166.

lym. jug., lymphatica jugularis; at the points marked * there is still a minute connection between the pronephric sinus and the lymphatic.

temp. s. max. prim., posterior tip of the temporal division of the sinus maxillaris primigenius.

lym. lat., lymphatica lateralis; this lymphatic in early stages is broadly plexiform and has a dorsal and a ventral division.

cor. lym. ant., cor lymphaticum anterius; the surrounding lymphatics are severing connection with the heart; the lymphaticovenous junction is on the ventral surface of the heart, and anterior to this are two small venous branches which later become consolidated with it to form the anterior vertebral vein.

v. jug. ext., vena jugularis externa.

d. Cuv., ductus Cuvieri.

gan. jug. (vag.), ganglion jugulare of the vagus group.

Other structures as in the preceding plates.

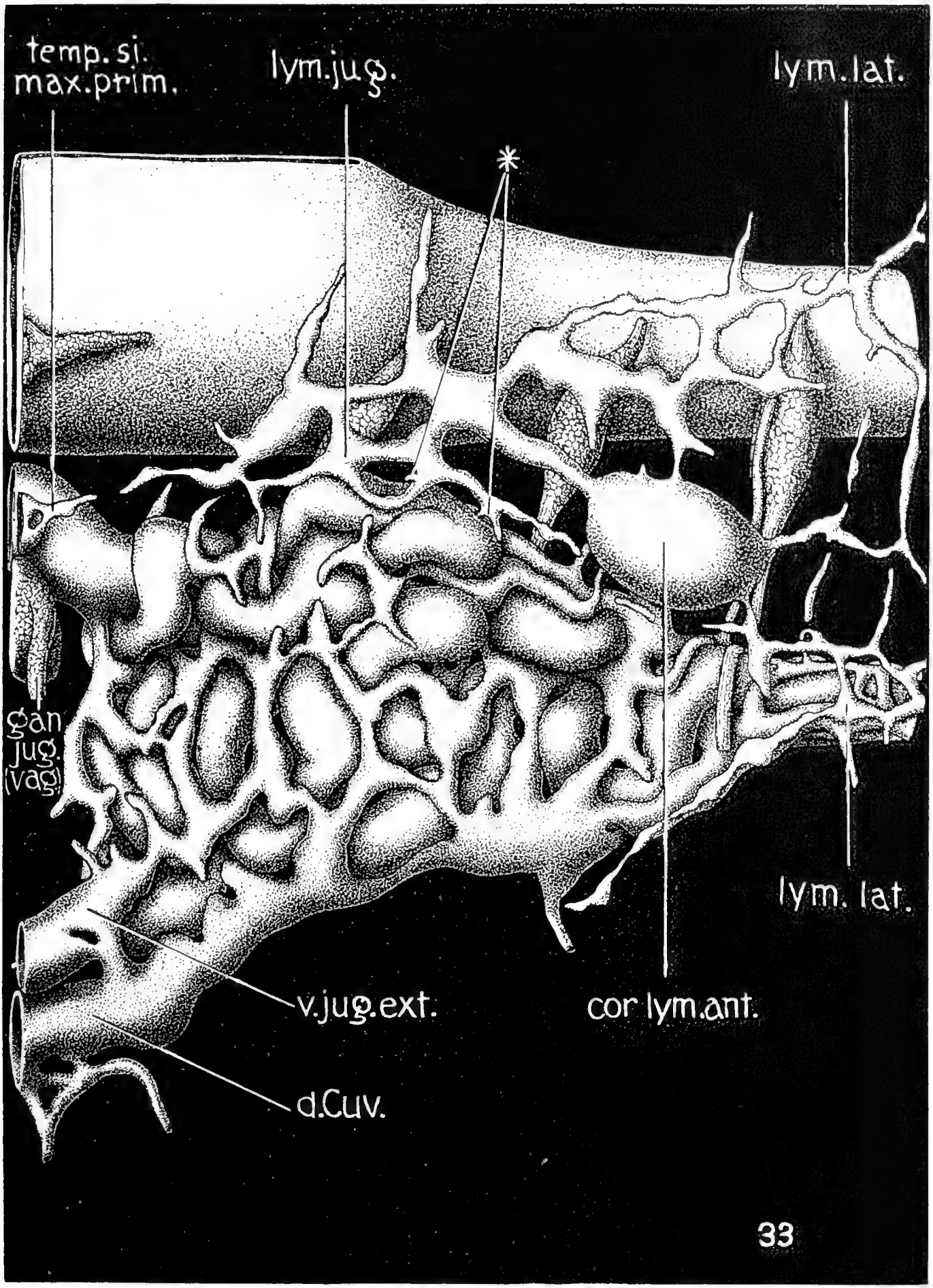

temp. si.
max.prim.
lym.jug.
lym.lat.
gan
jug.
(vag)
lym. lat.
v.jug.ext.
cor lym.ant.
d.Cuv.
33

PLATE 7

EXPLANATION OF FIGURE

34 Reconstruction of the vascular channels and other structures in the region of the left pronephros in an 8-mm. embryo of Bufo vulgaris (K. E. C., series B 49), lateral view; × 166.

lym. jug., lymphatica jugularis; the point marked * still shows a minute vestigial connection with the pronephric sinus.

temp. s. max. prim., temporal division of the primary maxillary sinus.

lym. lat., lymphatica lateralis (both dorsal and ventral divisions); the common channel of the jugular and the lateral lymphatic dorsal to the lymph heart is referred to in the text and photomicrographs as the common segment of these vessels.

cor. lym. ant., cor lymphaticum anterius; the surrounding lymphatic plexus has temporarily severed ts connection with the heart.

v. vert. ant., vena vertebralis anterior.

v. jug. int., vena jugularis interna.

v. jug. ext., vena jugularis externa.

v. card. post., vena cardinalis posterior.

d. Cuv., ductus Cuvieri.

med. spin., medulla spinalis.

gan. jug. (*vag.*), ganglion jugulare of the vagus group; *vag.* (cf. figs. 31–33).

Other structures as before.

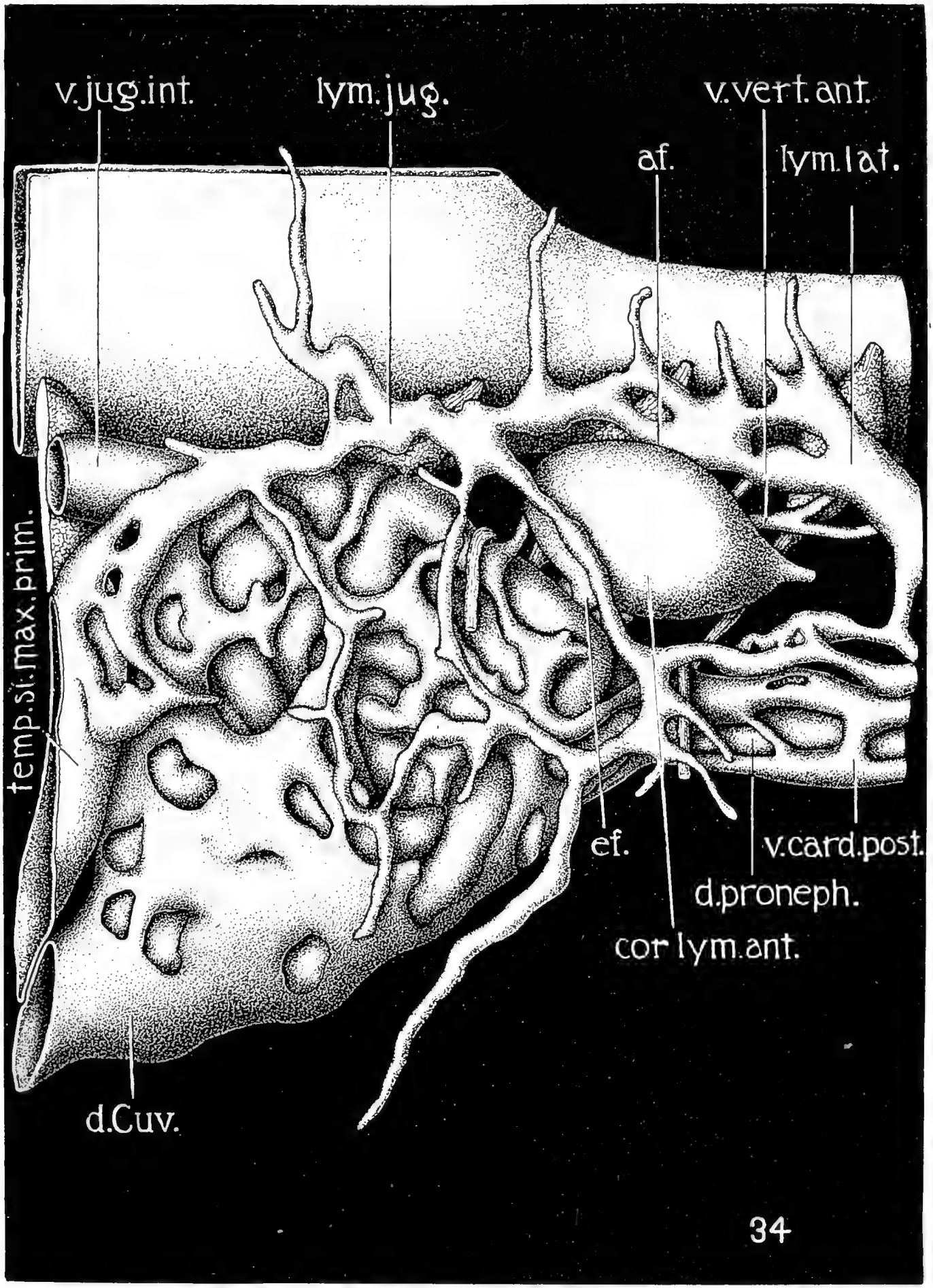

v.jug.int.
lym.jug.
v.vert.ant.
af.
lym.lat.
temp.si.max.prim.
ef.
v.card.post.
d.proneph.
cor lym.ant.
d.Cuv.
34

PLATE 8

EXPLANATION OF FIGURE

35 Reconstruction of the vascular channels and other structures in the region of the left pronephros in a 10-mm. embryo of Bufo vulgaris (K. E. C., series B 34), lateral view. × 166.

cor. lym. ant., cor. lymphaticum anterius; the lymphaticovenous junction or efferent portal is shown at *ef.*; *af.*, indicates the point at which the afferent portal is being established.

lym. jug., lymphatica jugularis.

lym. lat., lymphatica lateralis.

temp. s. max. prim., posterior portion of the temporal division of the primary maxillary sinus.

v. vert. ant., vena vertebralis anterior.

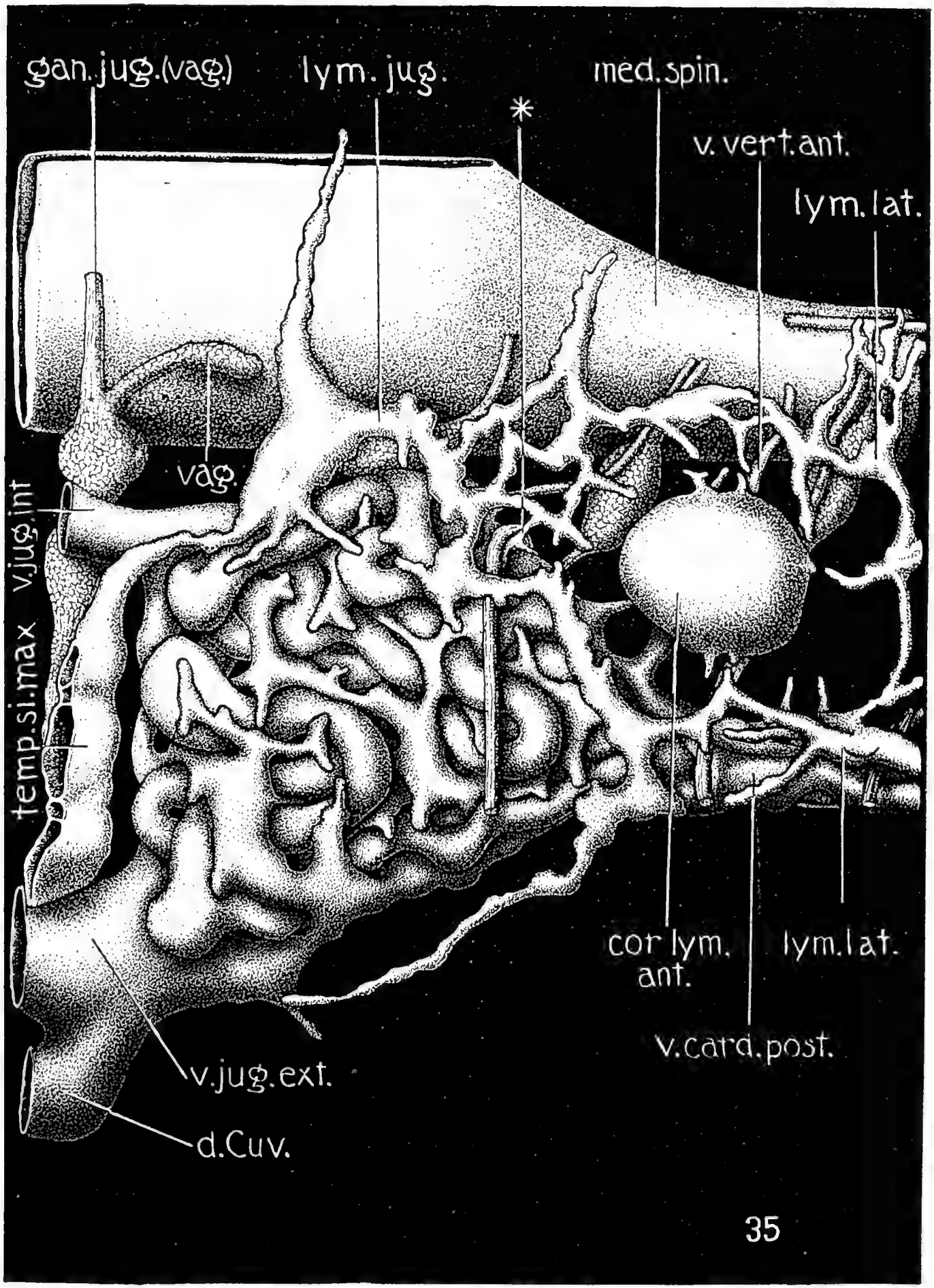
v. jug. int., vena jugularis interna.

v. card. post., vena cardinalis posterior showing both the medial (subcardinal) and lateral divisions.

d. Cuv., ductus Cuvieri.

d. proneph., pronephric duct.

Other structures as before.


gan.jug.(vag.)
lym. jug.
med.spin.
v. vert.ant.
lym.lat.
vag.
v.jug.int
temp.si.max
cor lym. ant.
lym.lat.
v.card.post.
v.jug.ext.
d.Cuv.
35

Resumen por el autor, Halsey J. Bagg

Trastornos en el desarrollo de los mamíferos producidos por las emanaciones de radio.

En un grupo de ratas adultas el autor las sometió antes y después de aparearse a las inyecciones de pequeñas dosis de soluciones radioactivas. Las inyecciones fueron subcutáneas e intravenosas. En un segundo groupo ratas preñadas, casi a término, fueron expuestas a la radiación de rayos gamma fuertemente filtrados. La dosis ordinaria fué unos 1350 milicuries horas. Generalmente una cantidad de emanaciones de radio inicialmente equivalente a más de un gramo de bromuro de radio fué empleada durante próximamente una hora. Los cambios característicos producidos por el radio fueron hallados en las crías de las hembras previamente inyectadas con soluciones radioactivas. Estos cambios fueron áreas subcutáneas de extravasación, situadas principalmente a lo largo de la linea media dorsal de los embriones. Es interesante notar que en tres crías diferentes se encontraron lesiones muy separadas en los jóvenes de madres que fueron tratadas varios dias antes de la fecundación. Muchos de estos cambios pueden notarse en embriones expuestos a la radio-actividad después de haber comenzado el desarrollo, pero generalmente los embriones murieron durante el tratamiento y fueron más tarde reabsorbidos o abortados. Las ratas jóvenes tratadas con la radiación de los rayos gamma tres días antes del nacimiento presentaban el mismo tipo de lesiones indicadas más arriba, pero en adición se encontraron marcadas interrupciones en el caso del sistema nervioso central, el sistema reproductor, y especialmente en la formación de los ojos. Varios de estos animales carecían prácticamente de neopalio, pero salvo por el hecho de ser ciegos no presentaron racciones neurológicas anormales. En este grupo varios animales vivieron durante más de un año y crecieron hasta alcanzar el tamaño normal.

Translation by José F. Nonidez
Cornell Medical College, New York

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DISTURBANCES IN MAMMALIAN DEVELOPMENT PRODUCED BY RADIUM EMANATION

HALSEY J. BAGG

Huntington Fund for Cancer Research, Memorial Hospital and the Department of Anatomy, Cornell University Medical College, New York City

TEN TEXT FIGURES AND THREE PLATES (FIGURES ELEVEN TO FIFTEEN)

The effects of radium on animal development has been the subject of several researches since the early work of Bohn (1), in 1903, upon the ova and larvae of the sea-urchin. Experiments on developing nematodes, molluscs, amphibians, fishes, and birds are associated with the names of Perthes (2), P. Hertwig (3), Schaper (4), O. Hertwig (5), and G. Hertwig (6). These investigators report developmental retardations following radiation of the ova and developing embryos. They found a particular susceptibility of the nuclei of the cells and a general slowing up in the developmental processes, especially in the case of the central nervous system. The total disturbances, depending upon the period of development when the radiation was applied, resulted in the formation of monstrosities conforming more or less to a general type.[1]

Similar experiments concerning the effects of x-rays on development have been conducted by many investigators. After exposure to x-radiation, Perthes (7) noted abnormal cell division and a retardation in the development of the ova of Ascaris mega-

[1] In connection with the above statement, and applying to x-ray treatments as well, the question of dosage is an important one. A survey of the literature shows that there was a very wide range in the severity of the dose employed, and in several cases the experimental settings were inadequately described (Bohn used 'some centigrams' of pure radium bromide for from twenty minutes to two hours). The amount of radium metal used in the investigations that have been mentioned varied from 2 mg. to 35.1 mg., and the time from a few seconds to several hours. The deleterious changes in the animal tissues varied with the amount of radium and the time of exposure.

locephala. Gilman and Baetjer (8), after radiating the ova of Amblystoma, and Baldwin (9), the fertilized ova of frogs, were able to produce a fairly constant type of developmental defect. Injurious results have followed in all cases where mammals have been exposed to x-radiation. It has been shown that when any particular part of a young animal is exposed to a sufficient amount of radiation, that part fails to reach its normal size and is unable to exercise a full degree of function.

Arrests in development and the production of abnormal types may be induced not only by radio-activity, but by many physical or chemical agents. Abnormal temperature changes, treatment by many chemicals, lack of oxygen supply, or the overabundance of carbon dioxide, etc., have produced marked changes in the developing embryo.

The present experiments are mainly concerned with disturbances in mammalian development, before and after birth, as a result of exposing the embryos of rats, at various times during the prenatal period, to irradiation from radium emanation. The effect on the embryos following radiation of the mother at varying intervals before mating was also determined. These experiments were designed not only to study the factors underlying the production of abnormal types, but through an examination of the abnormal to gain a clearer insight into the nature of normal development and differentiation.[2]

I acknowledge with pleasure my indebtedness to Dr. James Ewing for his aid in the interpretation of the pathological results.

METHODS AND APPARATUS

Two methods were used for applying the radium emanation. In the first method an 'active deposit' was obtained by exposing a definite quantity of common salt to a comparatively large amount of radium emanation, about 500 millicuries were used,

[2] Dr. J. F. Gudernatsch was a co-investigator with the writer during the year 1919. A preliminary report of the work done with him at that time is given in the Proceedings of the Society for Experimental Biology and Medicine, 1920, vol. 17, p. 183.

or the amount of radium emanation initially equivalent to one-half a gram of radium metal. To the radio-active salt thus produced sufficient water was added to make a physiological solution. The pregnant rats were injected subcutaneously in the shoulder region and intravenously through the caudal vein; 3 to 4 minims constituted the usual dose. Because of the rapid loss of radio-activity of these solutions, the injections were made immediately after the preparation. The details involved in preparing and measuring the doses, as well as the methods for protecting the experimenter, are described elsewhere (10 and 11). The activated solution exhibited all the known phenomena of radium metal itself; alpha, beta, and gamma rays were present, but the greatest physiological effects were probably due to alpha-ray activity. After long experimentation, a dose of 5 millicuries was found to be the maximum applicable to the aims of this experiment. In the second method gamma-ray radiation was applied through the ventral body wall of pregnant rats at nearly full term. A large amount of radium emanation was used, an amount equivalent to $1\frac{1}{2}$ grams of radium metal, filtered by 2 mm. of lead and $\frac{1}{2}$ mm. of silver. The source of emanation was 1 cm. away from the animal. The applicator, called a 'lead tray' in clinical usage, was 6 cm. in diameter and 1.5 cm. high. This was placed in the bottom of a small wire cage, 10 by 13 cm. in diameter and 10 cm. high, and was covered by a thin sheet of cardboard. The animal was placed on this paper immediately above the applicator.

Preliminary tests showed that a dose of about 1300 millicurie hours was sufficient to produce developmental arrests in the embryos without killing the pregnant animals. Doses as high as 2900 mc. hrs., however, were successfully used in some cases. The embryos were killed by ether, and histological material procured at various periods after the treatment. The tissues were fixed in Bouin's solution, cut in serial section, and stained with haematoxylin and eosin.

EXPERIMENTAL RESULTS

Series A. Injections of radio-active solutions

I. Subcutaneous injections after mating. Sixty-five full-grown, normal, pregnant rats were treated in this series. They were divided into four groups, each treated at different periods after mating. Ten pregnant females were injected 7 days after mating; twenty-four, 10 to 14 days after; twenty-one, 15 to 17 days after; and ten, 18 to 21 days after mating. Many of the animals were killed at weekly intervals after treatment, although some were allowed to reach full term.

Various degrees of developmental disturbances were noted, as shown in the following groups:

1. There was a large number of cases where no embryos developed, in others many began development, but were absorbed or aborted at an early time. The females in which no embryos were found, although they were definitely considered pregnant before treatment, occurred among cases treated soon after mating and in those instances where females were autopsied a considerable time after treatment. Figure 1 shows the remnants of maternal and embryonic structures; from the size of the placentae one can see that the foetuses had reached a fair degree of development before the radiation retarded the normal physiological processes. In one case (fig. 2) a small ovoid sac was found attached to the uterine wall by a thin stalk. This apparently represented the remnants of a former embryo and placenta. Extravasated blood and cell detritus were found in this sac and a great many large cells of an epithelioid nature that probably belonged to the former embryonic syncytium. The wall of this cyst was formed by fibrous connective tissue.

2. Embryos were killed by the treatment, but were removed from the mother and preserved before they were absorbed. These showed various extravasations from the vessels of the subcutaneous connective tissue, within the meningeal sinuses, and mainly along the dorsal midline of the body. Figure 3 shows a typical example of such a lesion which was situated in the mid-dorsal line. The mother of this embryo, no. 1167, was mated on

April 22, 1919, injected with 4.9 millicuries on May 7th, and was killed two days later. When the embryo was cut in serial section, it showed that the haematoma in the dorsal subcutaneous tissues had exerted sufficient pressure upon the spinal column to produce at one place a complete dislocation. Microscopical examination of the viscera showed no pathological changes. Not all the foetuses of a litter were affected in the same degree. In one case seven foetuses were found, three showing haemorrhagic lesions, two beginning to macerate, and two in the process of absorption. This variation in resistance was due either to the higher or lower vitality of the embryos themselves or to the amount of radioactivity which passed the placentae. In another case the foetuses, although injured, were carried to full term, and among a litter of six young, two were apparently normal and four showed haemorrhagic spots on the head, face, and along the dorsal midline of the body.

3. Several young of a single litter showed areas of extravasation and were born alive. Their mother died, however, and foster mothers refused to nurse them.

4. Eight litters gave normal living young. This number is low, because, as previously stated, many pregnant rats were killed by the experimenter at various intervals after treatment. The average number of young per litter was 4.8, as compared with 6.5 per litter for the control rats, but the probable errors indicate that this difference is, very likely, not significant. Only one litter, containing four young, survived a treatment given seven days after mating. Several of the rats of this group, which had apparently escaped the full radium exposure during the uterine period or perhaps they were more resistant to it, when mated inter se produced litters of apparently normal young of normal fertility. The offspring of these animals, about twenty in number, were observed for two generations, but no abnormalities were noted.

II. Subcutaneous injections before mating. Seventy-seven females were treated in this group, eleven died as a result of the injection before they were mated, while several were killed at weekly intervals after mating, and some were allowed to continue

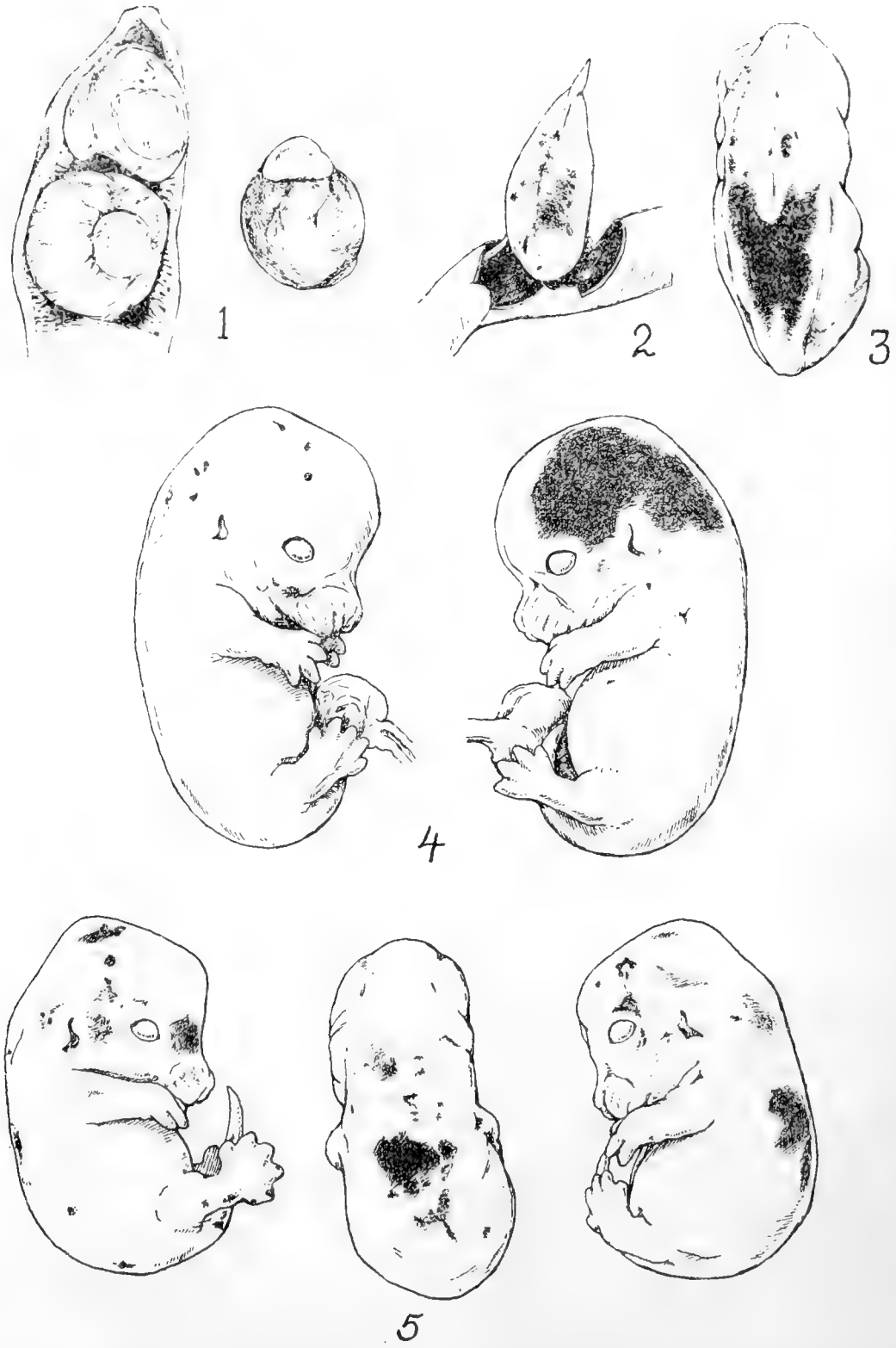

Fig. 1 Two well-developed placentae are shown at the left attached to a uterus which has been partly opened. The remnants of embryonic tissue are superimposed on the placentae. At the right is a placenta which has been dissected from the uterus, and shows more clearly the remains of embryonic material, here

to full term. Thirty-four animals were injected between 5 and 7 days before mating; seventeen, 10 to 14 days before, and fifteen, 20 days before mating.

Only three litters in this group showed abnormal young. The most interesting was a litter of seven, in which case the female was treated with 4.2 mc., 22 days previous to fertilization, and the foetuses, approximately 16 days old, showed very pronounced areas of extravasation, which in one case (fig. 4) covered a large area on one side of the head and a few small scattered areas on the other side. These areas were not only along the dorsal midline, but also on the lateral surfaces of the body as well (fig. 5). The lesions were much more widely distributed and more variable in size than in the cases recorded under section I. Although the conditions that produced these results were repeated many times, the above is the only case where positive data were obtained. Usually the female had either been rendered sterile or the young were killed and absorbed during early stages. There were two other cases, however, where young were found with haemorrhagic areas, and these occurred in a group of females that were treated seven days before mating. Female 85 was given a dose of 6.6 mc. on November 7, 1919. It was mated on November 14th, and

represented as a lighter area in the upper portion of the drawing. Female mated April 22, 1919, injected May 7th, killed May 16th. Dose = 4.6 mc. (subcutaneous).

Fig. 2 A stalked sac partly dissected from the uterus, showing the remnants of a former embryo and placenta. Female mated April 22nd, injected May 7th, killed May 16th. Dose = 4.8 mc. (subcutaneous).

Fig. 3 This is a dorsal view of a rat embryo, showing a characteristic area of extravasation due to the treatment of the mother during pregnancy. Female mated April 22nd, injected May 7th, killed May 9th, at which time seven foetuses were found about fifteen days in development. Two of the litter were macerated and two absorbed. Dose = 4.9 mc. (subcutaneous).

Fig. 4 Areas of extravasation are shown in the two views of this embryo, similar to the condition shown in figure 3, but in this case resulting from treating the mother twenty-two days before fertilization. There are a few small scattered areas over the right side of the head and a large area of extravasation on the left side. Female injected April 22nd, mated May 12th, killed May 30th. Seven foetuses were found, fifteen to sixteen days old. Dose = 4.2 mc. (subcutaneous).

Fig. 5 These are three views of another foetus, a litter mate of the one shown in figure 4, showing the wide distribution of the extravasated areas over both sides and back of the animal. The experimental conditions are the same as for figure 4.

as three young were born December 11th, fertilization took place about fourteen days after the treatment. Two of the young were apparently normal, but one showed a large haemorrhagic area, which involved most of the right side of the snout, the right eye, and a portion of the lower jaw on that side. This area disappeared after three days. Female 99, injected and mated at the same time with female no. 85, received a dose of 5.6 mc. Five young, three males and two females, were born on December 13th, making the date of fertilization about sixteen days after treatment. One male and one female showed definite haemorrhagic areas on the face. Consideration of these cases will be deferred until later.

Seventeen females following treatment were killed at varying intervals after mating and showed markedly haemorrhagic or cystic ovaries and congested uteri. In these cases radium emanation apparently had either so altered the maternal tissues as to prevent fertilization or development when started was soon followed by the death of the embryo and its absorption. Many nodules were found in the uteri in which it was impossible to differentiate between embryonic and maternal structures.

The remaining females (as previously stated, eleven died between the period of treatment and mating) produced either full-term normal young or young apparently normal at autopsy. Several of these living young grew normally and were mated inter se, but produced no abnormal offspring, although observed for two generations.

III. Intravenous injections after mating. The intravenous injections were primarily planned to act as a check on the series of subcutaneous treatments. The object was to determine the immediate reactions that might occur in the embryo as a result of injecting a comparatively large dose of radio-active solution into the circulation of the pregnant female, and whether these reactions would be similar to those already recorded for the subcutaneous series. The toxic reactions were so prompt and fatal that it was not necessary to treat many animals to settle this point. A typical case in that of female no. 123. This animal, of about nineteen days' pregnancy, was treated with 30 mc.

injected directly into the blood stream through the caudal vein. This was six times greater than the usual dose in the first two series. Three young were born dead twenty-four hours later. They showed very definite radium changes, typical of those already recorded for the subcutaneous series. Figure 6 shows a foetus still attached to an apparently normal placenta, but a characteristic area of extravasation was found over a considerable portion of the left side of the head. In figure 7A a dorsal view shows another embryo with two comparatively small haemorrhagic areas along the dorsal midline, and the placenta in this case is also normal. The third foetus in this litter was apparently normal, but the placenta (fig. 7B) had acted in the nature of a 'shock absorber' in protecting the foetus from exposure to the radio-activity, and it was so swollen and completely filled with blood as a result of its injury, that it had the appearance of a large haemorrhagic sac.

Series B. Results from radiating nearly full-term pregnant rats with gamma-ray radiation

Ten rats were treated at the end of about nineteen days of pregnancy. It was found that exposure to about 1350 mc.hrs. of radium emanation was sufficient to produce very decided changes in the embryo and yet leave the pregnant females sufficiently uninjured to be able to nurse their young and care for them until after the weaning period. When the dose was increased to 3378 mc.hrs., the young were severely injured, and were either killed outright or died two or three days after birth.

The following are the conditions that resulted in the first generation of animals treated in utero with a dose of about 1350 mc.hrs.:

1. The young of each litter were born two or three days after the treatment, alive and apparently normal.

2. About ten days after treatment, about half of each litter became markedly anemic, showed symptoms of diffuse edema, and promptly died. There was an easily recognizable slow development of meningeal and spinal-cord haemorrhages, similar to those already described as a result of treatment by radio-active

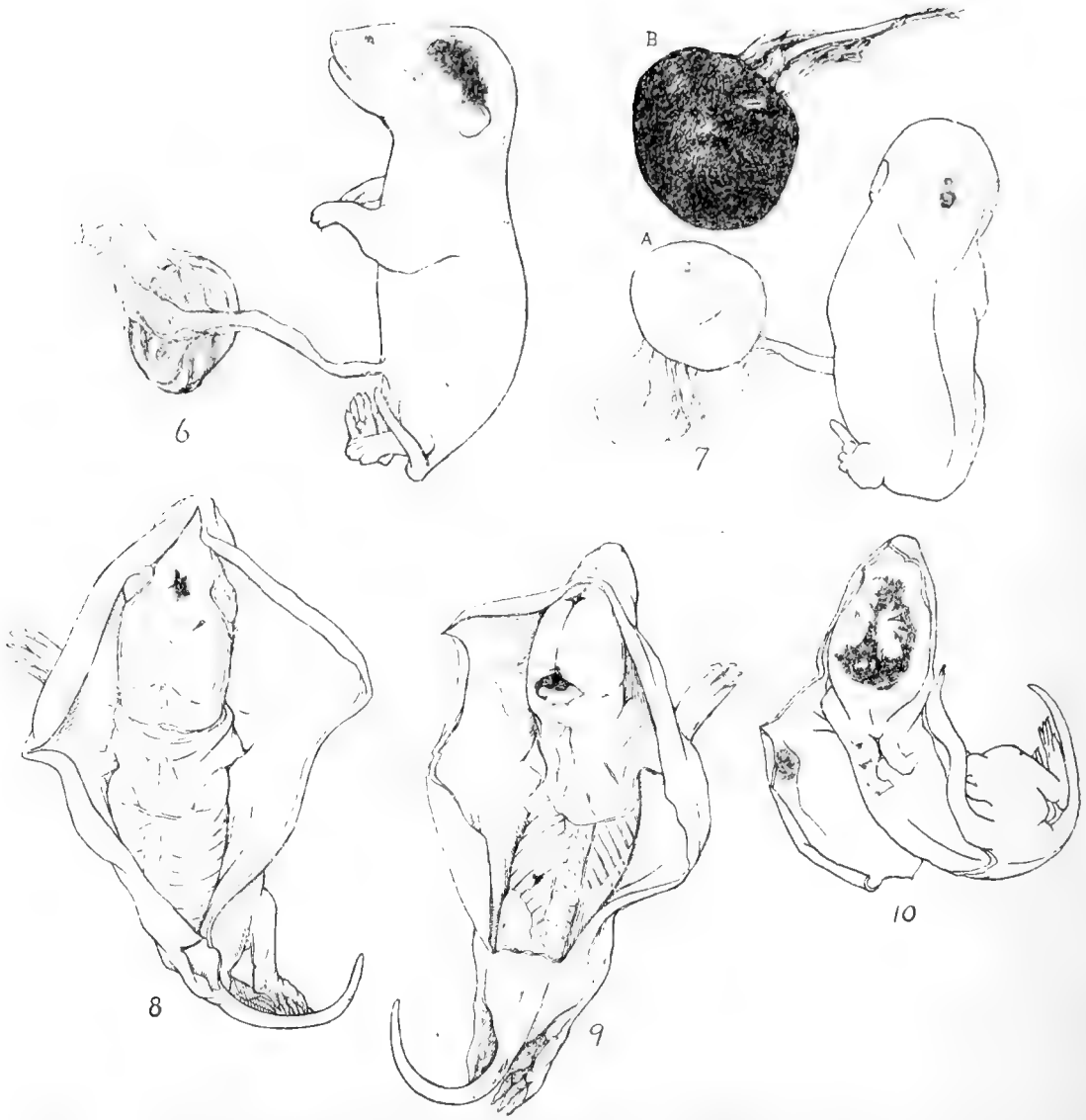

Fig. 6 There is a large and a small area of extravasation on the head of this foetus. The mother was injected intravenously with 30 mc. of radio-active solution and three young, about full term, were born twenty-four hours later. All were dead. In this case the attached placenta is apparently normal.

Fig. 7 The lower figure (A) shows an embryo with two dorsal head lesions and an apparently normal placenta. This is a litter mate of the animal shown in figure 6. At *B* is indicated a large haemorrhagic placenta from the third young of this litter, which itself was apparently normal.

Fig. 8 Dorsal view of a young rat with the skin dissected to either side. There is a prominent area of meningeal extravasation in the frontal region. This animal was treated in utero with 1350 mc.hrs. of gamma-ray radiation on February 21st and was born apparently normal on February 23rd. It died on March 3rd.

Fig. 9 Dorsal view of a young rat showing areas of frontal and occipital extravasations which were within the meningeal sinuses. There is a smaller lesion in the left dorsal thoracic region. This is a litter mate of the animal shown in figure 8, and the experimental conditions were identical. Death occurred on March 3rd.

Fig. 10 There is an extensive meningeal extravasation over a considerable portion of the hemispheres, and a haemorrhagic lesion is shown on the reflected skin from the dorsal interscapular region. This is a litter mate of the animals shown in the two preceding figures. Death occurred on March 2nd.

solutions. A series of these lesions is shown in figures 8, 9, and 10. Figure 8 shows a young rat with the dorsal integument partly dissected away, exposing a typical haemorrhagic area in the region of the frontal lobes. The slow development of this lesion could be easily noted through the thin, transparent scalp. This young was one of several treated in utero with 1350 mc.hrs. of gamma-ray radiation on February 21, 1920. It was born two days later, and died on March 3rd. The young rat shown in figure 9 was a litter mate of the previous animal. It shows the presence of three distinct haemorrhagic areas, a small frontal lesion, a fairly extensive one in the occipital region, and a small lesion in the subcutaneous tissues in the thoracic region, near the middorsal line on the left side of the body. This animal also died on March 3rd. A third animal belonging to the same litter is shown in figure 10. Here is seen a still more acute reaction, as shown by the fact that the animal died a day sooner than in the two cases above. There is an extensive area of meningeal haemorrhage which covers most of the dorsal portion of the brain, involving the frontal and occipital regions and the medial area between, as well as a considerable portion of the right temporal area. In addition, a distinct, rounded haemorrhagic lesion may be noted on the reflected skin on the left side of the body. This lesion occurred in the midshoulder region of the back.

The heads of several of the young rats showed marked lateral compression. In one case a haemorrhage so affected the spinal cord as to produce complete paraplegia. The tissues of these animals were studied histologically. Save for the mechanical disturbances produced by the presence of the extravasated areas, the most marked pathological conditions were seen in the liver and intestines. In the first case there was a pronounced fatty degeneration of the hepatic cells, and in the second, a desquamation of the lining cells of the intestinal mucosa.

3. It is interesting to note that the other half of each litter survived the treatment, grew to a normal size, and some animals have lived for over eighteen months. They showed the effects of the late uterine treatment by the following arrests in development:

a. The first pathological condition noted was that the eyes became smaller, the pupils opaque, and there finally was a complete, or nearly complete, closing of the lids and total blindness. This condition was first observed a short time after the eyes had opened. The photographs in figure 11 show three views of a female rat about one year old with typical eye deformities. The upper view shows the entire animal, which had grown to normal size and weight for its age. The left eye was nearly completely closed, as is shown more clearly in the lower right-hand view of the head at a higher magnification. Both pupils were opaque, but, as shown in the illustration, the right eyelids were slightly more opened than those of the other side. The animal was one of a litter treated in utero on March 8, 1920, was born six days later, and the photograph was taken on March 1, 1921. The dose in this case was 2920 mc.hrs. of gamma-ray radiation, which was a dose higher than that usually tolerated.

b. Mating tests showed that both the males and females were completely sterile in the first lots, but subsequently a first-generation female, that had been treated with 1350 mc.hrs., mated with a male similarly treated, gave birth to nine apparently normal young.

c. Before these adult offspring of treated animals were killed for histological examination, their neurological reactions were very carefully studied. The animals, being blind, when startled assumed various defensive attitudes, but save for these reactions their behavior was remarkably normal. There was no ataxia in locomotion or in any of the feeding reactions, auditory acuity was normal, and there was no cutaneous hypoesthesia or other sensory disturbances. Except for blindness, there was nothing to suggest abnormal sensory function.

d. When these animals were autopsied, marked developmental disturbances were noted in the condition of the central nervous system. The cerebral hemispheres were greatly reduced in size, and in several cases very little cortical material remained. Those portions of the brain that were ontogenetically older (the archiostriatum and the cerebellum) were apparently normal. The optic tracts were markedly atrophic. Correlated with this

disturbance in brain development, the skull was found to be asymmetrical, narrow, thicker than normal, and concave in the frontal region.

Figure 12 shows a dorsal view of a normal, untreated brain of an adult rat, magnified five diameters. In figures 13 and 14 are dorsal and lateral views of a brain of one of the rats which belonged to the same litter as those of section 2 of this series. This animal was treated with 1350 mc.hrs. on February 21, 1920, was born on February 23rd, and was killed December 31, 1920. This was one of the animals which (except for blindness) showed no abnormal neurological reactions. The magnification in figures 13 and 14 is the same as that for the control brain in figure 12. The dorsal view in figure 13 shows an apparently normal cerebellum and normal olfactory lobes, but the part of the brain which represents the rudiments of the hemispheres shows a great lack of development of cortical substance. In a side view of the brain in figure 14, the cortex may be seen to be very thin; indeed, not completely covering what should normally be the frontal, occipital, and lateral aspects of the brain. The remains of the hemispheres do not sufficiently approach each other in the median line to cover the colliculi beneath. In figure 13 the meninges on the left side of the brain have been removed, but on the other side they have been left in place. It was possible in this specimen to see the lateral ventricles through the transparent membranes. Several other brains have been studied which showed various degrees of developmental arrests resulting from radium treatment. In some cases the hemispheres were markedly reduced in size, were widely divergent in the median line, and yet the pallium was complete over the entire surface. In all these cases there was marked optic atrophy. These brains are now being sectioned, and a study of them in greater detail will be the subject of a separate communication.

e. A histological study of the eye showed that the eyeball was reduced to one-fourth the normal diameter. The retina was missing, but traces of the choroid remained as a few scattered pigment cells. The cornea was three times as thick as normal and covered with four or five layers of opaque squamous epithe-

lium. The optic nerve was extremely small, not more than one-third the normal dimensions.

f. The testes of the radiated animals were decidedly atrophic, and a comparison with the normal is shown in the photograph in figure 15. The diameters of the testicle alone (minus the epididymis) of the experimental animal was 14 mm. for the length and 7 mm. for the width, while the control measurements from normal animals of the same age and weight and with the same method of fixation were 21 mm. for the length and 11.5 mm. for the width. The epididymis of the radiated testis was practically missing. A small portion of the tail remained, but the head and body of the epididymis had failed to develop. Histological examination shows that there is little evidence of spermatogenesis. Some tubules seem to contain imperfect spermatoblasts and forming spermatozoa, but the great majority of tubules show complete degeneration and loss of epithelial cells, and contain loose granular material, which in places is calcified. Some spermatic tubules are greatly dilated and filled with granular material. Very few interstitial cells are visible.

The ovary of the radiated animals was reduced to one-fourth or one-fifth the normal size. The graffian follicles were entirely missing. Groups of lutein cells persisted in small numbers, but showed marked hydropic degeneration. Some of the large vessels about the ovary were sclerosed.

g. The liver, kidney, lungs, spleen, and the other organs were examined, but showed no pathological disturbance.

CONTROL GROUP

Pregnant rats of the same stock, the same age and weight, were injected subcutaneously and intravenously with equal amounts of solutions that previously had been strongly radio-active, but were allowed to 'decay,' until they had lost their radio-activity. These experiments gave absolutely negative results. As a control to the gamma-ray experiments, pregnant rats, sisters of the treated animals, were allowed to breed under exactly the same experimental conditions. No abnormal young were observed.

DISCUSSION AND SUMMARY OF RESULTS

It has been shown that when doses of radio-active solutions are injected into an animal marked physiological reactions take place. Large doses produce severe toxemia, resulting in pronounced pathological changes in the various viscera of the white rat (10). A study of metabolic changes in dogs, as determined by urine analysis, showed that, following intravenous injections of such solutions, there were very decided increases in the total nitrogen content of the urine, the urea, creatinine, uric acid, and the total phosphates (12). A prompt reduction occurred in the number of white blood cells of the dog after intravenous injections of these solutions, associated with a marked decrease in the relative percentage of circulating lymphocytes (13). In order to reduce as much as possible the severity of the reaction, very small doses of radio-activity were used in the experiments recorded in this article. But even with comparatively small doses, certain rats treated in utero showed very acute reactions. Many were killed by the treatment and were absorbed or aborted. Others were found showing pronounced areas of subcutaneous extravasations, mainly situated along the middorsal line of the body and within the meningeal sinuses. This condition was probably due to the destructive action of radium on the endothelium of the blood vessels, as well as a possible increase in blood pressure, as was shown to occur in the dog by Burton-Opitz and Meyer (14) after intravenous injections of very small quantities of radium bromide. A similar reaction of the blood vessels to radiation was previously reported by Halkin (15) for the skin of pigs, and by Danysz (16) for radiated mice. This destructive action of radium on the blood vessels is in line with clinical observations on the usual prompt regression of very vascular tumors (the angiomata, in particular) after exposure to irradiation.

The changes in the rat embryos of this experiment are interesting in so far as they show that a sufficient amount of radio-activity was able to pass the placenta and subsequently affect the developing embryo. This occurred after subcutaneous as well as intravenous injections of the mother. By far the most

interesting observation concerned the presence of lesions similar to those described above in rat embryos whose mother was treated with radio-active solutions a considerable time before mating. The writer has no explanation to account for this phenomenon. It would appear that the treatment of the mother several days previous to conception has lessened the faculty of the later-developing embryo to form proper endothelium of the blood vessels, and the wide distribution of these lesions over the body of the embryo (peculiar to this group of animals) would tend to substantiate this view. One female was injected twenty-two days before fertilization, and since the solutions lose their radio-activity very rapidly (there is about a 50 per cent reduction in the first hour after the preparation) the likelihood of any radio-activity remaining over during this period and affecting the egg at a later critical moment is remote. The amount of radio-activity remaining after twenty-two days, if present at all, should, as determined from physical computation, be infinitesimally small.

The series of intravenous injections again emphasize the specific action of radium emanation in the production of typical areas of subcutaneous emtravasations in the developing young, and in addition shows that the placenta may act in the nature of a 'shock absorber' and prevent the embryo from receiving the full effect of the radiation.

We now come to a consideration of the cases wherein pregnant females were treated with external applications of comparatively large doses of gamma-ray radiation. At this time a report is given only for embryos treated towards the end of pregnancy. The writer plans to continue this line of investigation and treat at earlier prenatal periods.

The results emphasize the well-known delayed reaction associated with gamma-ray radiation. There was approximately a ten-day interval following treatment during which no changes were noted in the embryo, and during this period the young animals were born in an apparently normal condition. Acute reactions promptly occurred at the completion of this time, killing half of each litter. The young rats died showing typical radium changes, such as anemia, diffuse edema, and meningeal,

spinal-cord, and subcutaneous extravasations. These extravasations were markedly similar to those already described for the series of solution treatments. The liver in these animals showed a fatty degeneration of the hepatic cells similar to the condition reported by Mills (17) after exposing a series of mice to gamma-ray radiation. The only other pathological change noted in these embryos was a desquamation of the lining cells of the intestinal mucosa. This observation is in line with the results emphasized by Hall and Whipple (18) in their experiments on Roentgen-ray intoxication in dogs.

While the animals described above died after showing acute reactions certain of their litter mates (half of the litter) continued to develop apparently normally. This difference in reaction may possibly be due to individual variability or tolerance for the radiation, but it probably can be explained by the fact that certain embryos were slightly farther away from the source of radiation than others, and as the intensity of radiation varies inversely as the square of the distance, even such slight differences in distances that did exist would be sufficient to subject the embryos to a considerable range in intensity of radiation. This is especially important in this case because the source of radiation was only 1 cm. from the body wall of the mother. The quality of radiation, however, remained the same for all the embryos.

It was soon apparent that the animals that lived over the ten-day period had not completely escaped the effect of the radiation, as was shown by a suppression of the full development of the eyes. Eye defects were noted, such as opaqueness of the pupil, atrophy of the lens, and closing of the lids, which resulted in complete blindness. These animals grew to a normal size, successfully competed with their cage mates for food, and showed absolutely no abnormal neurological condition, except those clearly incident to blindness. At autopsy, in some cases over a year after birth, very decided developmental arrests were noted in the structure of the brain. All grades of such maldevelopment were noted in the condition of the neopallium, from merely a decrease in the size of the hemispheres, which permitted the corpora quadrigemina to be clearly visible from above, to a more

marked absence of the cerebral cortex until only a very thin lamina of tissue remained to represent that structure, and there were large areas in the frontal, occipital, and temporal region where no cortex existed at all, so that when the meninges were removed the basal ganglia were clearly seen from without.

This correlation between defects in the development of the eye and the brain has been emphasized by Stockard (19) in his recent paper on developmental rate and structural expression. He states as follows: "The periods of arrest necessary to induce the eye and the brain modifications are so close together or so nearly the same, that one generally finds combinations and mixtures of the defects among the same experimental group of embryos." Again in the same article Stockard has shown that the type of deformity that results from experimental disturbance depends upon the developmental moment at which the interruption occurs. It is significant that the animals of this experiment showed arrests in the development of the neopallial portions of the brain and not in those regions which are ontogenetically older. Apparently the radium emanation, acting towards the end of pregnancy, had affected the development of the brain after the basal ganglia, the cerebellum, and medulla had become fairly well differentiated, and therefore those portions showed no gross changes. But the radium had slowed the developmental rate of the neopallium (which we know is one of the last portions of the brain to differentiate) during its period of active cell proliferation, and that portion of the brain was never able to reestablish its proper rate of development in relation to the other parts of the brain. If the period of treatment had occurred earlier in prenatal existence, other portions of the brain would probably have shown disturbances as well. The writer does not believe that the deformities in the brains of these animals were due to the early production of vascular disturbances later recovered from, but to an actual inhibitory effect of the radiation upon the developing nerve cells. If extravasations had occurred in this group of animals, and were so situated as to affect the development of the cerebral cortex in particular, they probably would have been detected as were even the comparatively small lesions

which were associated with the acute reactions. Also, if the effect was largely due to vascular disturbances, from the nature of the radiation employed, one would expect more generalized changes throughout the entire brain. However, on the other hand, Craigie (20), in his recent paper on the relative vascularity of various parts of the central nervous system of the albino rat, suggests that "the vascularization of the more recently evolved centers (of the cortex cerebri) is more susceptible than the more ancient regions to sexual, hereditary or environmental influences."

From a neurological point of view, it is interesting to consider that the animals with practically no cerebral cortex reacted so normally in their ordinary behavior. Except for blindness, there was no other apparent sensory disturbance, and motor coordination appeared perfect. The physiological functions localized in the cerebral cortex of the rat were in these animals apparently transferred to the basal ganglia and other paleokinetic portions of the brain, showing the remarkable degree of compensation possible in the mammalian brain when the disturbing element acts at an early period in its development. In this connection it is worth mentioning that the radium emanation did not produce a sudden traumatic effect, as is normally the case with the experimental production of brain lesions, and, in fact, the radium changes were probably prolonged over a considerable period. This condition favored the establishment of compensatory reactions, and exists (as shown by the writer in a recent article (21)) even in the case of radium lesions experimentally produced in adult mammalian brains.

The reproductive system completes its development a considerable time after birth, and so it is not at all surprising that these structures should have shown a considerable amount of atrophy due to the developmental arrest during the prenatal period. The ovaries and testes appeared to suffer with equal severity.

An interesting correlative relation was shown by the fact that the other viscera (digestive, excretory, etc.) of the animals that showed marked developmental arrests of the nervous and reproductive systems were apparently normal and the animals grew

to an average size. The lungs, liver, kidneys, etc., had differentiated before the physical agent was employed. Further studies with earlier prenatal treatments should throw some light on establishing the critical growth periods of the various embryonic structures.

As a final point, the results of this investigation may be of interest to the clinicians and the laboratory workers who handle large quantities of radium and utilize x-rays. Although the results of this paper deal only with irradiation of the female, there is no reason to believe that the germ cells of the male are more resistant to these destructive agents than those of the female, and, in fact, there is very good experimental evidence to show that spermatozoa of some animals are especially likely to produce abnormal young after exposure to comparatively small quantities of irradiation. Physicians should guard against the possibility of producing developmental arrests such as shown in this article when treating pregnant women, as well as the possibility of altering the human germ cells by irradiation previous to conception.[3]

CONCLUSIONS

1. The marked selective action of radium emanation on fast-growing embryonic structures was noted in these experiments.

2. Very decided developmental arrests occurred in the differentiation of the nervous and reproductive systems of mammalian embryos exposed to irradiation towards the end of pregnancy.

3. Experimental animals with greatly reduced, or practically no neopallium, gave apparently normal neurological behavior, except for blindness.

4. Radium emanation, used either in the form of a radio-active solution injected into the adult female, or employed as an external

[3] The writer does not mean to be understood as stating that present-day clinical irradiation treatments produce such effects in the developing young, but it is his personal opinion that such changes are biologically possible. It is not possible to obtain desired information by comparing the amount of exposure that a small mammal can stand with the corresponding dose that a man should tolerate, judging by comparative weights. The small mammal can tolerate very much more radiation in proportion to its weight than a man can.

gamma-ray radiation, produced marked areas of extravasation in the subcutaneous connective tissue of the developing young. This suggests that the action of radium emanation might be selective upon the endothelium of blood vessels.

5. Extravasations occurred in the developing young of females treated with radio-active solutions a considerable time before fertilization, and suggest that in some way the faculty of the later developing embryos to form proper blood vascular endothelium had been interfered with.

6. The results so far obtained indicate that gamma-ray radiation is a physical agent admirably adapted to the study of experimentally produced developmental arrests in mammalian embryos.

7. When women are subjected to therapeutic irradiation, especially during the early stages of pregnancy, the clinician should be forewarned concerning the possibility of producing very grave disturbances in the developing child.

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PLATES

PLATE 1

EXPLANATION OF FIGURE

11 The upper photograph shows an adult rat with eye deformities due to gamma-ray irradiation during the prenatal period. The size and weight are normal for its age. The lower figures show the lateral views of the head at a higher magnification. Both pupils are opaque and the left eyelids are nearly completely closed. Mother treated on March 8, 1920, young were born on March 14th, and photograph was taken on March 1, 1921. Dose = 2920 mc.hrs.

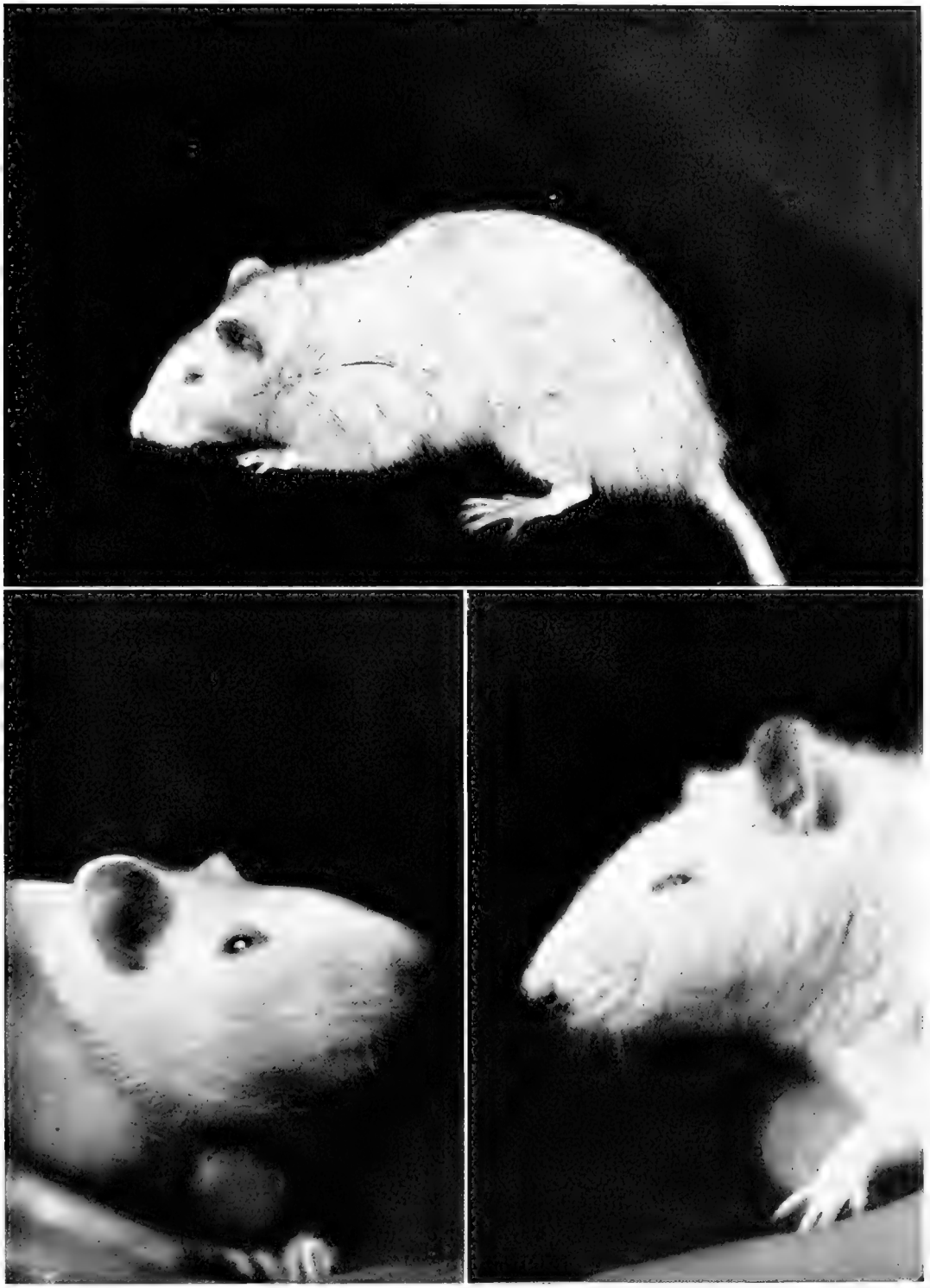


PLATE 2

EXPLANATION OF FIGURES

12 Dorsal view of a normal untreated brain of an adult rat.

13 Dorsal view of the brain of an adult rat showing the marked developmental arrest in the formation of the neopallium. The meninges on the left side of the brain are removed. Mother treated with gamma-ray irradiation on February 21, 1920, young were born on February 23rd. Brain from radiated young removed on December 31, 1920. Dose = 1350 mc.hrs. (For further reference see text.) The magnification is the same as for the drawing of the normal brain.

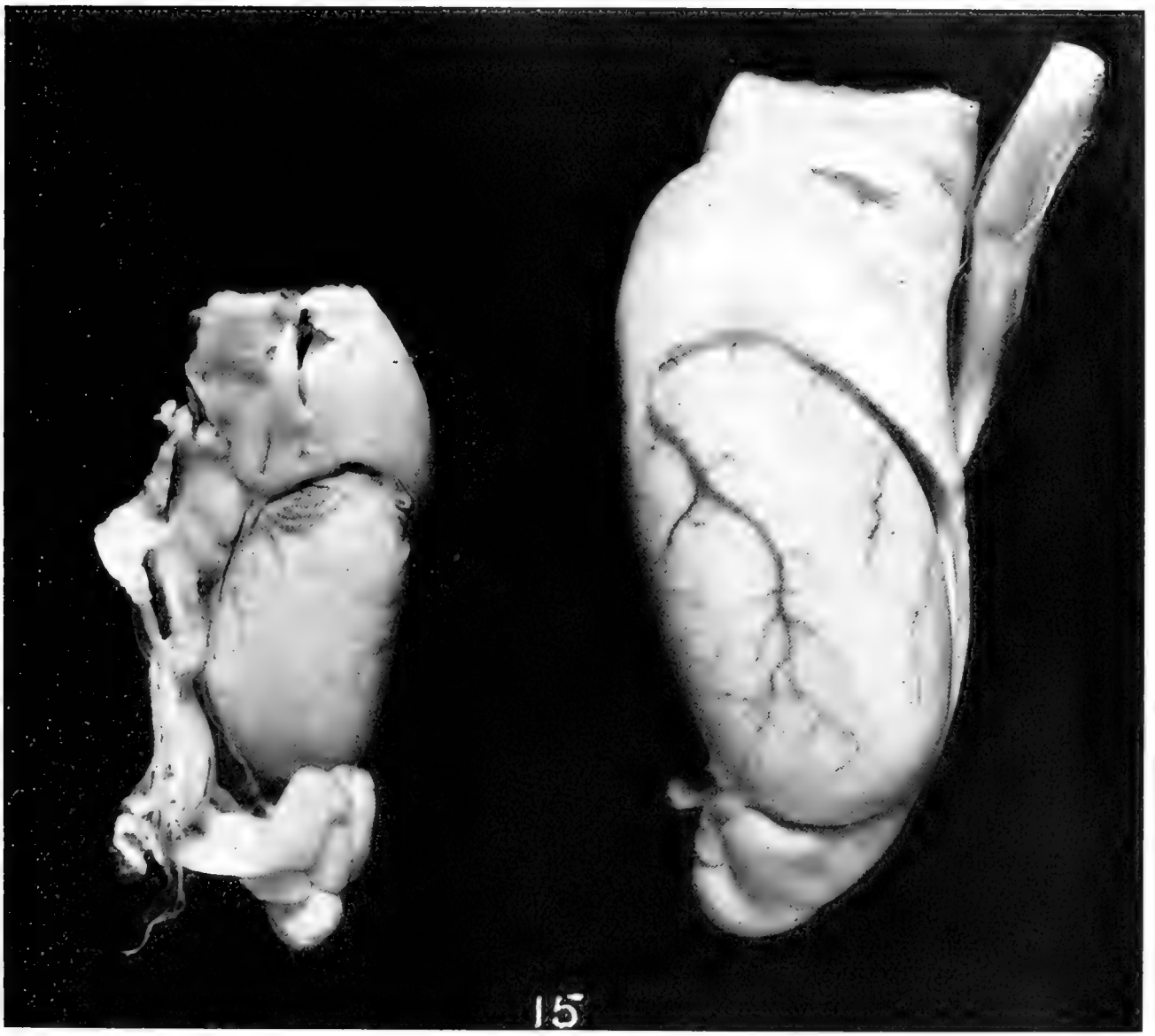
14 A lateral view of the brain shown in figure 13. This shows the thinness of the remains of the cerebral cortex and its total absence in the frontal and occipital regions. (For further reference see text.)


14
13
12

PLATE 3

EXPLANATION OF FIGURE

15 At the right is shown a normal untreated testicle of a white rat surmounted by a well-developed epididymis, which is partly obscured by fat. At the left is a radiated testis showing considerable atrophy. The single small lobe at the very bottom of the photograph represents the remains of the tail of the epididymis, the head and body of that part being completely missing. The animal was treated in utero on February 21, 1920, was born February 23rd, and was killed December 31, 1920. Dose = 1350 mc.hrs. of gamma-ray irradiation.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Resumen por el autor, E. A. Boyden.

El desarrollo de la cloaca de las aves, con especial mención del orígen de la bolsa de Fabricio, la formación de un seno urodeal y la presencia constante de una venta cloacal.

El presente trabajo contiene una revisión del desarrollo de la cloaca de embriones de pollo desde el tercer hasta el décimoquinto dia de la incubación, junto con observaciones sobre el desarrollo de la cloaca en otras tres especies de aves. El rasgo más notable es la presencia consetante de una ventana transitoria, producida por la desintegración de un área definidamente localizada del epitelio y su destrucción y absorción ulterior por los fagocitos, a raíz de cuyas transformaciones el contenido de la cloaca queda en contacto con el mesenquima durante un periodo de casi venticuatro horas. En opinión del autor esta particularidad susministra el único ejemplo, en la diferenciación de un órgano hueco, de una desaparición parcial de la pared epitelial como rasgo normal y constante del desarrollo.

La segunda parte de este trabajo trata de la formación de un seno transitorio situado hacia la región media del eje principal de la cloaca, el cual se ha interpretado como una repetición de la vejiga dorsal de los reptiles. Esta sección trata también de varias anomalías interesantes producidas el la desembocadura de los conductos wolfianos en la cloaca. Un tercer rasgo interesante es la presencia constante en los embriones de pollo, de un divertículo accesorio de la bolsa de Fabricio, el cual se origina probablemente mediante irregularidades consiguientes a la formación de la ventana cloacal. Por medio de este divertículo ha sido posible definir el esbozo de la bolsa con más exactitud que habia sido hecho hasta el presente y, por consiguiente, ofrecer nuevas ideas en relación con su orígen filogenético.

Translation by José F. Nonidez
Cornell Medical College, New York

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THE DEVELOPMENT OF THE CLOACA IN BIRDS, WITH SPECIAL REFERENCE TO THE ORIGIN OF THE BURSA OF FABRICIUS, THE FORMATION OF A URODAEAL SINUS, AND THE REGULAR OCCURRENCE OF A CLOACAL FENESTRA

EDWARD A. BOYDEN

Department of Anatomy, Harvard Medical School

FORTY-ONE FIGURES

The cloaca of the domestic fowl has been an object of interest to anatomists since early in the seventeenth century. It was discovered by Hieronymus Fabricius while investigating the urogenital apparatus of birds in connection with his pioneer study of the chick embryo. In his posthumous treatise, entitled "De Formatione Ovi et Pulli," Patavii, 1621, he describes as follows a blind sac lying behind the uterus of the fowl, but emptying into the cloaca close to its external orifice:

A third thing to be noted in the anus is a duplex vesicle,[1] which in its deepest part rises up to the os pubis, and is seen clearly and further back as soon as the uterus, already described, offers itself to view. Since the vesicle is pervious to the extent that a passage opens below from the anus to the uterus itself and from the uterus into the vesicle, as it were superiorly, the vesicle being closed at the other end, we have come to the belief

[1] The meaning of duplex in this passage is doubtful. The organ itself is never double. But in two species, a nestling raven (Osawa, '11) and the jay (Jolly, '15), it has been reported as bilobate. Osawa suggests that perhaps Fabricius may have had such a case before him when writing his description, but apparently Fabricius dealt only with the fowl, a species in which a bilobed condition has never been reported. The bursa is, however, two-walled, consisting of a mucous membrane and a muscular layer; and the recognized use of duplex to mean stout or thick, as applied to garments, may have been in the mind of Fabricius when he used this term. Unfortunately, its form is not recognizable in the woodcut in which he intended to show it.

that this is the place into which the cock injects his semen, and forces it in so that it is kept there.

This idea of a receptaculum seminis was discussed at length by his student Harvey, and by de Graaf, the latter publishing the first picture of the bursa. Both denied the function ascribed to it by its discoverer on the ground that it was equally well developed in both sexes. Beginning with the middle of the nineteenth century, it was subjected to microscopic examination and thereafter repeatedly studied, one group of investigators (Leydig and his successors) holding that it was purely a lymphoid organ; another group (Stieda and his school) maintaining, on embryological grounds, that it was primarily a glandular organ. Following Kölliker's description of the epithelial origin of the thymus, in 1879, these views were partially reconciled, but gave rise to a new discussion as to whether the epithelial primordium is replaced by invading tissue or whether it is itself transformed into a reticulum containing lymphocytes. Most authors since Wenckebach ('88) have held that the epithelium undergoes transformation without invasion. Recently Jolly ('15), in an elaborate summary of five years' work on the histogenesis, haematopoietic activity, and involution of the bursa of Fabricius, has advanced the theory that "the bursa represents an ancestral glandular organ, a cloacal caecum undergoing regression, which has become invaded by lymphocytes like other retrograding diverticula (the vermiform process of mammals and the intestinal caeca of birds), but in which, in view of a new function, a particular adaptation has taken place between the (persisting) epithelial tissue and the (invading) mesodermal, lymphoid tissue." In recognition of this symbiotic relation, Jolly would define both thymus and bursa as *lympho-epithelial* organs. Up to the present time, however, one must acknowledge that all attempts to analyze the function of the bursa or to find its counterpart in the hind-gut of other vertebrates have met with only partial success.

Modern investigation of the cloaca may be said to have begun with the embryological studies of Gasser ('73–'80) and of Wenckebach ('88), who reëstablished the view of Bornhaupt ('67) re-

garding the entodermal origin of the bursa. Since then only one paper has added any substantial increment to our knowledge of the general development of the avian cloaca, that of Pomayer ('02), in the Fleischmann series, dealing especially with the development of the phallus.

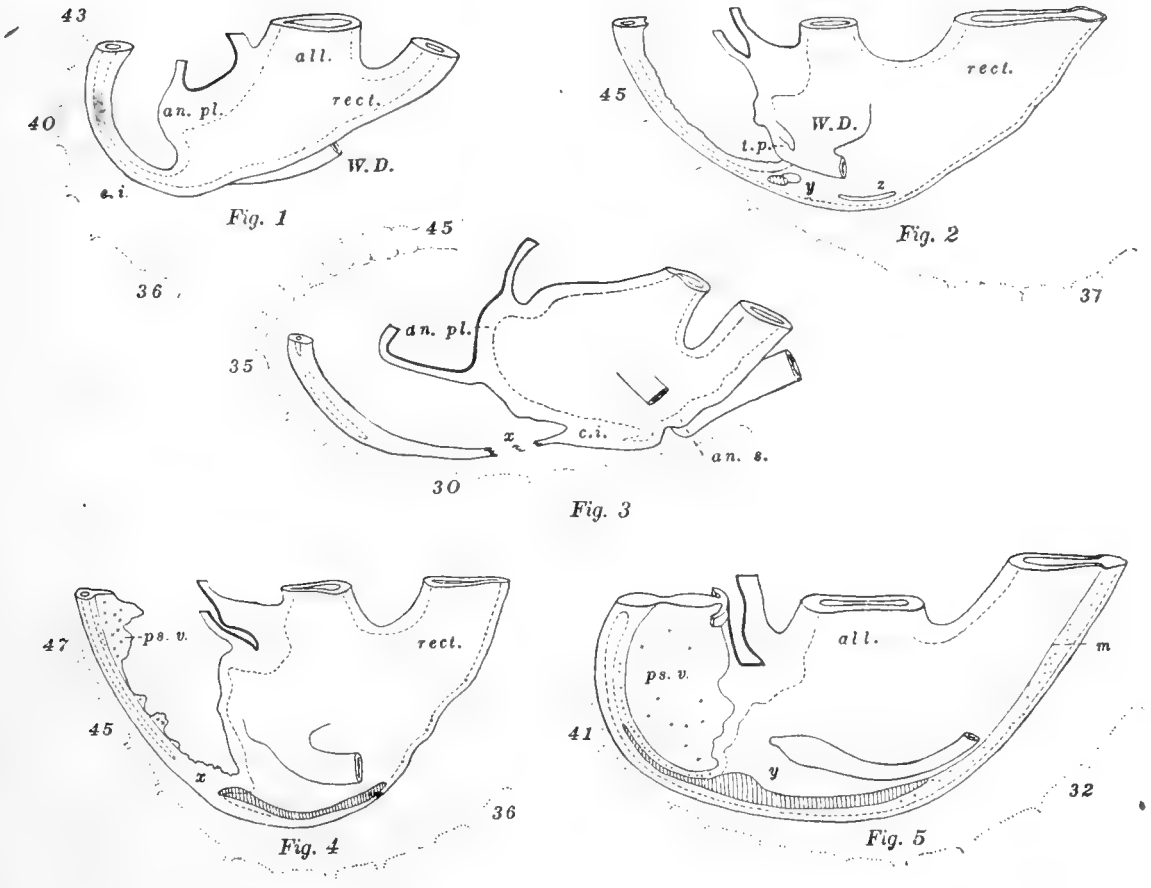
The present study originated with the discovery of a temporary foramen in the dorsal wall of the cloaca, produced by the disintegration of a definitely localized patch of epithelium and its subsequent removal by phagocytes, following which the contents of the cloaca are left in contact with the mesenchyma for a period of nearly twenty-four hours of incubation. This curious phenomenon was observed in over thirty embryos of the Harvard Collection, and its failure to occur has not been recorded in any embryos incubated approximately three days, the period at which the fenestra reaches its maximum size. My attention was first attracted to it by the presence of large numbers of embryonic phagocytes[2] similar to those found in the vestigial gill-filaments of chick embryos of corresponding age (Boyden, '18). Further study then demonstrated that this peculiar foramen was constant in its mode of development and invariably occurs, not only in chick embryos where it was first found, but in duck and pheasant embryos as well. It is of special interest not merely because it furnishes the only instance in the differentiation of a hollow organ, so far as I am aware, in which a gap occurs in the epithelial wall as a normal and constant feature of development, but also because it enables us, by virtue of the landmarks it establishes, to determine for the first time the exact point of origin of the bursa of Fabricius.

[2] These cells were first described as degeneration cysts, but they were subsequently seen in the underlying tissues into which they had been extruded from the epithelium, and were then recognized as embryonic phagocytes. It is a debated question whether these should be classed with the wandering cells of later embryonic stages and thus derived from the mesenchyma in general (the macrophages, clasmatocytes, etc., of numerous authors), or should be considered to have arisen in situ as reactions of the local mesenchyma, or even of the epithelium itself, to the presence of dead protein. This problem will be discussed in another paper in connection with the appearance of phagocytes in the anal plate at so early a period as forty-eight hours of incubation.

In following the origin and fate of this particular foramen, to which I have applied the name *cloacal fenestra*, it became necessary to review the entire chain of events in the development of the cloaca from the formation of the primitive streak to the period of histological differentiation, and to supplement a quantitative study of chick embryos with observations on other species, notably duck, pheasant, gull, and tern embryos. As a result of this study a number of other interesting facts have come to light. Those relating to the early development of the hind-gut and tail have been reserved for a subsequent publication.

DEVELOPMENT OF THE CLOACAL FENESTRA

In describing the origin of this foramen it will be necessary to refer occasionally to a peculiar tissue in the sacrocaudal region of young chick, pheasant, and duck embryos, which up to this time has not been observed in other birds or vertebrates. I refer to an indifferent cell-mass in the proximal end of the tail which persists long after the adjacent region has been differentiated—as late as the beginning of the fourth day of incubation in chick embryos. As seen in figure 5 (*ps. v.*), this inert mass lies within the angle formed by the cloaca and the caudal intestine, to both of which structures it is fused in a sagittal plane. Laterally it passes over into the mesenchyma of the tail, but rather abruptly, so that its limits can be approximately defined and the whole mass modeled in relation to surrounding structures, as displayed in figure 13. Beginning at the proximal end of the tail, this tissue is seen to be directly fused with the wall of the cloaca in the territory included between the anal plate and the junction of the caudal intestine with the cloaca (this being the wall of the cloaca which will later give rise to the bursa of Fabricius). Dorsally, this tissue is fused with the ventral border of the caudal intestine, and so intimately that the latter never has a chance to differentiate into an epithelium before it is resorbed. Ventrally, it fuses with the ectoderm bordering the anal sinus, while caudally it merges with the tail-bud mass—a fusion of three germ-layers extending across the tip of the tail. Thus the core of the tail is composed of an indifferent cell-mass, the whole of which can



GRAPHIC RECONSTRUCTIONS ILLUSTRATING INITIAL STAGES IN THE FORMATION OF THE CLOACAL FENESTRA

(Dotted lines and arabic numerals refer to somites; dash lines, to cavities of the cloaca; crosses, to the primitive-streak mass; periods, to scattered phagocytes; cross-hatching, to concentrated areas of disintegration on left side of embryo.)

Fig. 1 Tern embryo (Sterna hirundo) H.E.C. 2167: 5.5 mm. × 42. *all.*, allantois; *an. pl.*, cloacal membrane; *c. i.*, caudal intestine; *rect.*, rectum; *W. D.*, wolffian duct.

Fig. 2 Duck embryo (Anas domestica) H.E.C. 2193: 3 days, 21 hours. × 42. *t. p.*, terminal portion of W. D.; *y* and *z*, primary and secondary foci of disintegration (*z* restricted to right side of embryo in this stage).

Fig. 3 Turtle embryo (Chrysemys marginata) H.E.C. 1067: 6 mm. × 42. after R. F. Shaner. *an. s.*, primordium of anal sacs (cf. div. *c.* of chick embryo in plate 3); *x*, point of rupture of caudal intestine.

Fig. 4 Duck embryo (Anas domestica) H.E.C. 2194: 3 days, 21 hours. × 42. *ps. v.*, ventral half of primitive-streak remnant; *x*, occluded segment of caudal intestine.

Fig. 5 Chick embryo (Gallus domesticus) H.E.C. 2071: 3 days, 18 hours. × 42. (Compare with model of same embryo, fig. 13.) *m*, marginal sulcus separating thin-walled roof from thick-walled sides of cloaca.

now be defined as representing a persistence of the primitive streak in the form of a primitive-knot mass.[3] From the cloaca to the tip of the tail it forms a deeply staining homogeneous mass differentiating above and below into epithelial structures and on the sides into the mesenchyma of the tail. The portion occupying the distal end of the tail is an active tissue giving rise to the medullary tube, caudal intestine, notochord, and other caudal tissues. The proximal half, on the other hand, is degenerating. Some of it may contribute to the mesenchyma of the tail, but most of it, as indicated by the presence of innumerable phagocytes gorged with pycnotic nuclei, is undergoing resorption. This latter portion, representing an excess tissue, is absent from saurians and mammals, the caudal intestine in these forms lying close to the inner curvature of the tail. In this respect the cloaca of the tern (fig. 1) resembles that of lizards and snakes more than it does that of the gallinaceous birds.

A second process which must be considered in relation to the formation of the cloacal fenestra is the disintegration of the caudal intestine.[4] In all reptiles and mammals that I have examined and in one species of bird embryos (Sterna hirundo, the common tern) the caudal intestine undergoes reduction in the following manner. It appears to be pulled out, as if by the elongation of the tail, so that it tapers uniformly from the newly formed dilated portion at the tip of the tail to a slender tube at the oldest portion—the region adjacent to the cloaca. As the latter por-

[3] The details of the process by means of which the primitive streak is segregated in the tail of the embryo will be described in a subsequent paper. At this time it is sufficient to state that the area described above is derived from that portion of the primitive streak which is included between the rhomboidal sinus and the anal plate of a fifteen-somite embryo. In consequence of the folding of the blastoderm, and of the accompanying overgrowth of the tail, the dorsal portion of the primitive streak, lying under the ectoderm, is folded into the outer curvature which forms the tip of the tail and thus becomes the tail-bud mass. The ventral half, lying above the entoderm, and therefore on the inner curvature of the fold, is tucked under the tail and compressed into the angle between the anal plate and the caudal intestine.

[4] This term of Koelliker's seems more appropriate than 'post-anal gut' introduced by Balfour, since the gut-tract of the tail is an outgrowth of an area which originally lies anterior to the anal plate. As applied to mammals, the term is still less appropriate, as the caudal intestine disappears long before the anus is formed.

tion becomes more slender the lumen becomes occluded and the solid strand thus formed soon after ruptures (fig. 3, *x*). At least some of the cells disintegrate and are removed by phagocytes, but pycnotic nuclei are inconspicuous here as compared with the abundance of necrotic cells to be found in the degenerating caudal intestine of the chick. This process, which begins at the cloacal end of the gut, progresses slowly in a craniocaudal direction until the entire caudal intestine disappears. In duck, pheasant, and chick embryos, however, the reduction of the caudal intestine is greatly complicated by the disintegrating process going on in the primitive-streak mass, as referred to above, and by the disintegration of the adjacent cloacal wall, the latter process resulting in the formation of the cloacal fenestra.

The developmental history of this foramen, which is thus intimately associated with the removal of the caudal intestine, is divided into two phases, a period of active *disintegration*, beginning at about the 41-somite stage (chick embryo, 2 days, 18 hours), and lasting approximately twelve hours, and a period of *closure*, beginning somehere near the 50-somite stage (7-mm. embryos, of approximately 3 1/3 days), and ending in embryos of about 9 mm., incubated 3 days and 18 hours. Expressed in terms of embryonic growth, the first trace of the process appears just before the wolffian ducts fuse to the cloaca. The final stage in closure occurs about the time the ultimate somite is formed (I have found as many as fifty-three); that is, before the resorption of caudal somites begins.

The initial phase, as illustrated by the first text plate (figs. 1 to 5), is based upon two embryos. In consequence of the great rapidity with which the degenerative process is initiated, a far greater number of specimens of the same age than were available would have had to have been sectioned in order to have provided more than the two stages referred to. For there is not the slightest indication of the process in an embryo only one somite younger than the one shown in figure 5, where the entire area of the cloacal wall which is to be denuded has already begun to degenerate.

The first indication of impending disintegration appears in a duck embryo of forty-five somites (fig. 2). Two paired foci of degeneration (*y* and *z*) are here disclosed in the cloacal wall, one near the junction of the caudal intestine and cloaca, the other just anterior to the orifice of the wolffian duct. It is probable that area *y* is the first to develop as it is present on both sides of the cloaca, while area *z* is present only on the left side. This specimen, if corroborated by more examples, would seem to indicate that the degenerative process, which later involves the caudal intestine, begins in the wall of the cloaca near its junction with that structure.

In the next stage (fig. 4, of a duck embryo two somites older), the two areas on each side have grown together, presenting a continuous line of degeneration. In addition the lumen of the caudal intestine has become occluded (fig. 4, *x*), in the region which corresponds to the point of rupture in other vertebrates. This observation is important as indicating the independent origin of the two processes—the resorption of the caudal intestine and the formation of a cloacal fenestra—and shows that in the duck, at least, the caudal intestine becomes detached slightly in advance of the production of the fenestra. In this specimen, what remains of the undifferentiated primitive streak (*ps. v.*) is appended to the caudal intestine. In the embryo shown in figure 2, which is younger in other respects, all the primitive streak has been removed, its former presence being indicated only by the roughened and irregular ventral margin of the caudal intestine.

The third stage, illustrated by a chick embryo of forty-one somites (fig. 5), shows an extension of the area of degeneration both caudal and cephalad,[5] and the appearance within this area

[5] The cephalic extension contains only scattered phagocytes (represented by periods in the figure) and does not usually become denuded of epithelium, although the fenestra has been observed to extend that far in a few cases. If the cut end of the rectum in figure 5 be examined, it will be noticed that the periods are limited to a zone of the cloacal wall which is thinner than the adjacent zones. This area, together with the dorsal wall of the caudal intestine with which it is continuous and homonymous, represents a persistence of the primitive condition of the hind-gut which, like the roof of the foregut, is always thin-walled when first

of discontinuous holes where complete resorption of the epithelium has taken place (fig. 13, from a wax model of the same embryo). The perforated walls of the cloaca at this period thus simulate in appearance a fenestrated membrane. Almost immediately, however, the holes run together, forming a continuous rift along the cloaca and caudal intestine. In this manner the dorsal wall of the cloaca becomes detached from the sides and thus isolated as a trough-shaped structure, is slowly resorbed. Its histological appearance will be described later in the paper.

An invasion of the caudal intestine also occurs from another region in chick embryos and, to a lesser extent, in ducks. This is an extension of the degeneration process going on in the primitive-streak mass (fig. 5, *ps. v.*) into the ventral wall of the caudal intestine, and involves only that part of the intestine which is adjacent to the primitive streak. Thus, in the undifferentiated epithelium of the inner curvature of the caudal intestine are found phagocytes (again represented by periods, fig. 5) which are coextensive and continuous with the primitive-streak mass, which is itself undergoing rapid phagocytosis. The occurrence of these has nothing to do with the invasion of the caudal intestine from the cloacal end, except that the two processes cooperate in destroying that end of the gut.

The resorption of the caudal intestine in birds can now be summarized as follows. In chick embryos the flanks of the caudal intestine are invaded by a degenerative process originating in the cloaca, which removes the epithelium before the cavity of the anal gut can be occluded. In duck embryos the two processes take place nearly simultaneously, the cloacal invasion slightly preceding the occlusion of the caudal intestine. Finally, in terns, the cloacal fenestra is not present at all, and the caudal intestine undergoes reduction by the method already described as common to most amniotes.

formed. The side walls are the first to thicken. As development proceeds, the latter are brought closely together, buckling the flat, thin-walled area into a steep-pitched roof. But for some time there is an abrupt transition between thick- and thin-walled portions, and it is along this thin area, and its continuation into the cloaca, that resorption of epithelium first appears.

The final stage in the formation of the fenestra, ending the period of disintegration, is shown in figures 14 and 15, of an 8-mm. embryo of forty-eight somites (3 days and 6 hours). The entire roof of the cloaca, between the wolffian ducts and the anal side of the caudal intestine, has been denuded of epithelium, leaving a considerable gap bounded only by mesenchyma (dash line, fig. 14). The connection of the cloaca with the caudal intestine has been lost, and the latter, together with the primitive-streak mass, is now rapidly disintegrating at the ruptured ends. As a rule, degeneration does not spread any farther cephalad than recorded in figure 5. But occasionally it extends much farther, and is probably instrumental in producing irregularities in the dorsal wall, which will be discussed later, in the section dealing with accessory diverticula.

The *cytological changes* involved in the formation of the fenestra include the necrosis of the epithelial cells, their removal by phagocytes, and the reaction of the surrounding mesenchyma to the denuded area. As seen in ordinary serial sections, the first step in the disintegration of the flanks of the cloaca is a slight oedema of the epithelium which causes the cells to spread apart. As these become necrotic, the cytoplasm becomes finely granular and then vesicular and the nuclei pycnotic. At this stage the epithelium presents a confused histological picture due to the simultaneous degeneration of so many cells. But almost immediately the cells in regions *y* and *z* (fig. 2) are resorbed, leaving a gap in the wall covered only by mesenchyma. At first the mesenchymal cells appear to congregate about the region, as if to plug up the opening, and this continues as long as there is an abundance of necrotic tissue. During the stage when the wall is a fenestrated membrane the mesenchyma may even invade the cavity. This is especially true of the caudal intestine which is eventually replaced by mesenchyma which has grown in through rifts in the sides and filled the cavity before the walls have been completely removed.

The most favorable time for observing the cytological changes is after the gap has been formed on each flank of the cloaca, but before the roof of the cloaca thus isolated has itself been removed.

The degenerating epithelial cells bordering the gap may then be studied in less crowded condition. Such a picture is presented in figure 19—an obliquely frontal section passing through the fenestrated area at right angles to the back lines of the cloaca; that is, in a plane cutting the allantoic duct lengthwise. In this figure the following features should be noted: the isolated roof of the cloaca, rows of necrotic epithelial cells on either flank, the concentration of mesenchyma about the gap on either side, and the rounded margins of the epithelium conspicuous by their failure to regenerate. In the epithelium bordering the gap are occasional pycnotic nuclei, and here and there a phagocyte, indicating a slow resorption in contrast to the sudden removal characteristic of initial stages. When the degenerative process slows down and finally comes to an end, a single large foramen is left in the dorsal wall of the cloaca extending from behind the level of the wolffian duct to the site of the caudal intestine, having a lenticular shape when viewed from below (fig. 15). As seen in microscopic section (fig. 20) the epithelium of the roof of the cloaca has been entirely removed, leaving in its place a line of mesenchymal cells which have flattened out into a surface layer as if under compression by the fluid in the cavity, in a manner recalling the formation of the false epithelium which lines the joint cavities.

Even before degeneration stops, however, the process of closure sets in. This consists of a fusion of the epithelial margins of the gap beginning at the caudal angle of the aperture, so that in the space of another twelve hours, only a slender cleft remains at the anterior end of what was once a big fenestra (fig. 23, *fen.*). This process of closure seems to be aided if not caused by a progressive approximation of the sides of the cloaca, beginning at the anal plate, which results in the fusion of opposite walls and the formation of a urodaeal membrane. Figure 21, of a cross-section of the fenestra in the last stage of closure, shows that even to the end of closure no regeneration of the cloacal lips has taken place, but that rather the free margins of the walls have been pushed down into the mesenchymal cavity, as if by lateral compression exerted upon the side of the cloaca. By the middle of

the fourth day of incubation all signs of the cloacal fenestra have disappeared, and its site cannot be accurately located except in such general terms as lying between the accessory bursa and the urodaeal sinus.

In concluding this chapter one may say that the most conspicuous feature of the entire process is the rapidity with which it takes place—both the sudden appearance of a gap and the rapid closure of it—all occurring within a period of twenty-four hours. Although the evidence presented would lead one to infer that the disintegration of the cloacal wall precedes the reduction of the caudal intestine, and is thereby independent of it, and calls for a separate explanation, it is still possible that the cloacal fenestra represents a modification or extension of the process by which the caudal intestine is reduced in other vertebrates. Any attempt, however, to explain the significance of this foramen in the domestic fowl, duck, and pheasant, must take into account an equally peculiar feature, likewise found only in birds with a fenestra, namely, the undue persistence of the primitive streak in the proximal end of the tail. It is well known that the tail in modern birds, and of fowls in particular, is shorter than in the Archaeornithes. It is conceivable that the degenerating primitive-streak mass in the tail of the chick embryo represents a persistence of material once utilized in tail-building but now superfluous. It would also seem, from a comparison of the cloacas in the first text plate, that the persistence of this indifferent tissue has delayed the differentiation of the caudal intestine and perhaps of the whole tail itself. For figure 5 represents a chick embryo in which the ventral wall of the caudal intestine has not been differentiated into an epithelium, but is still continuous with the primitive streak throughout its length. Yet that chick is older in other respects than the tern embryo of figure 1, as evidenced by the lesser number of somites in the chick, and by its greater maturity of form. If it be granted that the development of the caudal intestine in the chick has been retarded by the persistence of the primitive-streak mass, it is not inconceiv-

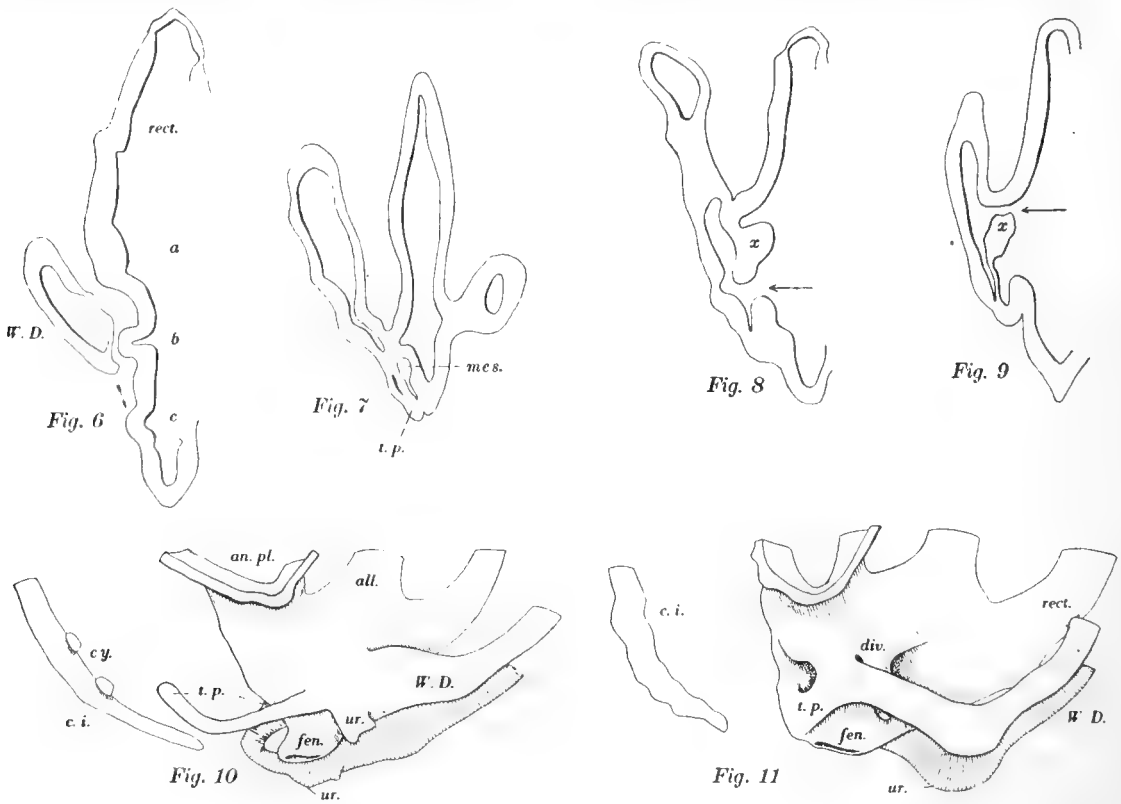
able that the development of the corresponding region in the adjacent cloacal wall has likewise been interfered with, and that when reduction of the caudal intestine does occur, both of these areas are subjected to a retrograde process more rapid and extensive than obtains in other vertebrates.

DEVELOPMENT OF THE UROGENITAL APPARATUS

Anomalies arising in connection with the wolffian ducts

About the time that the primary excretory ducts reach the level of the cloaca in their downgrowth from the pronephros, an eruption of diverticula appears on each flank of the cloaca opposite the distal portion of the ducts. Since these outpocketings of the cloaca seem to develop in response to the presence of the wolffian ducts, and later fuse with them, I have named them *complemental diverticula*. A surface view of this stage, such as is shown in figure 13 of a 41-somite chick embryo (62 hours), reveals the presence of two groups of diverticula—a circlet of five or six small ones opposite the terminal portion of the duct, and a single larger one farther up on the shaft, as broad as the whole field of smaller ones. In this embryo the duct of the left side has not fused with the cloaca, although fusion on the right side has taken place. In a 40-somite embryo neither duct has fused. My observations would therefore differ somewhat in detail from the statement of Lillie that the wolffian duct "reaches the cloaca (with which it unites) about the 31-som. stage" and that "at about the sixtieth hour the ends of the ducts (described in the preceding sentence as solid) fuse with broad lateral diverticula of the cloaca, and the lumen extends backwards until the duct becomes viable (?) all the way into the cloaca (at about 72 hours, 35 somite stage)." For a frontal section (fig. 6) of the cloaca shown in figure 13, at the place where the left wolffian duct makes the nearest approach, shows that the duct has not yet fused with the cloaca, that its terminal portion is patent, and that the mesial wall of the duct is thinning out in anticipation of fusion. The section through the left side happens to

pass through three diverticula, the broad one (*a*), and two smaller ones (*b* and *c*, members of the terminal circlet of diverticula). The arrow indicates that the duct in sections higher up would reach as far as the point *c*. In subsequent stages the mesial wall of the duct would fuse with the cloacal diverticula forming



TEXT PLATE ILLUSTRATING ANOMALIES OF THE WOLFFIAN DUCT

Fig. 6 Chick, H.E.C. 2071 (section 661): 2 days, 18 hours. × 77. *a*, proximal complemental diverticulum; *b* and *c*, distal complemental diverticulum; arrow indicates extent of wolffian duct in other sections. Note thinning out of mesial wall of duct in preparation for fusion with cloaca.

Fig. 7 Duck, H.E.C. 2194 (section 680): 3 days, 21 hours. × 77. *mes.*, mesenchyma interposed between distal and proximal attachments of duct.

Fig. 8 Chick, H.E.C. 2073: 2 days, 21 hours. × 77. *x*, plate formed by fusion of mesial wall of W. D. with complemental diverticula of cloaca; arrow shows where plate has been ruptured, through distal diverticulum.

Fig. 9 Chick, H.E.C. 2072: 2 days, 22 hours. × 77. Arrow shows where plate has been ruptured through proximal diverticulum.

Fig. 10 Model of duck embryo, H.E.C. 2197: 4 days, 8 hours. × 40. *all.*, allantois; *an. pl.*, cloacal membrane; *c. i.*, caudal intestine; *cy.*, epithelial cysts of unknown origin; *fen.*, fenestra; *t. p.*, terminal portion of W. D.; *ur.*, primordium of ureter; *W. D.*, wolffian duct.

Fig. 11 Model of duck embryo, H.E.C. 2195: 4 days, 8 hours. × 40. *div.*, aberrant complemental diverticulum.

a continuous plate (figs. 8 and 9, *x*) from *a* to *c*. In some cases the plate ruptures first through the distal diverticulum (see arrow in fig. 8); in others at first through the proximal one (fig. 9). But in all chicks of older stages that I have examined, the plate is resorbed, leaving a single large opening from *a* to *c*. It is probable that phagocytes aid in this resorption, as I have found them within the thin plate as soon as the duct has joined the cloaca. As development proceeds, the lateral walls of the cloaca beginning with the anal plate gradually come together, forming a solid membrane comparable to the urethral plate of mammals, so that finally the opening of the wolffian duct becomes restricted to the middle of the cloaca at the level *a* of figure 6 (cf. figs. 14 and 16). Not all of the complemental diverticula, however, fuse with the ducts. Some of them, no doubt, are soon suppressed. Others of them persist for a longer or shorter time, growing out as accessory diverticula (figs. 11, 22, 24, and 32, *div.*).

The most interesting anomalies occur in duck embryos, and are due to the excessive length of the wolffian duct, which normally grows down to the very end of the cloaca (fig. 7). In one case observed, only the proximal portion of the duct had fused with the cloaca, the terminal portion growing out as an aberrant diverticulum (fig. 10, *t.p.*, left). In other cases both terminal and proximal portions fuse, but not continuously, so that an area of mesenchyma is left between the two attachments (fig. 7, *mes.*). If, then, the basal ends of the ducts begin to grow, a ring-shaped (fig. 10, *t.p.*, right) or a U-shaped (fig. 11, *t.p.*, left) attachment of the ducts is formed, opening into the cloaca at two points, representing the original points of fusion. A similar anomaly has been found in a chick embryo (H.E.C. 99), and it would seem almost certain that a larger number of specimens would show many indications of aberrance resulting from the fusion of the wolffian duct to the complemental diverticula. The further changes in the form of the wolffian ducts and their incorporation into the wall of the cloaca will be considered in the next chapter.

Formation of the urodaeal sinus

In discussing the origin of urinary bladders Felix defines four main types: 1) mesodermal bladders, arising from the fusion or dilation of the caudal ends of the wolffian duct; 2 and 3) dorsal and ventral cloacogenic bladders, outgrowths or dilations of the dorsal and ventral walls of the cloaca, respectively, and, 4) allantoidogenic bladders formed by the retention of the proximal end of the allantois. The first type in its pure form is realized only in selachians, the second type only in amphibians, both groups being devoid of an allantois. The bladders of all other vertebrates, according to Felix, are of mixed origin. When we examine birds, it appears that they are the only class among amniotes without one or more bladders, yet curiously enough, reptiles, from which birds have descended, constitute the class with the greatest number and diversity of bladders. Thus, according to Felix, lizards derive their bladders from three sources, dorsocloacogenic, allantoidogenic and mesodermal; and in turtles the bladder is formed from dorsocloacogenic, ventrocloacogenic, allantoidogenic, and mesodermal origins (Keibel and Mall, II, p. 869). It would be strange, then, if the bird did not exhibit some traces of bladder formation in its ontogeny, and such, in fact, may be found. The most conspicuous of these is the intra-embryonic expansion of the allantois shown in figure 39. It is almost identical at this stage with the primordium which develops into the ventral bladder in most reptiles. But it is completely resorbed in adult birds.

The other structure in bird embryos which recalls the reptilian bladders (this time those of dorsocloacogenic and mesodermal origin) is the *urodaeal sinus*, a name which I have applied to the cavity of the urodaeum at its maximum extent (figs. 40 and 41 *urod.*). Minot in 1900 called attention to the peculiar relations of this cavity as follows: "From the closure of the intestinal opening by the entoderm (occluded rectum), and of the anal opening by the anal plate (meaning urodaeal membrane), there is left a clear passage from the wolffian duct across (to) the opening of the allantois." And he quotes the suggestion

offered by G. H. Parker that "the physiological purpose of this arrangement is to secure the transmission of the excretion from the embryonic kidney to the allantois, and to prevent the escape of the excretion, either into the intestine or into the amniotic cavity, where it might prove injurious to the embryo." That the urodaeal sinus is a mechanism inherited directly from reptiles was revealed two years later by the comparative studies of Fleischmann and his students on the cloaca and phallus of lizards, snakes, turtles, birds, and mammals. He notes that in the Sauropsida the urodaeum is divided into two portions, a distended oral portion always in relation to the wolffian ducts, and an elongated caudal portion which forms an open passageway (even in young embryos) to the anus. The shutting off of the urodaeal sinus from below in birds is due to the fact that the second half of the urodaeum never elongates, but remains short and impervious through the formation of a urodaeal membrane.

While the posterior portion of the urodaeum becomes elongated and subject to great modification in various reptiles, the anterior chamber (urodaeal Kammer of Unterhössel) is always associated with bladder formation. It becomes chiefly dilated in a dorsolateral direction, so that the entire cavity and associated mesodermal ducts assume the appeance of a dorsal bladder (cf. Fleischmann, Taf. VIII, figs. 1, 2 and 4). This striking feature appears temporarily in bird embryos as the urodaeal sinus, and is as convincing a repetition of reptilian ancestry as the allantoic bladder previously referred to in figure 39. But since it was studied chiefly in older embryos, and then largely by means of sagittal sections, its extent and composition was not fully appreciated even by Fleischmann.

As seen in figures 40 and 41, the urodaeal sinus (*urod.*) is a greatly inflated segment of the cloaca, placed athwart the main axis of the hind-gut, between the occluded rectum and the urodaeal membrane. Its lumen from front to back is reduced to the size of a fissure, but is greatly expanded laterally and dorsoventrally, extending from the wolffian duct of one side to that of the other and from the dorsal side of the cloaca to the allantois. Although existing as a single structure at this stage, it has been formed

by the confluence of three originally separate elements. The first of these to appear is the median diverticulum designated as diverticulum *c* in the reconstructions shown in plate 3. It arises as early as the beginning of the fourth day and maintains its identity as a distinct and conspicuous feature of the cloaca as late as the seventh day, at which time it is incorporated in the urodaeal sinus. This structure has been figured in descriptions of the avian cloaca as far back, at least, as the work of Bornhaupt ('67). But I question whether its existence as a separate rounded diverticulum has ever been appreciated. Pomayer, in the Fleischmann series, labeled it "Urogenitaltasche" in a sagittal section of a duck, giving it the same designation as the paired urogenital pockets of the snake, Tropidonotus, which are dilated outpocketings on the dorsal wall of the cloaca into which the wolffian ducts empty. A median diverticulum occurs in the same place (as diverticulum *c*) in the turtle embryos modeled by R. F. Shaner (fig. 3, *an. s.*), and has been interpreted by that author as the primordium from which the respiratory sacs (bursae anales) of turtles develop. In view of its position between the two wolffian ducts in both chicks and turtles, it seems not improbable that diverticulum *c* represents the dorsal outpocketing of the cloaca of reptiles from which the wolffian ducts have shifted in course of their migration to the allantois.

The second and third components of the urodaeal sinus arise more or less together. As seen in figures 14 and 6, the wolffian ducts, when they first reach the level of the cloaca, fuse to the cloaca along a broad area extending from the caudal margin to near the allantois (*a* to *c*). The fusion at *c* approximates the primary position of the excretory ducts in lower vertebrates. In consequence, however, of the fusion of the two side walls of the cloaca, beginning with the anal plate, to form the urodaeal membrane, the outlet of the wolffian ducts at *c* and *b* in figure 6 is suppressed. The broad complemental diverticulum (fig. 6, *a*) thus becomes the main channel, and in course of development is enlarged into a wing-like expansion of the cloaca connecting the wolffian duct with the neck of the allantois (fig. 16). Meanwhile the segment of the wolffian duct between the orifice

of the ureter and the cloaca begins to develop irregular enlargements sometimes suggesting diverticula (fig. 17), which eventually result in the widening of that segment. By the eighth day the distended ends of the wolffian ducts have been taken up in the urodaeal sinus as far as the origin of the ureters, the latter ducts in this process rotating from the dorsal to the mesial border of the wolffian duct. From this period on, the original components lose their identity in the sinus. In the adult the depth of this cavity is greatly reduced, the whole forming a shallow transverse segment, the definitive urodaeum, the latter being separated from the coprodaeum by the urorectal fold of Retterer and from the proctodaeum by the uro-anal fold. The position of these folds in the embryo is evident as early as the beginning of the fourth day of incubation.

Another interesting feature of the urogenital apparatus which occurs at this time is the constriction of the metanephric pelvis at its lower third into a narrow isthmus (fig. 39). This was figured by Schreiner ('02), who noted its relation to the umbilical arteries. As is well known, the adult kidney of birds is constricted into three lobes. The cause of the upper constriction is yet to be determined; the lower constriction is accounted for by the mechanical obstruction offered by the umbilical arteries. The developing kidneys of the pig, as shown by Lewis and Papez, are similarly caught in the bifurcation of these vessels, but instead of becoming notched as in the bird, they escape by moving upward, sometimes, however, being brought so near together as to fuse from side to side, forming a 'horseshoe kidney.'

In closing this chapter I wish to call attention to the changes which have been taking place in the terminal segment of the intestine. In figures 35 and 40 its lumen is shown to be occluded for some distance, the solid tube thus formed joining the urodaeal sinus by a thin linear attachment. By the fifteenth day the cavity of the coprodaeum has been reëstablished and considerably distended except at the solid linear attachment. This greatly dilated chamber at the end of the intestine (fig. 41, *copr.*) is unquestionably homologous with the lower end of the rectum of the human foetus, as figured by Johnson ('14).

This includes a rectal ampulla passing below into a plicated 'zona columnaris.' In the chick embryo it is bounded above by a single transverse plica and below by the urorectal fold already mentioned. Since this ampulla functions as a part of the cloaca in the adult bird, being the chamber in which both fecal matter and urine are retained, it seems better to keep the name coprodaeum, which Gadow applied to the most anterior of the three divisions of the cloaca.

DEVELOPMENT OF THE BURSA OF FABRICIUS AND ASSOCIATED DIVERTICULA

The primordium of the bursa is usually described as a swelling in the caudal wall of the cloaca, caused by the coalescence of vacuoles arising within the urodaeal membrane during the fifth and sixth days of incubation (figs. 31 and 18, *bursa*). While modeling earlier stages of the cloaca in relation to the development of the fenestra, I was much surprised to find that all chick embryos which had been incubated about four days showed a conspicuous diverticulum at the site of the caudal end of the cloacal fenestra, measured by its greatest extent (figs. 24 and 27, *a*; cf. figs. 16 and 18). The picture was further complicated by the occurrence, in several cases, of a second diverticulum (fig. 24, *b*), arising as an outpocketing of the cloaca at the site of the cephalic end of the fenestra. Furthermore, diverticulum *a*, while originally developing as an invagination of the cloaca, soon became solid, then vacuolated, in continuity with the vacuoles in the developing urodaeal membrane (fig. 28, *a*), and then, by fusion of vacuoles, appeared to develop into the bursa itself (fig. 30, *bursa*). In view of these facts, it seemed not improbable that diverticulum *a* represented an earlier and more significant stage in the origin of the bursa than had hitherto been reported—a stage which had been overlooked because the cloaca had never been modeled during this period of its growth. This interpretation, if true, would be of importance as bringing the origin of the organ into line with other derivatives of the gut. For it would show that it originated as an invagination of the entodermal tube, thus removing one more difficulty in the interpretation of an

organ which has been a bone of contention among anatomists since its discovery by Fabricius. The chief obstacle to this conclusion, however, arose from the examination of a single specimen pictured in figure 29. In this figure diverticulum *a* seemed farther removed from the anal plate than in other specimens, thereby leaving a vacuolated area between it and the anal plate (labeled *bursa* in the drawing) which might well develop into the *bursa* of figure 30, there recognized as the definitive bursa by the coalescence of the vacuoles. To solve this difficulty it became necessary to collect a series of graded embryos of other species of birds. Subsequent reconstruction of domestic duck and pheasant embryos left the matter still more confused, as in these forms the diverticula were present and similar to those in the chick, but less pronounced. Finally, an examination of tern embryos, birds some distance removed from the gallinaceous tribe, brought the desired results. In these forms, as can be seen in figure 36 to 38 and reconstructions of earlier stages, no diverticula are developed at all, and the bursa arises directly from the region adjoining the anal plate, as a thickening of epithelium in continuity with that plate and restricted to the territory lying between it and the site of the caudal intestine (fig. 1). A reexamination of chick embryos in the light of these facts has led to the following conclusions. The bursa of Fabricius in the chick begins soon after the rupture of the caudal intestine, as early as the beginning of the fifth day, as a proliferation of entodermal epithelium on the caudal border of the cloaca adjoining the anal plate (fig. 26, *bursa*), but it does not develop from the epithelial elements which originally belonged to the caudal intestine, as maintained by Stieda. As the two walls of the cloaca, beginning at the anal plate, progressively fuse to form the urodaeal membrane, vacuoles appear in the solid plate thus formed (figs. 27, 28, and 29, *bursa*). Those on the free border adjoining the anal plate coalesce and distend the cloaca, forming the definitive bursa of Fabricius (fig. 30, *bursa*). Previous to these events, however, a diverticulum may appear at each end of the area marking the site of the cloacal fenestra. The caudal diverticulum (*a*) is always present in chick embryos,

where it is associated with the bursa of Fabricius (figs. 33 and 34). The other diverticulum (*b*), when present, becomes associated with the urodaeal sinus (fig. 32, *div. c*). Both of them are probably to be regarded as irregularities produced at either end of the fenestra by the removal of intervening epithelium. They are present only in those birds which exhibit a fenestra, and are most conspicuous in that species which has the largest fenestra—the domestic fowl. The regularity with which diverticulum *a* appears may be explained by the fact that the posterior end of the fenestra is always larger, and that diverticulum *a*, when first formed, arises from the prominence to which the primitive streak of earlier stages was attached (cf. figs. 21 and 23).

The later stages of development, which have been partly described by previous authors on the basis of sagittal sections, are shown in figures 34 and 39, 35 and 40, and 41. These models illustrate the development of the bursa up to the period of histological differentiation. The successive steps leading to this period are: 1) the continued outgrowth of the bursa and simultaneous enlargement of its cavity through further coalescence of vacuoles; 2) the projection of the anal sinus (proctodaeum) in a ventrodorsal direction across the flanks of the urodaeum on its way to connect with the bursa (cf. figs. 18 and 39); 3) the breaking through of the thin plate separating the cavity of the bursa from the proctodaeum (cf. figs. 34 and 35), and, lastly (fig. 41), the differentiation into three parts of the passage-way thus made continuous from anus to the end of the bursa. At this stage (eleventh day) this passage-way is still separated from the rest of the cloaca by the urodaeal membrane, which does not rupture until after the seventeenth day (Gasser). As seen in figure 41, the first of its three parts, the proctodaeum of ectodermal origin, has assumed the shape of a compressed chamber with broad flange-like expansions. By the fifteenth day ectodermal glands have begun to differentiate around its circumference. The second and third parts, of entodermal origin, have developed, respectively, into a short bursal stalk and a greatly expanded but plicated sac, the bursa itself (fig. 41). The cavity

of the latter is subdivided by longitudinal plicae into eleven (or twelve) grooved chambers. A cross-section of the bursa during the fifteenth day (fig. 12) shows that in the interval between the eleventh and fifteenth days some of the primary plicae have cleft the central cavity deeper than others, so that the eleven primary cavities have become tributary to six or seven secondary channels, opening into the main cavity after the manner that minor and major calyces open into the pelvis of the kidney.

Fig. 12 Transverse section of a model of a 55-mm. chick embryo, H.E.C. 1968: 14 days and 5 hours. × 28. *bl. v.*, blood vessel; *cav.*, cavity of bursa; *cor.*, cortex of follicle, derived from tunica propria; *med.*, medulla of follicle, derived from epithelium; *musc.*, muscularis; *t. p.*, tunica propria.

Histogenesis begins with the appearance of the primary plicae and ends in the formation of spherical masses of lymphoid tissue (the 'follicles' of Stannius). Each follicle consists of a cortex and a medulla, the medullae or cores of the follicles (the 'Follikelkeime' of Stieda) being the first to appear. These grow out into the tunica propria as solid buds of epithelium which soon become clothed peripherally with a cortical layer derived from the subjacent connective tissue (fig. 12, *cor.* and *med.*). In the course of development the follicles grow larger and larger until they meet, the resulting pressure molding them into a polyhedral shape. The walls of the bursa thus become greatly thickened, resembling somewhat in gross appearance the walls

of the proventriculus (glandular stomach of birds) to which the bursa was compared in 1829 by Berthold. In the region next to the stalk, according to Schumacher ('03), the follicles are neither so thick nor so sharply limited, but look more like a diffuse infiltration of tunica propria with lymphocytes. To these finger-like processes, which in my model of the fourteen-day chick are restricted to the dorsal wall of the bursa where it joins the stalk, Schumacher has applied the term mucosal villi.

The nature of the epithelial transformation has received several interpretations. Wenckebach ('88) and Schumacher ('03) maintain that the entodermal epithelium constituting the medulla of each follicle is differentiated directly into lymphoid tissue, and that this process is followed by a differentiation of the mesenchymal cortex into a similar tissue, the border-line between the two layers becoming ill-defined in later stages. Retterer, in his latest paper ('13), extends the activity of the epithelium still further, stating that "the cortex of the follicles of the bursa is likewise of epithelial origin." The most comprehensive account, however, is that of Jolly ('15), who based his conclusions not merely upon histogenesis, but also upon the involution of the organ (both natural and induced) and upon examination of tissues in vitro. Beginning with the eleventh day of incubation, he finds numerous amoeboid cells, formed directly from the mesenchymal network, accumulating in the vicinity of the epithelial buds. These they soon invade, the most active phase of penetration occurring between the fourteenth and eighteenth days. Although at first the epithelial cells give way to the new arrivals, by becoming detached from one another and in some cases by even degenerating, the majority of them, he maintains, enter upon a symbiotic relation with the invaders by means of which both cell strains continue to divide actively, the amoeboid cells giving rise to large numbers of small lymphocytes, the epithelial cells forming a reticular network within which the lymphocytes reside. Simultaneously the cortex becomes differentiated into a highly vascularized lymphoid tissue.

In involution the order of events is reversed; the lymphocytes in the medulla die and the epithelial cells close their ranks, tend-

ing to reconstitute themselves into a compact epithelial bud—a process which Jolly has compared to the production of Hassal's corpuscles in the thymus. As involution continues the follicles separate from the epithelium and become replaced by fibrous tissue, the whole process taking place progressively from apex to base of the bursa in such a way that a gradual but rapid diminution of volume and weight ensues. During the eighth month the bursa loses all possibility of functioning, and in the course of the next two months becomes reduced to a thin-walled cyst, still opening into the cloaca at its posterior end, but so completely fused to the aponeurosis of the rectum that it can be detected only by careful dissection. In this condition it may persist until old age. Only in the Ratitae, according to Forbes, does it remain as an undiminished organ throughout life where, by virtue of its broad opening into the proctodaeum, it becomes a repository for the urine. In these birds, according to Gadow, micturition and defecation are separate processes, whereas in most other birds the urine backs up into the coprodaeum and there mixes with the faeces until evacuated.

The following table, arranged from data submitted by Jolly, is introduced to summarize the growth and involution of the bursa in the fowl:

Age	*Length mm.*	*Weight grams*
Hatching	5	0.05
1 month	10	0.50
2 months	15–18	0.50–1.0
3 months	20–25	1.5
4 months	30	3.0 ($\frac{1}{400}$ of body)
4½ months		2.51
5 months		0.97
6 months		0.22
7 months	10–20	0.26
12 months		0.12

The function of the bursa has never been satisfactorily explained. Jolly's description of the haematopoietic foci of the bursa, from which he derives not merely lymphocytes, but also red corpuscles and granular leucocytes, has added something to our knowledge of its activity, but, as he well recognized, this

function is not peculiar to the bursa, but is an attribute common to the mesenchyma of certain other organs. He does, however, propose a specific function when he suggests that the bursa contributes substances to the organism which bear a causal relation to the inception of sexual maturity. He bases this theory on two facts: 1) that the maximum development of the bursa is attained at the time when spermatogenesis is just getting under way; 2) that involution of the bursa corresponds exactly with the appearance of sexual maturity, as measured by the sudden increase of testicular weight and the appearance of ripe spermatozoa. Before accepting this theory, however, one would like to know to what extent the precocious involution, which Jolly produced in the bursa by means of the x-ray, affected the differentiation of the testis. That some such line of experimentation as this would be profitable seems almost certain when we consider the history of such organs as the thymus. For it is far from inconceivable that the bursa may also be a glandular organ in process of transformation into an endocrine gland, if it has not already arrived at that estate.

The phylogenetic interpretation of the bursa is equally obscure. An extensive number of investigators distributed over three centuries have tried to solve this problem and during this period have proposed numerous hypotheses, all of which have been rejected (see Retterer, '13 b, for list). Forbes, after examining the bursae of over ninety species of birds and covering the literature, came to the conclusion that the bursa was a glandular outgrowth of birds sui generis. Wenckebach limited the problem by establishing the entodermal origin of the bursa, thus making obligatory the origin of homologous structures (with which it is to be compared) from the dorsal wall of the vertebrate cloaca. Its origin has been still further limited by this paper to the area between the cloacal end of the caudal intestine and the anal plate.

These limitations render untenable the hypothesis put forth by Stieda ('80) that "the bursa develops from the epithelial elements which originally belong to the caudal intestine." Equally untenable is the modification of this theory, presented by

Fleischmann ('02).[6] Recently Stieda's point of view has been revived again, this time by Jolly ('15), who has made it a basis for the theory that the bursa represents a recrudescence of the cloacal end of the ruptured caudal intestine.

"The first anlage of the bursa," he writes in his conclusion," occupies exactly the situation of the post-anal intestine and it is orientated like it; it may be said, even, that the anlage blends with what remains of the post-anal intestine. One may consider that the bursa represents the remainder of the caudal intestine which rises up again posteriorly and, turned toward the head, undergoes a further development under the form of a true cloacal caecum, in the walls of which lymphoid tissue develops."

In refutation of this theory, new evidence, presented in the first section of this paper, shows that the entire region of junction between caudal intestine and cloaca, together with the adjacent wall of the latter, has been removed by the process which forms the cloacal fenestra. There is, therefore, nothing left of this end of the caudal intestine which Jolly assumes to be present and which he describes as growing out, in an unusual direction, to form the bursa. Furthermore, even after the closure of the fenestra, the bursa does not arise at the site of the former caudal intestine, but on the anal side of it, beyond diverticulum *a* (figs. 25 to 33).

Another theory, presented during the last ten years, is that of Osawa ('11), who has revived the hypothesis of Martin St. Ange ('56). He believes that the bursa is homologous with the prostate gland even though the latter is well developed in the male only. Osawa bases his conclusions on the ground that the "bursa occupies the place where the ureter and ductus deferens discharge themselves, and its follicles are laid out after the manner of glands." In refutation of this view, it may be stated that the

[6] In a foot-note to his paper (p. 58) Fleischmann suggests that "the caudal process of the primitive urodaeum of mammals, which now bears the perplexing name caudal intestine, is comparable morphogenetically with the bursa of Fabricius." This conjecture has recently called forth the following rejoinder from Keibel ('21): "The caudal intestine of birds has not the slightest thing to do with the bursa of Fabricius."

point of origin of the group of glandular outgrowths that constitute the prostate gland is rather remote, embryologically, from that of the bursa; also that the prostate develops much later and is radically different in its histological nature. Physiologically it becomes functional with sexual maturity, at the time when, as Jolly has shown, the bursa degenerates.

The only other vertebrate structures thus far proposed, which in any way meet the requirements of the homology, are the anal sacs (bursae anales) of turtles. Gadow, in the Cambridge Natural History Series, 1909, describes these organs in the adult as highly vascularized, thin-walled sacs which are incessantly filled and emptied with water through the vent, and act as important respiratory organs. Forbes, in 1877, objected to the comparison of these sacs with the bursa of Fabricius on the ground that they were paired, lateral structures. Wenckebach also saw this objection, but considered that the anal sacs were the only diverticula which in any way could be compared in point of origin with the bursa, and, in view of the almost total ignorance regarding the embryology of the sacs, held that the objections to the comparison should not be conclusive. During the last year a graded series of models of the turtle cloaca have been made in this laboratory by R. F. Shaner as a part of an anatomical study of the 9.5-mm. Chrysemys embryo. As a result of this study he is of the opinion that the anal sacs arise from a single median diverticulum (fig. 3, *an. s.*). Through the courtesy of Doctor Shaner, I have had the pleasure of studying the models upon which his paper is based and concur in his opinion. Another feature which at first seemed to favor the comparison between the bursa and the anal sacs is the striking similarity of the process by means of which the outlet of each diverticulum is taken over by the proctodaeum. In each case lateral expansions of the proctodaeum (fig. 39) grow down across the flanks of the cloaca until they establish communication with either the bursa or the anal sacs. But the description of the saurian cloacas in the Fleischmann series seems to indicate that this invasion of some point of the urodaeum by the lateral proctadaeal invagination is not restricted to reptiles equipped with anal sacs,

but occurs in most other reptiles. Another objection to this homology is based upon the fact that the anal sacs arise on the cephalic rather than on the anal side of the caudal intestine. They are thus more nearly comparable to diverticulum *c*, which unquestionably represents the urodaeal Kammer or dorsal bladder of the saurian cloaca, than to the bursa of Fabricius.

In Unterhössel's account of the saurian cloaca another diverticulum is represented which, as a possible homologue of the bursa, seems much more promising. This is an invagination of the dorsal wall of the cloaca, defined by Unterhössel as lying at the junction of the urodaeum and the proctodaeum. It is figured in models of late embryonic stages of three different species, and would seem to be a modification of the same structures. The first is a vaulted portion of the roof of the urodaeum of the lizard Platydactylus guttatus (Taf. VIII, fig. 1, *st*). The second is a comb-shaped diverticulum occupying the same position in the cloaca of the snake Anguis fragilis (Taf. VIII, fig. 2, not labeled). The third consists of a pair of dorsal diverticula lying behind the urodaeal chamber and described as outpocketings of the proctodaeum in the snake Tropidonotus natrix (Taf. VIII, fig. 4, *s*). But it will be remembered that the bursa for a long time was described as an outgrowth of the proctodaeum, and the author in this case admits the lack of younger stages. From an examination of the account of the saurian cloaca I am convinced that the key to the homology of the bursa of Fabricius lies in the study of the reptilian cloaca, and am optimistic enough to believe that such a careful study of the younger stages of the reptile cloaca as Fleischmann and his students have made of older stages will bring the desired results. The comparison which Schumaker has lately made with the tonsiloid tissue discovered by Keibel in the cloaca of the mammal Echidna does not seem to meet the problem. At best it can only be considered a vestige of a reptilian prototype, and to reptiles we must again direct our attention for interpretation of the bursa of Fabricius.

SUMMARY

This paper represents a review of the development of the cloaca in bird embryos from the third to the fifteenth day of incubation. It is based on the study of a large number of chick embryos supplemented by observations on three other species of birds. The most striking feature to be recorded is the regular occurrence of a temporary fenestra in the wall of the cloaca, caused by the disintegration of a definitely localized area of epithelium and its subsequent removal by phagocytes, following which the contents of the cloaca are left in contact with the mesenchyma for a period of nearly twenty-four hours. It is of interest not merely because it furnishes the only instance in the differentiation of a hollow organ in which a gap occurs in the epithelial wall as a normal and constant feature of development, but also because it enables us, by virtue of the landmarks it establishes, to determine for the first time the exact point of origin of the bursa of Fabricius.

The second part of this paper deals with the formation of a temporary sinus, placed athwart the main axis of the cloaca, which sinus has been interpreted as a repetition of the dorsal bladder of reptiles. This section also deals with some interesting anomalies growing out of the attachment of the wolffian ducts to the cloaca.

A third feature of interest is the regular occurrence in chick embryos of an accessory bursal diverticulum (*div. a*), probably arising from the irregularities consequent upon the formation of the cloacal fenestra. By means of this diverticulum it has been possible to define the primordium of the bursa more accurately than has hitherto been done and therefore to offer new suggestions regarding its phylogenetic origin.

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PLATE 1

EXPLANATION OF FIGURES

Models illustrating the formation of a cloacal fenestra and the early development of the cloaca in chick embryos. All figures are drawn to the same scale (magnification, × 50). H. F. Aitken, del. (plates 1 and 4).

13 H. E. C. 2071: 2 days, 18 hours (cf. with text fig. 5, a sagittal reconstruction of the same embryo). In passing across the picture from left to right at its upper level the organs are encountered in the following order: medullary tube, notochord, caudal intestine, primitive-streak mass, proctodaeum, allantois, rectum, dorsal aortae. This stage shows the persistence of a mass of primitive-streak tissue in the angle between the cloaca and caudal intestine; the mergence of four structures (medullary tube, notochord, caudal intestine, and anterior half of primitive-streak remnant) with the indifferent tail-bud mass; a circlet of five or six complemental diverticula around the unattached terminal portion of the W. D.; a larger complemental diverticulum opposite its shaft; and the isolated foramina (in the back wall of the cloaca and adjacent portion of the caudal intestine) which mark the first step in the disintegration of the cloacal wall and the formation of a fenestra.

14 and 15 H.E.C. 1953: 3 days, 6 hours; 8 mm. (cf. with fig. 22, a sagittal reconstruction of the same embryo). At the left of figure 13 are the remnants of the caudal intestine and primitive streak, each detached from the cloaca by a process of disintegration. The dash line indicates that portion of the cavity of the cloaca which has been denuded of epithelium. It is bounded by mesenchyma only, and indicates the maximum extent of the cloacal fenestra, shown to better advantage from below in figure 15. At the cephalic end of the fenestra in both figures is an aberrant diverticulum probably derived from one of the complemental diverticula shown in figure 12.

16 H. E. C. 1942: 4 days, 3 hours; 10.5 mm. (cf. with fig. 27, a sagittal reconstruction of the same embryo). Note diverticula lettered *a* and *c* in fig. 26, together with accompanying legend.

17 H.E.C. 2097: 4 days, 3 hours; 10.5 mm. (cf. with fig. 28, a sagittal reconstruction of the same embryo). Note accessory diverticulum lettered *b* in figure 27.

18 H.E.C. 1951: 5 days, 13 mm. (cf. with fig. 31, a sagittal reconstruction of the same embryo). Note the swelling (bursa of Fabricius) caused by coalescence of vacuoles at the bottom of the cloaca; the distended cavity of the urodaeum connecting allantois and excretory ducts; the flattened and occluded area between the urodaeum and bursa (urodaeal membrane); the down-growing proctodaeum astride the cloacal membrane, reaching out to connect with the bursa; the constriction in the metanephric pelvis marking the future division between the second and third lobes of the adult kidney (cf. with fig. 39).

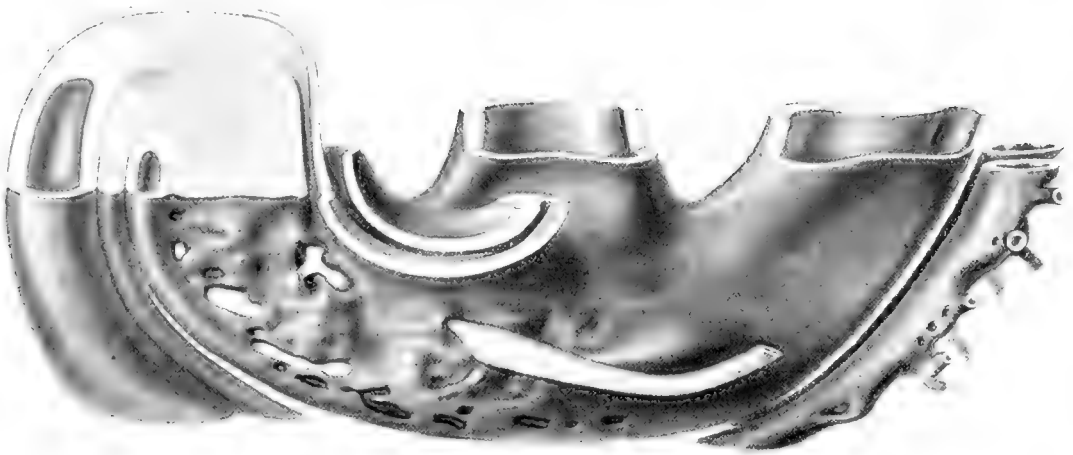

Fig. 13

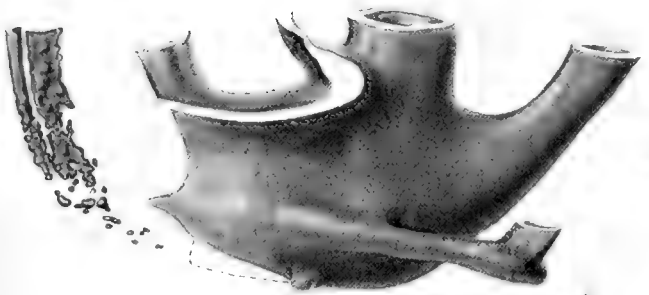

Fig. 14

Fig. 15

Fig. 16

Fig. 17

Fig. 18

PLATE 2

EXPLANATION OF FIGURES

Projection-lantern drawings of microscopic sections through the cloacal fenestra of chick embryos, drawn to the same scale (magnification, × 340).

19 H.E.C. 512 (section 121): 2 days, 20 hours? Obliquely-transverse section passing through cloaca at right angles to the long axis of the fenestra (cf. with imaginary line connecting letters *y* and *all.* in text fig. 5). Note bilaterally symmetrical gaps in cloacal wall; the concentration of mesenchyma around the gaps; the isolated floor of the cloaca, with necrotic cells on margin; the phagocytes in the cavity and the pycnotic nuclei in the epithelium bordering the gap.

20 H.E.C. 2057 (section 736): 3 days, 4 hours; 6.8 mm. Section through fenestra during period of maximum extent (cf. embryos shown in figs. 14, 15 and 22). Note complete resorption of disintegrating epithelium shown in preceding figure, the rounded epithelial margins which fail to regenerate, the flattening out of the mesenchyma bordering exposed cavity.

21 H.E.C. 2124 (section 749): 3 days, 18 hours; 8.5 mm. Last stage before closure showing section through fenestra reduced to small slit (same age as embryos shown in figs. 23 and 24). Note approximation of two side walls, the complete absence of regeneration along epithelial margins.

Fig. 19.

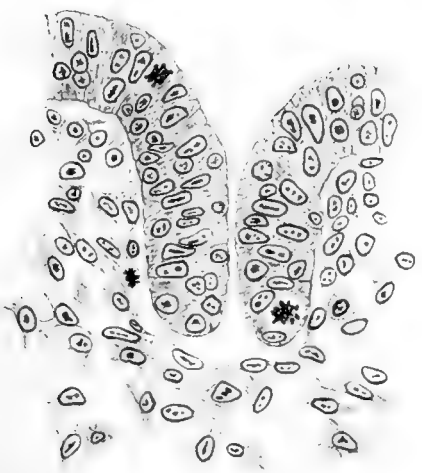

Fig. 21.

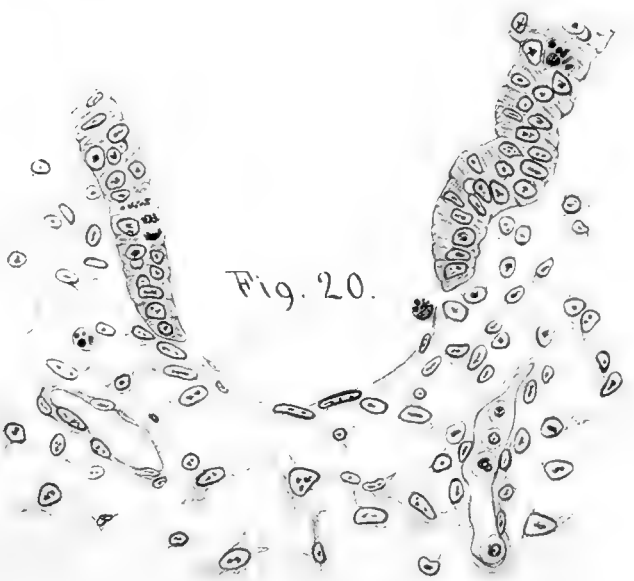

Fig. 20.

PLATE 3

EXPLANATION OF FIGURES

Graphic reconstructions of the cloaca of bird embryos drawn to the same scale (magnification, × 35). Dash lines indicate cavity; dotted lines, vacuoles. This plate represents chiefly a quantitative study of chick embryos made to demonstrate the origin of the bursa of Fabricius together with the identity and significance of a series of diverticula occurring on the back wall of the cloaca between the anal plate and the rectum. Diverticulum *a* represents an accessory diverticulum, arising from the caudal angle of the cloacal fenestra, which regularly becomes appended to the bursa of Fabricius; *b* represents an accessory diverticulum, only occasionally present, which arises from the cephalic angle of the cloacal fenestra and which becomes associated with the urodaeal sinus; *c* represents a diverticulum which regularly forms the medial component of the urodaeal sinus.

22 Chick embryo, H.E.C. 1953. 3 days, 6 hours, 8.0 mm.
23 Chick embryo, H.E.C. 2120. 3 days, 18 hours, 9.2 mm.
24 Chick embryo, H.E.C. 2126. 3 days, 18 hours, 9.5 mm.
25 Chick embryo, H.E.C. 2058. 4 days, 4 hours, 11.0 mm.
26 Chick embryo, H.E.C. 2098. 4 days, 3 hours, 10.0 mm.
27 Chick embryo, H.E.C. 1942. 4 days, 3 hours, 10.5 mm.
28 Chick embryo, H.E.C. 2097. 4 days, 3 hours, 10.5 mm.
29 Chick embryo, H.E.C. 2100. 4 days, 22 hours, 13.0 mm.
30 Chick embryo, H.E.C. 1943. 4 days, 3 hours, 12.0 mm.
31 Chick embryo, H.E.C. 1951. 5 days, 0 hours, 13.0 mm.
32 Chick embryo, H.E.C. 2059. 4 days, 23 hours, 14.0 mm.
33 Chick embryo, H.E.C. 2074. 5 days, 23 hours, 15.0 mm.
34 Chick embryo, H.E.C. 2076. 6 days, 7 hours, 17.3 mm.
35 Chick embryo, H.E.C. 1962. 8 days, 1 hours, 21.5 mm.
36 Sterna hirundo (common tern) H.E.C. 2169. 8.0 mm.
37 Sterna hirundo (common tern) H.E.C. 2115. 10.4 mm.
38 Sterna hirundo (common tern) H.E.C. 2173. 13.4 mm.

ABBREVIATIONS

all., allantois
an., anus
an. pl., cloacal membrane (anal plate)
bursa, bursa cloacae (of Fabricius)
cauda, inner curvature of tail
cau. A., caudal artery
c. i., caudal intestine
copr., coprodaeum (ampulla recti)
d., accessory rectal diverticulum
div., complementary diverticula
fen., cloacal fenestra
Mull., Müllerian duct
pelv., pelvis of kidney
phal., phallus
proct., proctodaeum
ps. v., ventral half of primitive streak
rect., ampulla recti (coprodaeum)
umb. A., umbilical artery
ur., ureter
urod., urodaeum
ur. m., urodaeal membrane
W. D., Wolffian duct

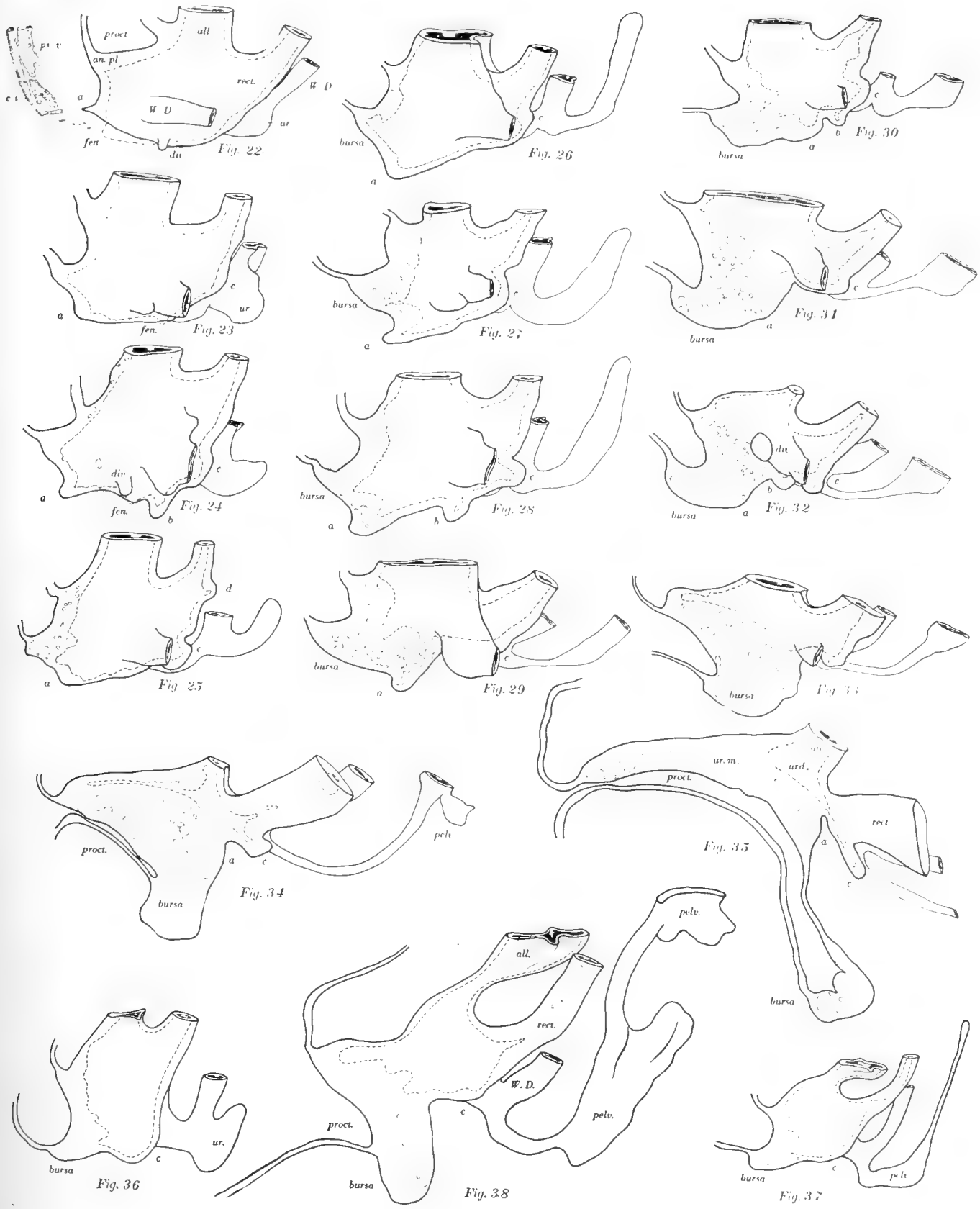
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Fig. 37

PLATE 4

EXPLANATION OF FIGURES

39 Model of chick embryo, H.E.C. 1945: 5 days, 15 hours; 15 mm. × 37. Showing especially the bladder-like enlargement of the allantois in the intra-embryonic body cavity, the lateral invaginations of the proctodaeum to meet the bursa of Fabricius (*proct.*), and the constriction of the metanephric pelvis into two parts by the umbilical artery.

40 Model of chick embryo, H.E.C. 1962: 8 days, 1 hour; 21.5 mm. × 37. Note the occluded rectum, the prominent urodaeal sinus (*urod.*), and the elongating bursa.

41 Model of chick embryo, H.E.C. 1967: 11 days; 31 mm. × 21. Note differentiation of bursa into stalk and plicated gland, also division of cloaca into the three transverse parts characteristic of the adult: proctodaeum (ectodermal origin); urodaeum, cloaca proper, receiving urogenital ducts; and the coprodaeum, rectal ampulla, with its 'zona columnaris.'

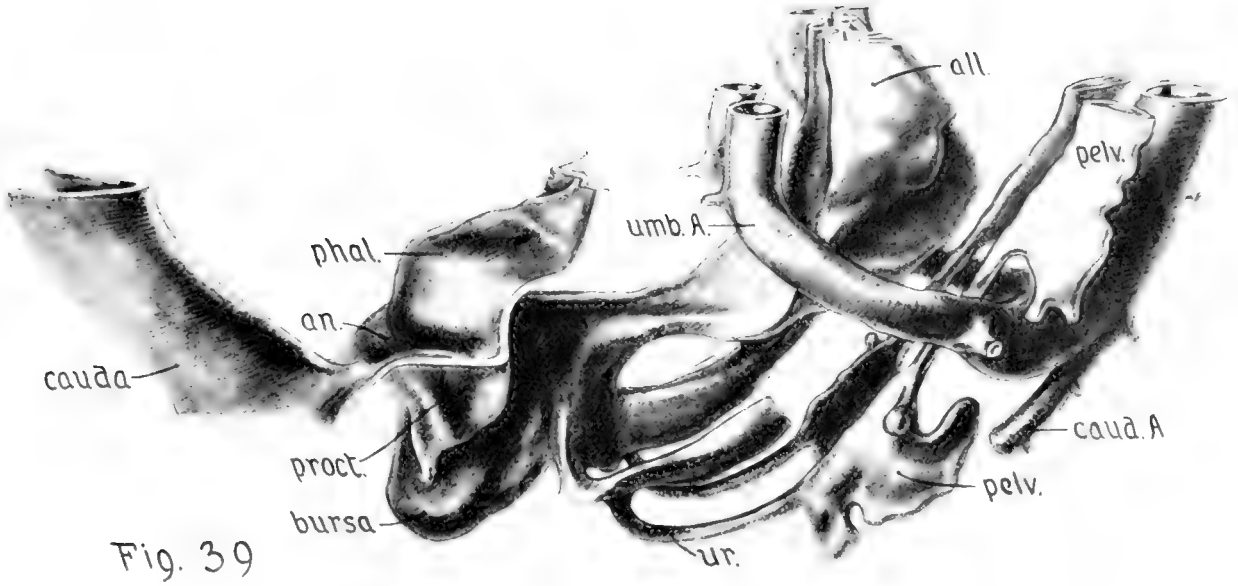

Fig. 39

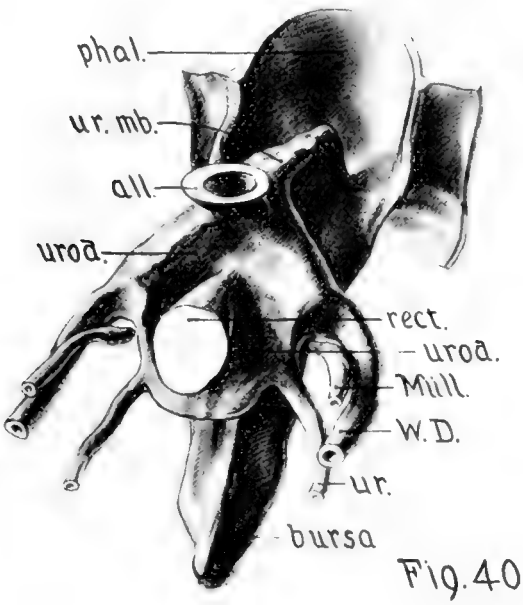

Fig. 40

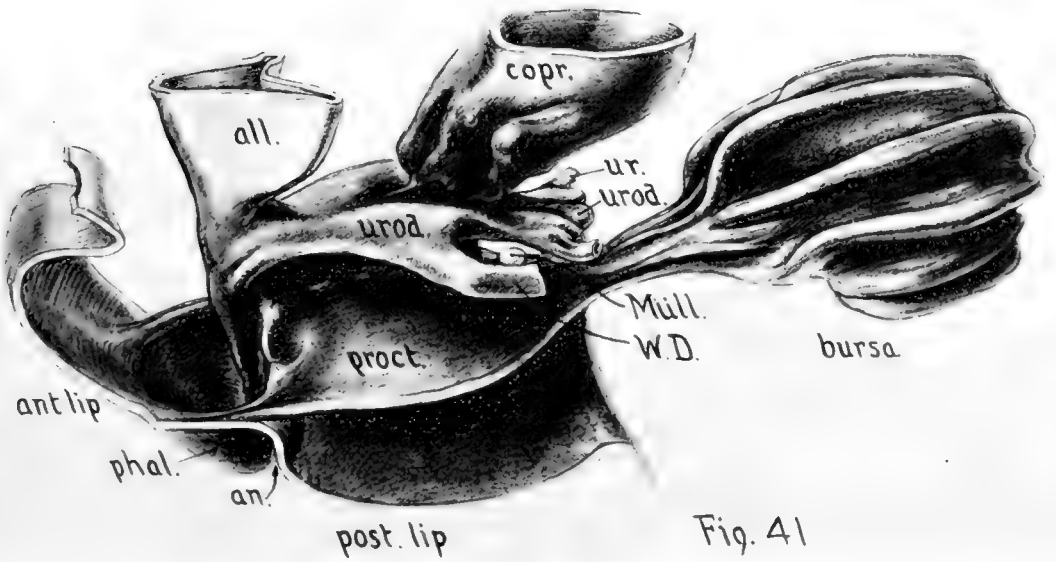

Fig. 41

Resumen por el autor, Ivan E. Wallin.

Sobre la naturaleza de las mitocondrias.

I. Observaciones sobre los métodos de teñido de las mitocondrias aplicados a las bacterias.

El autor ha teñido bacterias mediante los métodos más empleados para el teñido de las mitocondrias, especialmente el verde janus. Todos los métodos procados tiñen las bacterias. Los métodos para el teñido de las mitocondrias, incluso el de coloración vital mediante el verde janus, no son específicos para las mitocondrias sino que tiñen también bien las bacterias.

II. Reacciones de las bacterias a los tratamientos químicos.

El objeto de estos experimentos ha sido buscar una diferencia fundamental en el comportamiento de las bacterias y las mitocondrias bajo la acción de ciertos agentes químicos empleados para determinar la naturaleza química de las mitocondrias. El autor no ha encontrado diferencia fundamental alguna en estas reacciones.

Translation by José F. Nonidez
Cornell Medical College, New York

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ON THE NATURE OF MITOCHONDRIA

I. OBSERVATIONS ON MITOCHONDRIA STAINING METHODS APPLIED TO BACTERIA

II. REACTIONS OF BACTERIA TO CHEMICAL TREATMENT

IVAN E. WALLIN

Department of Anatomy and the Henry S. Denison Research Laboratories, University of Colorado, Boulder

ONE PLATE (NINE FIGURES)

INTRODUCTION

The publication of Altmann's 'Bioblast theory' ('90) stimulated a new interest in the investigation of cytoplasm. The minute bodies observed by Altmann in the cytoplasm were thought by him to be the ultimate units of life, and the cytoplasm itself was considered a more or less passive and lifeless substance. This conception of cytoplasm and the contained bodies or granules has received no support from recent investigators. The bodies in question have come to be considered normal cytoplasmic organs by most investigators. They have been described by a great number of authors under various names. More recently the term 'mitochondria,' first used by Benda ('98), has come into general usage.

Following the pioneer work of Flemming ('82), Altmann ('90), and Benda ('98), a massive literature on mitochondria has accumulated. This literature has dealt chiefly with the presence or absence of mitochondria in the various types of cells in both plants and animals. Cowdry ('18) has given an exhaustive review of mitochondrial literature and has summed up the total of our knowledge of mitochondria.

It is quite apparent, from a perusal of Cowdry's excellent review, that we have an exceedingly limited knowledge concern-

ing the fundamental properties of mitochondria. Attempts have been made to investigate their physiological properties, but aside from a possible relationship to chloroplast formation in plants, nothing definite, apparently, has been established concerning their function. Regarding the chemistry of mitochondria investigators generally agree that they are of the nature of phospholipins and lipoids and perhaps contain some albumin. The theory of their chemical nature is based on their reactions to staining methods and various chemicals. 'Artificial mitochondria' were produced by Löwschen ('13) by the use of lecithin in different salt and albumin solutions.

Considerable study has been given to the morphology of mitochondria. The result of this type of work has led to the conclusion by Cowdry ('18) and others that the form of mitochondria is variable and after all of little importance. Two forms of mitochondria predominate, namely, rod-shaped and globular forms. Besides these two predominating types various irregular forms may be found.

An important consideration in the demonstration of mitochondria is the technique. This technique warns to the exclusion, in the chemicals employed, of various solvents of mitochondria, chief of which are ether, alcohol, and acetic acid. It has not been claimed for the majority of mitochondria staining methods that they are specific for mitochondria. This 'specificity,' apparently, has reference to other materials in the cell. However, it must be assumed that these methods, if they are to be of value, must have a relative specificity for mitochondria. The janus green vital staining method has been definitely placed in a class of specific stains for mitochondria by Cowdry ('18, p. 43).

The striking resemblance of mitochondria to bacteria is apparent to all who are familiar with the two groups of structures. This resemblance has been noted by various authors and has led Cowdry to suggest a division of mitochondrial literature into two periods: an older literature in which mitochondria were observed in cells and mistaken for bacteria and a newer literature in which they have been observed and recorded under various names.

The chief methods employed in cytological studies are based on the reactions of stains and chemicals on protoplasm. In many cases a differentiation between cells and cell structures is demonstrated solely by staining reactions. While such methods may be criticized on account of the absence of a definitely indicated specificity, their value cannot be denied, especially in cases where the difference is pronounced. It is fair to demand when structures bear so close a resemblance to each other as mitochondria do to bacteria that some method must be employed that will differentiate between the two if they are to be considered distinct structures.

Cowdry ('18 p. 72) says: "It occasionally happens that tissues prepared for mitochondria have been invaded by bacteria, in which case the bacteria stain just like the mitochondria by the Benda method, with iron hematoxylin and with fuchsin methyl green. I have found that large bacilli contain granules which stain intensely and apparently specifically with janus green. They resemble in distribution the so-called polar granules. Smaller forms often stain diffusely." It is not clear from this statement whether Cowdry means to limit this staining reaction of bacteria to those forms that have invaded cells or if he implies that bacterial smears fixed and stained by mitochondrial methods will give the same results. In another place, Cowdry ('18, p. 135), referring to mitochondria, says: "Fortunately, *they may be easily distinguished from bacteria by their staining reactions* (particularly to janus green), by their occurrence in almost all cells, by their behavior and by their lack of independent motility."

This latter statement would appear to imply that all bacteria possess independent motility. This would be contrary to established fact in bacteriology. Just what 'behavior' of bacteria is specifically characteristic is not indicated by Cowdry.

Concerning the staining reaction of bacteria to janus green, I cannot agree with Cowdry that "mitochondria are easily distinguished from bacteria" by this staining method.

The practically universal occurrence of mitochondria in plant and animal cells points to a fundamental property of these struc-

tures. Their nature remains as much a puzzle to-day as when they were first discovered. It is with a desire to point out certain similarities between mitochondria and bacteria besides the similarity of form as well as seek a specific differentiation between the two structures that these studies have been undertaken.

MATERIAL AND METHODS

The materials used in this investigation have included a large number of strains of bacteria, some from known pure cultures and others from various mixed infections. The mixed specimens were obtained from sputum from hospital patients, pus centrifuged from urine, pus from a carbuncle, cultures made from the intestinal contents of rabbits and kittens, cultures made from lymph nodes, and from various other sources.

The staining methods employed were: Bensley's acid fuchsin methyl green method, Schridde's modification of Altmann's method, Benda's crystal violet method, the copper hematoxylin method and the vital janus green method.

In the second part of this study a number of strains of bacteria were subjected to the action of alcohol, ether, chloroform, acetic acid, formaldehyde, potassium bichromate, osmic acid, and heat. The object of these experiments was not to determine the exact nature of the response of the organisms to these chemicals and heat, but to determine the effect on the staining reaction of the bacteria after such treatment. In every case controls were stained with the same stain used on the experimental preparations.

The janus green used in the vital staining was one of two lots that were kindly donated to the author by Professors Bensley and E. V. Cowdry. This opportunity is taken to express appreciation for this helpful courtesy. Viable cultures of human and bovine tubercle bacilli were supplied by Dr. Harry Gauss, of the National Jewish Tuberculosis Sanitarium in Denver.

I am especially indebted to my colleague Dr. Severance Burrage, of the Department of Pathology, for valuable assistance and suggestions in this work and also for generous use of bacterial cultures in his laboratory.

I. OBSERVATIONS ON MITOCHONDRIA STAINING METHODS APPLIED TO BACTERIA

In the following staining methods in which a fixation preceded the staining, smears were made in the usual way on the slide. Before the smears had time to dry they were immersed in the fixatives of the different methods and later treated according to the procedure for the particular method. In a few instances bacteria were centrifuged, fixed en masse, embedded, and sectioned.

The procedure in the janus green vital staining followed the method used by Cowdry ('14) for blood cells.

a. Bensley's acid fuchsin methyl green method

This method was used according to the directions given by Bensley ('11). It was found that the time for both fixation and staining could be shortened considerably with excellent results, obviously due to the more rapid penetration in the bacterial smears. In a number of instances the method was altered with a modified Flemming's fixation. This modified fixative consisted of osmic and chromic acids in the following proportions: 4 cc. 2 per cent aqueous solution of osmic acid and 6 cc. 1 per cent aqueous solution of chromic acid. This modification appeared to give a more rapid fixation and also good staining results with bacterial smears.

Besides a large number of unknown bacteria, the following strains were subjected to this method: human and bovine tubercle bacilli, Bacillus coli communis, Bacillus bulgaricus, Bacillus megatherium, Bacillus subtilis, Staphylococcus pyogenes aureus, Staphylococcus albus, and a pneumococcus.

In every case where this method was used the bacteria were well stained. In the majority of cases they were sharply stained.

b. Schridde's modification of Altmann's method

This method was used only to the extent of determining a positive staining in a few cases. The same difficulty experienced in demonstrating mitochondria with this method was experienced

with bacteria. Bacillus bulgaricus, Bacillus coli, and Staphylococcus pyogenes aureus were definitely stained by this method.

c. Benda's crystal violet method

A fairly large number of known and unknown bacteria were subjected to this method. Bacteria responded to this method just as mitochondria do. It gave the sharpest differentiation of bacteria obtained in any case where mitochondrial methods were used. Compared with Gram's stain, for example, on sputum smears, it gave a sharper differentiation. Here, also, it was found that the time for fixation and mordanting may be reduced considerably.

d. Copper hematoxylin method

This method was applied to only a few strains of bacteria. In some cases the staining of the bacteria was quite faint. This was particularly true after fixation with Zenker and the formalin-Mueller used with the Altmann-Schridde method. After Bensley's and the modified Flemming fixations the bacteria were stained very sharply by the copper hematoxylin method.

e. Janus green vital staining method

This method was used as prescribed by Cowdry ('14) in a 1:10,000 dilution in physiological salt solution. The dye was first tested by applying it to lymphocytes from a lymph node of the rabbit. It was found to stain the mitochondria of the lymphocytes as described by Cowdry.

The following results will serve to indicate the staining reaction on bacteria:

1. Human bacillus tuberculosis, viable strain. The bacilli stained rather faintly, the granular forms were easily recognized on account of the more intense staining of the granules. Observed ten hours after the preparation was made, the bacilli appeared to be stained slightly deeper.

2. Bovine bacillus tuberculosis, viable strain. The bacilli stained perhaps a little fainter than the human strain. Observed

three hours after the preparation was made, the bacilli did not appear to have absorbed any more of the dye.

3. Bacillus subtilis. A few moments after the preparations were made, deeply stained granules could be observed in the bacilli, while the cytoplasm of the bacilli was very faintly stained. In some bacilli the granules were very small, in others they were quite large. Figures 1 to 3 are camera-lucida drawings of some bacilli from these preparations after different lengths of time in staining.

4. Bacillus megatherium. The preparations contained a great number of spores besides the bacilli. The spores appeared to be tinted by the dye. The staining reaction of the bacilli varied in different preparations, apparently depending upon the age of the culture. In some cases the cytoplasm was distinctly stained, while in other cases it was not stained, but contained intensely stained granules. Figures 4 to 6 represent camera-lucida drawings of bacilli from various cultures with different lengths of staining time. In one preparation the cytoplasm was quite intensely stained immediately after application of the dye. When it was examined three hours later, the majority of the bacilli had swelled to about three times the normal size and contained very large intensely stained granules. A drawing was not made of this preparation and I have been unable to get the same results again.

5. Unknown bacilli and cocci from a mixed culture. Both the bacilli and cocci were intensely stained immediately after preparation was made. There were a number of bacilli that were unstained. Obviously, it could not be determined in the preparation if they belonged to the same strain that did absorb the dye. Observed ten hours after the preparations were made, a number of the bacilli were swollen and contained large intensely stained granules, other bacilli were unstained.

6. Unknown bacilli and spores, apparently a pure culture, made from the intestinal contents of a rabbit. The bacilli were intensely stained immediately after the dye was applied. The spores appeared to be tinted by the dye.

7. Unknown bacilli and spores, apparently a pure culture made from the intestinal contents of a five-day-old kitten. The bacilli were moderately stained, no granules apparent. The spores did not appear to have absorbed any of the dye.

8. Unknown cocci, culture made from a human throat swab. The cocci were moderately stained, no granules apparent.

9. Unknown bacilli, culture made from a lymph node of a rabbit. Apparently not a pure culture. Some bacilli stained faintly, others quite intensely. Some large bacilli that were faintly stained contained intensely stained granules.

10. Unknown bacilli and spores, culture made from a lymph node of a rabbit. The bacilli were moderately stained. The spores were decidedly tinted by the dye.

11. Unknown bacilli and cocci, culture made from a lymph node of a rabbit. Bacilli and cocci were moderately stained.

12. Unknown cocci, culture made from a lymph node of a rabbit. Preparation contained cocci of two sizes. Larger cocci were intensely stained, the smaller forms were moderately stained. The difference in staining was also demonstrated in the two forms when they were stained with Loeffler's methylene blue.

13. Bacillus coli, pure laboratory culture. The bacilli stained intensely immediately after the dye was applied, a few forms were only faintly stained. After the stain had acted for five and a half hours, the majority of the bacilli were swollen and contained a single large intensely stained granule. Figures 8 to 9 are camera lucida drawings of the preparation immediately after it was made and five and a half hours later.

DISCUSSION

The results recorded above demonstrate that the mitochondrial methods used are not specific for mitochondria, but that they also stain bacteria. The intensity of the stain varied with the different strains of bacteria used and apparently there was a variation in intensity with the different methods on the same strain of bacteria. Such variations apparently, also occur with mitochondria. The janus green vital staining method appeared to be the most delicate of the methods used.

The effect of janus green on tubercle bacilli was contrary to expectation. On account of the fatty envelope of these forms, it was to be expected that they might stain more intensely than any other bacteria. This would imply that fats, waxes, and lipoids should respond in a like manner to a given stain. Such an inference may not necessarily be true. However, the proof that mitochondria are of a lipoidal nature is far from conclusive. While there is nothing specially indicated as to the chemical nature of the bacteria that were stained by janus green, it would appear that one is justified in concluding that these bacteria and mitochondria do have something in their chemical structure that is common to all.

The different reactions of a strain of bacteria at different periods in the life of the culture to janus green is suggestive. It would appear that janus green has possibilities as a delicate indicator of the physiological state of certain strains of bacteria.

II. REACTIONS OF BACTERIA TO CHEMICAL TREATMENT

The behavior of mitochondria when subjected to various chemicals and heat has been one of the chief methods used in determining the nature of these bodies. N. H. Cowdry ('17) made a detailed study of the comparison of mitochondria in plant and animal cells. The behavior of the two groups of mitochondria under the influence of various chemicals (ether, alcohol, formaldehyde and acetic acid) as well as their morphology was the method employed in this comparative study. Cowdry concludes that there is no difference between the mitochondria of plants and animals.

It must be admitted at the outset that in most instances there is nothing specifically indicated in the reaction of minute microscopic particles to chemicals. With perhaps a few exceptions, these reactions are only relative. For example, ether acting upon tubercle bacilli for a limited time will extract a fat (supposedly forming an envelope for the bacillus) from the organism. From such a reaction there is nothing indicated as to the particular kind of fat that has been dissolved. However, inasmuch as these

methods have been used not only in comparing the mitochondria of plants and animals, but also in determining the approximate chemical nature of mitochondria, it is necessary in this comparative study of bacteria and mitochondria to also determine the reaction of bacteria to these chemicals. It must also be admitted that there is no basis for supposing that all strains of bacteria should respond in the same way to a given chemical. It has been indicated by Cowdry and others that all mitochondria do not respond to a given chemical in the same way.

The methods employed in this study of the reactions of bacteria to chemicals were designed to retain as much as possible of the materials resulting from the chemical action. Metal rings coated with paraffin were sealed to microscopic slides, smears of the bacteria were then made inside of the rings, and after the chemicals were added cover-glasses were sealed over the rings to prevent evaporation. After a given time the cover-glasses were removed and the chemical was permitted to evaporate. When the smears had thoroughly dried and the paraffin around the smears had been removed with xylol, a thin film of celloidin was painted over the smear. The smears were then stained, using the carbol-fuchsin method for tubercle bacilli preparations and Pappenheim's pyronin-methyl green and Loeffler's methylene blue for the other preparations. With careful handling in the staining and washing, the celloidin membrane remains intact on the slide. Control preparations were made in connection with every chemical preparation.

For determining the action of ether, chloroform, and heat on bacteria it is obvious that the paraffin rings could not be used. In these experiments large quantities of bacteria were placed in vials and the ether and chloroform added. After four hours the ether and chloroform were permitted to evaporate considerably. The remains in the vials were then withdrawn with a pipette, placed on slides and permitted to evaporate to dryness. For the heat determinations the organisms were placed in vials with normal salt solution. The vials were then kept at a constant temperature in an incubator. After half an hour portions of the emulsion were withdrawn with a pipette and permitted to evaporate on slides.

The experiments recorded below were repeated a number of times. In some cases the results were not identical in one set of experiments. These differences in results were only slight and apparently of no particular consequence to the object of the experiments.

The main object in all of the experiments that follow was to determine the staining reaction of bacteria after treatment with chemicals and heat.

A. Action of alcohol on bacteria

Alcohol of various strengths was permitted to act on five different strains of bacteria for a period of five hours.

a. After 95 per cent alcohol. 1. Human tubercle bacilli. Stain the same as control, granules appear more distinct than in control.

2. Bovine tubercle bacilli. Stain the same as control, some crescent forms apparently not observed in control.

3. Bacillus megatherium (with spores). Bacilli stained fainter than controls, spores tinted.

4. Bacillus subtilis. Stained more intensely than control.

5. Unknown cocci and bacilli from a lymph-node culture, two strains of cocci, one intensely stained and the other very faintly in controls. The cocci appear to be destroyed. Two strains of bacilli (different in length) not observed in controls were intensely stained.

b. After 50 per cent alcohol. 1. Human tubercle bacilli. Some bacilli are very faintly stained, others appear to be slightly swollen.

2. Bovine tubercle bacilli. Some indication of disintegration, the bacilli intact were decidedly shrunken.

3. Bacillus megatherium. Bacilli could not be demonstrated by staining. The spores were decidedly swollen and in many parts of the field they were coalesced (partially dissolved).

4. Bacillus subtilis. Bacilli could not be demonstrated by staining. Field contained intensely stained granular debris.

5. Unknown cocci and bacilli from lymph-node culture. Field full of very minute well-stained cocci (granules?), also a few

large well stained cocci. Some of the larger cocci coalesced. Few exceedingly small well-stained bacilli. Large bacilli unstained.

c. After 25 per cent alcohol. 1. Human tubercle bacilli. Bacilli stain very faintly and appear shrunken. Granules in bacilli not visible.

2. Bovine tubercle bacilli. Great number of bacilli disintegrated.

3. Bacillus megatherium. Bacilli could not be demonstrated by staining. Some unstained bacillus-like forms partially coalesced. Spores coalesced, very few distinct in outline.

4. Bacillus subtilis. Granular debris, stained.

5. Unknown cocci and bacilli from lymph-node culture. Cocci could not be demonstrated by staining. Few small bacilli stained.

d. After 10 per cent alcohol. 1. Human tubercle bacilli. Bacilli appear more granular than control. Some disintegration.

2. Bovine tubercle bacilli. Most bacilli are granular, some crescent-shaped, some swollen, and some disintegrated. Some bacilli intact have a purple color.

3. Bacillus megatherium. Some unstained swollen bacilli present. Spores coalesced.

4. Bacillus subtilis. Field contains granular debris which has the appearance of minute cocci.

5. Unknown cocci and bacilli from lymph-node culture. Few intensely stained cocci, bacilli unstained.

e. After 5 per cent alcohol. 1. Human tubercle bacilli. Only a few bacilli intact and stained in thick part of smear, rest of field contains debris of disintegration.

2. Bovine tubercle bacilli. Some disintegration. Appear better preserved than after action of 10 per cent alcohol.

3. Bacillus megatherium. Bacilli could not be demonstrated by staining. Spores completely coalesced.

4. Bacillus subtilis. Only slight indication of a few unstained bacilli.

5. Unknown cocci and bacilli from lymph-node culture. Granular debris that appears like minute cocci. Few minute bacilli stained.

f. After 2 per cent alcohol. 1. Human tubercle bacilli. Almost completely disintegrated. Few swollen poorly stained bacilli present in field.

2. Bovine tubercle bacilli. Almost completely disintegrated. Few swollen poorly stained bacilli present in field.

3. Bacillus megatherium. Bacilli could not be demonstrated by staining. Spores coalesced.

4. Bacillus subtilis. Few intensely stained fragmented bacilli present in field.

5. Unknown cocci and bacilli from lymph-node culture. This preparation appears very much like the control. Bacilli apparently not stained.

B. Action of chloroform and ether on bacteria

a. After chloroform. 1. Human tubercle bacilli. Bacilli intact, but appear shrunken and more granular than control.

2. Bovine tubercle bacilli. Bacilli more faintly stained and appear more granular than controls.

3. Unknown bacilli, culture from intestinal contents of five-day-old kitten, two strains of bacilli, large and small. Large bacilli more granular than control, smaller forms clear and faintly stained.

4. Bacillus megatherium and spores. Few poorly stained and shrunken bacilli present. Spores not visible.

5. Staphylococcus albus. No normal cocci visible. Remains appear like exceedingly minute cocci.

b. After ether. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, granular debris.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, granular debris.

3. Unknown bacilli, culture from intestinal contents of five-day kitten. Bacilli could not be demonstrated by staining. Granular debris appears like minute cocci.

4. Bacillus megatherium and spores. Few disintegrated bacilli, remains mostly granular debris. Spores were not visible.

5. Staphylococcus albus. Remains, granular debris.

C. Action of acetic acid on bacteria

Acetic acid of various strengths was permitted to act on bacteria for a period of six hours. Glacial acetic acid was diluted with distilled water for the various dilutions.

a. After 0.5 per cent acetic acid. 1. Human tubercle bacilli. Bacilli disintegrated. Granular remains intensely stained (black).

2. Bovine tubercle bacilli. Many bacilli retain their form, others disintegrated. Intensely stained.

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli unstained, swollen, and coalesced.

4. Bacillus megatherium. Bacilli could not be demonstrated by staining. Spores swollen.

5. Staphylococcus albus. Swollen, unstained, and partially coalesced.

b. After 1 per cent acetic acid. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains granular, intensely stained.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Remains granular, faintly stained.

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli unstained, swollen, and distorted.

4. Bacillus megatherium. Bacilli unstained and distorted. Many forms contain 'bleb'-like swellings at end or center of bacillus, some contain two or three outpushings. Figure 7 is a free-hand drawing of a few of these bacilli.

5. Staphylococcus albus. Appear partially dissolved and coalesced. Unstained.

c. After 3 per cent acetic acid. 1. Human tubercle bacilli. Form of bacilli partially preserved. Faintly stained.

2. Bovine tubercle bacilli. Bacilli appear quite normal and well stained. Some appear to have vacuoles.

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli could not be demonstrated by staining. Remains, amorphous and faintly stained.

4. Bacillus megatherium. Few unstained bacilli that appeared partially dissolved. Spores coalesced.

5. Staphylococcus albus. Cocci unstained and coalesced.

d. After 5 per cent acetic acid. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, minute, intensely stained granules.

2. Bovine tubercle bacilli. (Accidentally destroyed.)

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli could not be demonstrated by staining. Remains, amorphous and faintly stained.

4. Bacillus megatherium. Bacilli and spores unstained and coalesced.

5. Staphylococcus albus. Cocci unstained and coalesced.

D. Action of formaldehyde on bacteria

Formaldehyde of various strengths (diluted in distilled water) was permitted to act on bacteria for a period of six hours.

a. After 1 per cent formaldehyde. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, intensely stained amorphous masses.

2. Bovine tubercle bacilli. Poorly stained bacilli that appear shrunken.

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli could not be demonstrated by staining.

4. Bacillus megatherium. Few unstained and swollen bacilli. Spores greatly swollen.

5. Staphylococcus albus. Cocci faintly stained and swollen.

b. After 3 per cent formaldehyde. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains intensely stained amorphous masses.

2. Bovine tubercle bacilli. Bacilli distorted in various ways: shrunken, crescent-shaped, and some with large intensely stained granules.

3. Unknown bacilli, culture from intestinal contents of kitten. No distinct bacilli stained. Unstained spore-like forms partially dissolved.

4. Bacillus megatherium. Bacilli could not be demonstrated by staining. Spores partially dissolved.

5. Staphylococcus albus. Cocci unstained and smaller than control.

c. After 5 per cent formaldehyde. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, intensely stained, large granules.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, granular.

3. Unknown bacilli, culture from intestinal contents of kitten. Some large and small bacilli present that stain more intensely than control, also some swollen and unstained forms.

4. Bacillus megatherium. Bacilli unstained and partially dissolved. Spores unstained and coalesced.

5. Staphylococcus albus. Cocci swollen, unstained, and coalesced.

E. Action of potassium bichromate on bacteria

Various strains of bacteria were subjected to the action of potassium bichromate in various concentrations for a period of four hours. The potassium bichromate was dissolved in distilled water.

a. After 0.5 per cent solution of potassium bichromate. 1. Human tubercle bacilli. Bacilli intact could not be demonstrated by staining. Remains, intensely stained granules.

2. Bovine tubercle bacilli. A few bacilli still retain form. Remainder of remains intensely stained granules.

3. Staphylococcus pyogenes aureus. Normal cocci could not be demonstrated by staining. Remains, intensely stained minute 'cocci.'

4. Bacillus megatherium and spores. Bacilli could not be demonstrated by staining. Spores swollen and stained.

5. Unknown cocci. Some cocci swollen and stained.

b. After 1 per cent potassium bichromate. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Granular remains intensely stained.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Granular remains intensely stained.

3. Staphylococcus pyogenes aureus. Cocci swollen and partially destroyed, also stained.

4. Bacillus megatherium and spores. Bacilli could not be demonstrated by staining. Spores unstained, some swollen.

4. Bacillus megatherium and spores. Bacilli and spores preserved, but unstained.

5. Unknown cocci. Few swollen and stained cocci. Clumps of stained granular debris.

c. After 2.5 per cent potassium bichromate. 1. Human tubercle bacilli. Bacilli intact could not be demonstrated by staining. Granular remains minute particles and intensely stained.

2. Bovine tubercle bacilli. Few faintly stained bacilli intact.

3. Staphylococcus pyogenes aureus. Cocci could not be demonstrated by staining. Granular remains.

4. Bacillus megatherium and spores. Few swollen and unstained bacilli. Outline of spores very faint.

5. Unknown cocci. Cocci very faintly stained and swollen.

F. Action of osmic acid on bacteria

Various strains of bacteria were subjected to the action of 1 per cent and 2 per cent osmic acid for a period of four hours.

a. After 1 per cent osmic acid. 1. Human tubercle bacilli. Bacilli well preserved, stain purple.

2. Bovine tubercle bacilli. Bacilli well preserved, stain deep red.

3. Staphylococcus pyogenes aureus. Cocci preserved, but unstained.

4. Bacillus megatherium and spores. Bacilli and spores preserved, but unstained.

5. Unknown cocci. Could not be seen on the slide.

b. After 2 per cent osmic acid. 1. Human tubercle bacilli. Poorer preservation than with 1 per cent osmic, faintly stained.

2. Bovine tubercle bacilli. Well preserved and intensely stained.

3. Staphylococcus pyogenes aureus. Cocci could not be seen on the slide.

4. Bacillus megatherium and spores. Bacilli and spores unstained, outlines difficult to see.

5. Unknown cocci. Preserved, but unstained.

G. Action of moist heat on bacteria

Various strains of bacteria were placed in vials containing physiological salt solution and kept in an oven at a constant temperature of 49°C.

a. After thirty minutes at 49°C. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Field apparently contained fat globules.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Field contained granular remains, stained amorphous masses and apparently fat globules.

3. Staphylococcus pyogenes aureus. Cocci could not be demonstrated by staining. Remains very minute granules.

4. Bacillus megatherium and spores. Bacilli could not be demonstrated by staining. Spores coalesced.

5. Unknown cocci. Cocci could not be demonstrated by staining. Remains, stained amorphous masses.

DISCUSSION

The results obtained from these experiments demonstrate that bacteria may lose their staining properties when subjected to the action of certain chemicals ordinarily used in microscopical technique. The degree to which the staining reactions were affected varied with the different chemicals and also with the strain of bacteria.

In many cases the bacteria retained their form, but were unstained, and in other experiments the bacteria were fragmented. In the cases where the organisms could not be seen they apparently had been dissolved or fragmented. In the majority of experiments where the remains on the slide were granular and fragmented these remains were stained. The possibility suggests itself that mitochondria may behave in the same way and that some of the irregularly shaped mitochondria sometimes observed may be the fragments resulting from chemical action.

The visibility of unstained bacteria varies with the difference in refraction of the bacteria and the surrounding medium. In those cases where the bacteria could not be seen the granular remains indicated the destruction of the organism. Unstained bacteria lodged in the cytoplasm of tissue cells cannot be distinguished easily and in some cases they are not visible. It is generally supposed that mitochondria are dissolved by the action of certain chemicals. It is possible that in many cases where they cannot be demonstrated by staining their form has been retained, but unstained and consequently not readily observed.

Bacteria apparently respond to heat in the same way that mitochondria do. The end-product from the action of heat was not the same for all the strains of bacteria that were used for this experiment. In some cases the remains were granular, in others they were amorphous. The amorphous material apparently represented the residuum of a solution after evaporation.

Cowdry ('18, p. 68) has noted the presence in some secreting cells of mitochondria with 'bleb-like' swellings and in egg cells of 'dumb-bell-shaped' mitochondria. The action of 1 per cent acetic acid on Bacillus megatherium is significant in this connection. The imitation of such 'bleb-like' and 'dumb-bell-shaped' mitochondria by bacteria as the result of chemical action suggests the possibility that mitochondria of these types may be due to the action of the chemicals used in fixation.

CONCLUSIONS

The results obtained in subjecting bacteria to mitochondrial staining methods and to the chemicals that have been utilized to determine the chemical nature of mitochondria appear to demonstrate that these methods are not specific for mitochondria, but have a similar reaction on bacteria. *To the degree that these staining methods and chemical reactions are not specific, bacteria and mitochondria have a similar chemical constitution.*

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PLATE 1

EXPLANATION OF FIGURES

All the figures, with the exception of figure 7, were made with the aid of the camera lucida. They were all drawn to the same scale. The lenses used were: 2-mm aproch. oil-immer. obj., comp. ocular no. 8.

1 Bacillus subtilis from an old culture one hour after the application of janus green.

2 Bacillus subtilis, *a*) $5\frac{1}{2}$ hours after application of janus green; *b*) 20 hours after application of janus green.

3 Bacillus subtilis from a 48-hour culture 24 hours after application of janus green.

4 Bacillus megatherium from an old culture $5\frac{1}{2}$ hours after application of janus green.

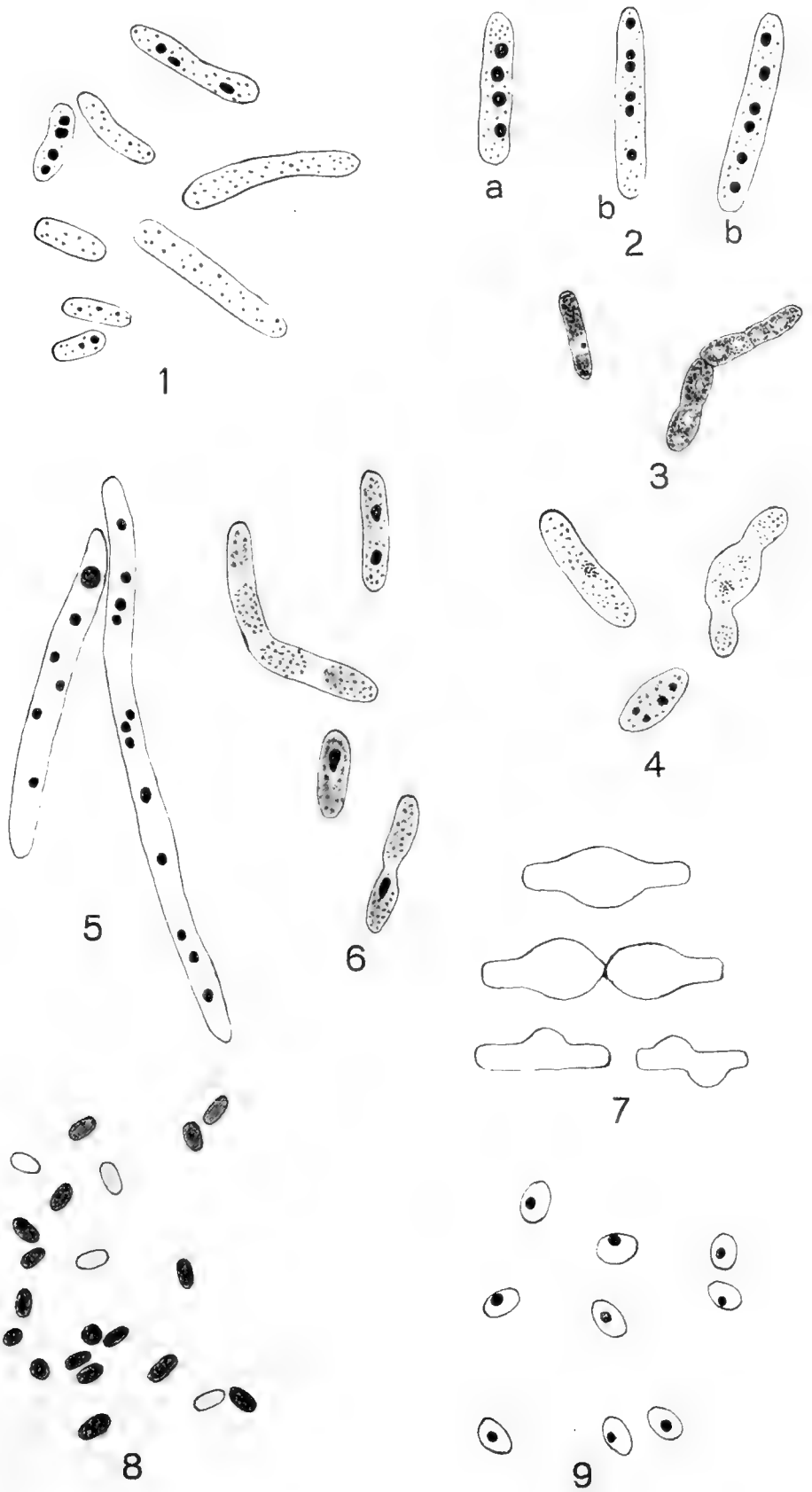
5 Bacillus megatherium from a 20-hour culture 15 minutes after application of janus green.

6 Bacillus megatherium from an old culture $\frac{1}{2}$ hour after application of janus green.

7 Free-hand drawing of bacillus megatherium after action of 1 per cent acetic acid for a period of six hours.

8 Bacillus coli 15 minutes after application of janus green.

9 Bacillus coli (same specimen as in fig. 8) $5\frac{1}{2}$ hours after application of janus green.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ADDENDUM

After this paper was submitted for publication, my attention was called to a work by Portier ('18) entitled, "Les Symbiotes," and to criticisms of Portier's book by Regaud ('19) and Guilliermond ('19).

Unfortunately, I have not been able to secure a copy of Portier's book in time to review it in this article. However, I have perused Regaud's and Guilliermond's criticisms. From these criticisms it is apparent that Portier in 1918 stated a theory regarding mitochondria that coincides with a conception of these bodies that has been growing in my own mind. I was not ready to state this hypothesis until I had collected more evidence in its support. A brief consideration of Regaud's and Guilliermond's criticisms is pertinent at this time.

Obviously, the details of Portier's evidence cannot be considered in this discussion. Regaud quotes the following resumé from Portier's "Les Symbiotes": "Chaque cellule vivante renferme dans son protoplasme des formations que les histologists désignent sous le nom de mitochondries. Ces organites ne seraient pour mio autre chose que des bactéries symbiotiques, ce que je nomme des symbiots. . . . . La bactérie symbiotique vient du milieu extérieur: elle peut, dans certains cas, y retourner et vivre d'une vie indépendante. Les bactéries seraient donc les seuls êtres simples; tous les autres seraient doubles."

Regaud indicates various characteristics of mitochondria that are supposedly not shared by bacteria. He mentions the inconstancy of form of mitochondria, their behavior towards acids and metallic salts, their albumin-lipoid constitution, their fragility, the impossibility of mechanical extraction of mitochondria from the living cell, and the synthetic properties of mitochondria. He also indicates the following characteristics of bacteria that presumably, are not shared by mitochondria: bacteria are definite organisms having a stable form (difficult to change in shape); bacterial life is generally resistant to chemical and physical agents; bacteria are easily extracted mechanically from living cells without alteration of the form of the bacteria; the form and structure of bacteria and even their staining qualities are indifferent to fixation.

I shall discuss briefly each of these characters that Regaud considers distinctive.

1. Inconstancy of form of mitochondria. This cannot seriously be considered a characteristic of mitochondria. It is a well established fact in bacteriological technique that certain bacteria assume different forms in different media (Jordan, '20, p. 67).

2. The behavior of mitochondria towards acids and metallic salts. In the second section of this paper I have given sufficient evidence that

some bacteria behave like mitochondria to acetic acid and other chemicals. The behavior of bacteria after fixation with a fixative containing potassium bichromate is definitely indicated by their staining reaction and is not unlike mitochondria.

3. The albumin-lipoid constitution of mitochondria. The chemical nature of mitochondria is unknown. I have shown in the second section of this paper that the chemical reactions used in attempting to determine the chemical nature of mitochondria have a similar effect on some bacteria.

4. The fragility of mitochondria. Mitochondria vary in fragility. This, I believe, has been assumed by various investigators. In a paper in preparation I will definitely demonstrate this difference. This character of mitochondria, per se, is in favor of a bacterial nature of mitochondria. Biological data furnish many examples of plants and animals that have become 'fragile' as a result of well-developed symbiosis and parasitism. The tapeworm is an example of a comparatively fragile organism. Its relationship to host is not as intimate as an intracellular symbiotic bacterium would be to its host. Surrounded by a living cytoplasmic environment, it is to be expected that a well-established symbiotic organism would lose many properties that the genetic type possessed. Further, one would expect the symbiotic form to acquire new properties.

5. The impossibility of mechanical extraction of mitochondria from the living cell. This apparent difficulty is undoubtedly dependent upon the fragility of mitochondria and consequently is irrelevant to the real problem.

6. The synthetic nature of mitochondria. Regaud argues that mitochondria are unlike bacteria in that they exhibit synthetic properties in the cytoplasm. He quotes himself and other investigators to support the contention that mitochondria produce secretion granules, pigment, etc. It appears to me that Regaud's argument is at least, equally convincing evidence that mitochondria are organisms.

7. Bacteria are definite organisms having a stable form. Bacteriological evidence does not support this contention. "The tubercle bacillus, for example, under ordinary conditions, is a typical rod, but sometimes produces branching filaments, and has been placed by some writers with the *trichomycetes*" (Jordan, '20, p. 64).

8. Bacterial life is generally resistant to chemical and physical agents. The chemical and heat experiments recorded in the second section of this paper refute this statement. Bacteria are not only affected by these agents, but in some cases are extremely sensitive to them.

9. Bacteria are easily extracted mechanically from living cells without alteration of the form of the bacterium. This argument has no bearing on the problem for, a priori, it must be admitted that a bacterium that develops an intracellular symbiotic existence would acquire fragility.

10. The form and structure of bacteria and even their staining qualities are indifferent to fixation. This is not in agreement with the results recorded in the second section of this paper. It was found that not only the form was altered by the fixation, but the staining qualities were also distinctly altered. In the paper in preparation I shall give further evidence regarding this point.

Regaud states that absolute differences between bacteria and mitochondria are incontestable. Further, he demands that if Portier will not admit that there are differences, he will have to demonstrate that these two structures (mitochondria and bacteria) can change from one to the other. I cannot find in Regaud's criticism the 'absolute differences' which he claims are incontestable. Regarding the demand for a demonstration of reversibility of mitochondria and bacteria as a proof that mitochondria are organized entities, it seems to me that one has just as much ground to demand that it be demonstrated that a tapeworm can revert to a free-living organism to establish its individuality.

Portier ('19) answers the arguments advanced by Regaud. Not having a full knowledge of Portier's data, I shall not attempt to consider Portier's rebuttal. However, one argument advanced by Portier in explaining the source of his 'symbiotes' (mitochondria) invites a critical consideration. Portier found bacteria in the intestine of the rabbit and apparently found similar bacteria in the cytoplasm of the intestinal epithelial cells. From this observation Portier concludes that the source of 'symbiotes' is from the intestinal contents. Regaud, justly, refuses to accept this interpretation of the phenomenon. I have observed the same phenomenon in the intestinal contents and the intestinal epithelial cells in a one-day-old kitten after mitochondrial fixation and staining. The fact that mitochondria may be demonstrated in the cells of embryos before the intestine is formed excludes the possibility of such an origin. The question of the origin of mitochondria is a major problem that may well rest until the nature of mitochondria has been established.

Guilliermond ('19) discusses three sections of Portier's book. I shall briefly discuss his criticisms in the order given.

1. Analogous forms. Guilliermond admits the analogy of form and further admits that mitochondria exhibit the property of division. He calls attention to the fact that the slightest upset in osmotic equilibrium suffices to change the character of mitochondria. In hypotonic media mitochondria immediately swell and transform into large vesicles. Of most important value as evidence, he argues that mitochondria have very little resistance to alcohol, chloroform, and acetic acid, and that it has been shown that a temperature of 40°C.[1] is sufficient to destroy the mitochondria in a few moments. He also says: "Up to the present time bacteria are not known that exhibit such fragility."

[1] In a correction (Compt. rend. des Soc. Biol., T. 82, p. 396), Guilliermond changes the temperature at which mitochondria disappear to 47°C.

This criticism deals with the fragility of mitochondria. I have answered this criticism above. However, I again insist that even if such a difference were a reality, it has no bearing on the problem. It must be admitted, on the basis of known biological behavior, that fragility is an accompaniment to well-established symbiosis and parasitism.

2. Bacteria are stained like mitochondria by Regaud's method. Portier's 'symbiotes' resist alcohol and acetic acid, are stained easily without change after fixation. Guilliermond states that this fact is an excellent means of differentiating between mitochondria and 'symbiotes.' Guilliermond admits that some mitochondria are more resistant than others to acetic acid and alcohol, but maintains that the more resistant forms are no longer mitochondria, but plastids differentiated from mitochondria.

In the first section of this paper I have shown that bacteria are stained like mitochondria by a number of mitochondrial methods, including the vital janus green method. From Guilliermond's criticism it would appear that Portier's 'symbiotes' are not mitochondria, but some other organism. In my work on staining of bacteria with mitochondrial methods I have found no fundamental difference in the staining reactions of mitochondria and bacteria. Guilliermond's statement that the more resistant mitochondria are no longer mitochondria needs elucidating evidence. If mitochondria metamorphose into organs that exhibit synthetic properties, then they assume properties that are characteristic of organisms. Numerous investigators have observed that mitochondria differ in their power of resistance to acetic acid and alcohol. I have shown in the second section of this paper that bacteria also differ in their reactions to these chemicals.

3. Mitochondria may be cultivated in certain cases. Guilliermond does not accept Portier's statement that he has grown mitochondria. He concludes with the statement: "We cannot conceive that anyone can culture such fragile elements."

I am not in a position to intelligently consider this latter criticism at this time. On the basis of evidence that I shall submit in a paper in preparation, I feel confident that mitochondria may be transferred intact to culture media. While it must be admitted that a demonstration of mitochondria growing as independent organisms in a culture medium would be absolute proof of their organized or bacterial nature, *the lack of such a demonstration is not proof that they are cytoplasmic organs and not organisms.*

The writer will discuss Regaud's and Guilliermond's criticisms at more length in a paper in preparation. From the preceding discussion, it is apparent that the fundamental aspects of the problem are not clearly defined. This confusion is apparently due to the vagueness of mitochondrial and bacterial definitions. Mitochondrial literature has not supplied a satisfactory definition of mitochondria. Jordan's "General Bacteriology" does not contain a definition of bacteria.

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Resumen por la autora, Beatrice Whiteside.

El desarrollo del saco endolinfático de Rana temporaria Linné.

Este trabajo trata del desarrollo del saco endolinfático de la rana desde la época en que aparece esta estructura hasta que adquiere la forma hallada en el adulto. La autora ha escojido la rana porque en este animal el saco es más grande y complicado de forma que en cualquier otro vertebrado investigado hasta el presente. La primera parte del trabajo consiste en una descripción de los estados sucesivos del desarrollo con especial referencia a la histología de la estructura mencionada y a su situactión respecto a las meninges espinales. Despues pasa a describir la topografía e histología del órgano después de haber completado su desarrollo. La segunda parte del trabajo está dedicada a una descripción de la estrcutrua anatómica del saco en todas las clases de los vertebrados.

Translation by José F. Nonidez
Cornell Medical College, New York

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THE DEVELOPMENT OF THE SACCUS ENDOLYMPHATICUS IN RANA TEMPORARIA LINNÉ

BEATRICE WHITESIDE

Zoological Laboratory, University of Zurich

NINETEEN FIGURES

INTRODUCTION

The membranous labyrinth of vertebrates has often been made the object of close study. There is one structure, however, connected with the ear which has hitherto received very little attention, namely, the saccus endolymphaticus. It is true that the topography of this organ is well known in most animals, but we are as yet badly informed as to its development, histology, and function.

Under these circumstances, I have, at Professor Hescheler's suggestion, undertaken an investigation of the development of the saccus endolymphaticus in Rana temporaria Linné. The work was done in the Zoological Laboratory of the University of Zurich, Switzerland, under the guidance of Prof. K. Hescheler, and I wish to take this opportunity of thanking him for the assistance he has so kindly given me. I also wish to express my thanks to Prof. J. Strohl, Dr. Marie Daiber, and Dr. H. Steiner, of Zurich University, for their many helpful suggestions, and I am indebted to Mr. A. Bychowsky for the execution of figures 11 to 19.

The present paper is divided into two principal parts: First, a description of the development of the saccus endolymphaticus in Rana temporaria Linné, and, second, an account of the topography of this structure in the different classes of vertebrates and the conclusions to be drawn from the same.

In order to show the relation of the saccus endolymphaticus to the other parts of the labyrinth, I shall preface my report with a short summary of Gaupp's ('04) description of the frog's ear.

In the membranous auditory organ of the frog the following parts can be distinguished: (fig. 1) the utriculus (*utr.*) with the sinus superior (*si.sup.utr.*) and posterior (*si.post.utr.*), the recessus utriculi (*rec.utr.*), the three semicircular canals (*ca.s.c.*) with the ampullae (*amp.*), the sacculus (*sac.*) with the ductus (*d.e.*) and saccus endolymphaticus (*s.e.*), the pars neglecta (*p.negl.*), the pars basilaris (*p.bas.*), the lagena cochleae (*lag.*), and the tegumentum vasculosum (not visible in the figure.)

The utriculus is irregularly cylindrical in form, with its long axis running horizontally from back to front. It is situated close to the median side of the bony labyrinth. On one side the utriculus passes into the recessus utriculi; on the other, into the narrow part of the sinus posterior. The three semicircular canals arise from the utriculus and each one is connected with this organ by two ends, the crus simplex and the crus ampullare (*amp.*), the former not being any wider than the canal itself, whereas the latter expands into an oval sac. The utriculus communicates with the sacculus through the foramen utriculo-sacculare and is connected at the latter's circumference with the sacculus and the pars neglecta. According to Gaupp, the sacculus of the inner ear has the shape of an oval sac, and possesses four pouch-like enlargements, namely, the lagena, the pars basilaris, the pars neglecta, and the tegumentum vasculosum. The auditory nerve enters the labyrinth with its two branches, the ramus anterior (*r.ant.*) and the ramus posterior (*r.post.*). It ends in the three cristae acusticae of the ampullae and also in the macula recessus utriculi, the macula sacculi, the macula lagenae, the macula neglecta, and the papilla basilaris. Each of the three first-mentioned maculae is covered with a membrane, on which many lime crystals are deposited. At its upper median side the wall of the sacculus expands into the ductus endolymphaticus (recessus labyrinthi, aequaeductus vestibuli). This structure is a very long and narrow canal which runs upwards median to the utriculus, and leaves the bony auditory capsule through a special aperture, the foramen endolymphaticum (apertura aequaeductus vestibuli). Inside the cranial cavity it widens (Hasse, '73) into the large saccus endolymphaticus,

which surrounds the brain and extends caudally into the vertebral canal. The part lying within the vertebral canal sends out processes through the intervertebral foramina which come to lie upon the spinal ganglia (Coggi, '90). All the different parts

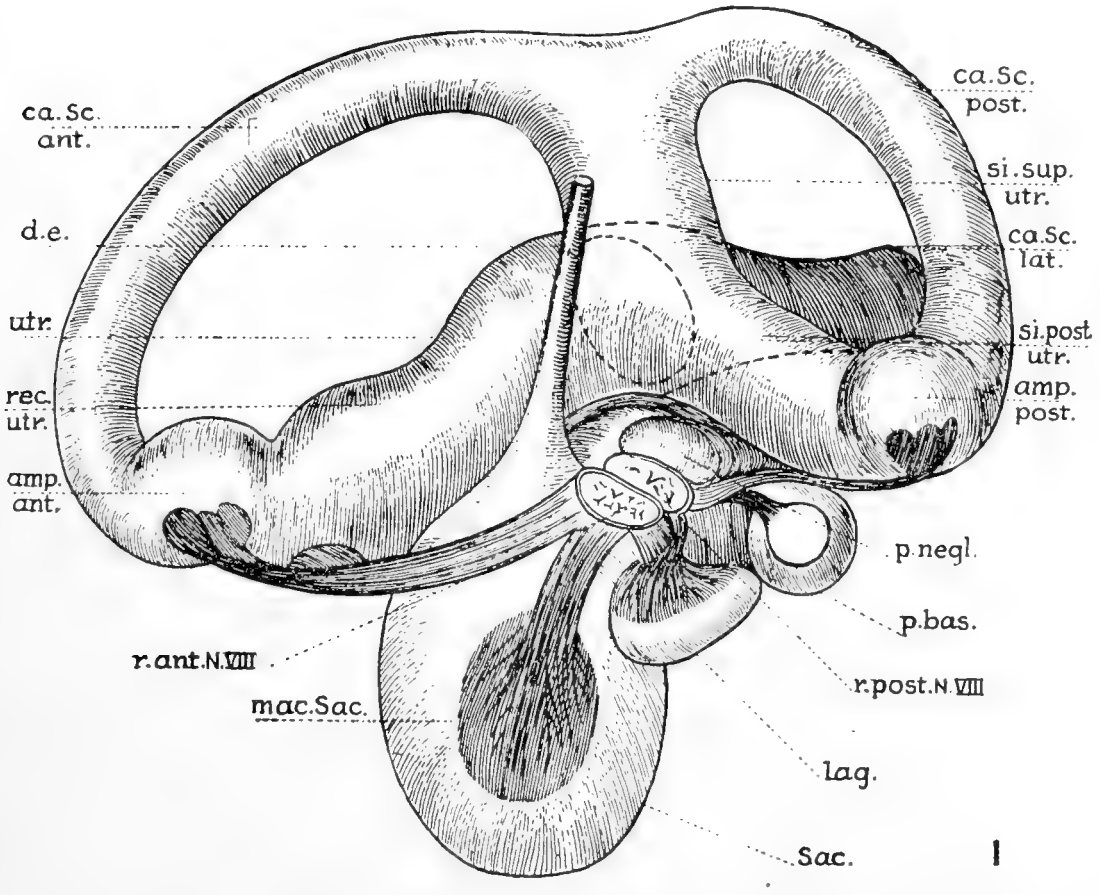


Fig. 1 Membranous labyrinth of Rana, according to Retzius (taken from Gaupp '96). *amp.ant.*, ampulla anterior; *amp.post.*, ampulla posterior; *ca.sc.ant.*, canalis semicircularis anterior; *ca.sc.lat.*, canalis semicircularis lateralis; *ca.sc.-post.*, canalis semicircularis posterior; *d.e.*, ductus endolymphaticus; *lag.*, lagena; *mac.sac.*, macula acustica sacculi; *p.bas.*, pars basilaris; *p.negl.*, pars neglecta; *r.ant.*N. VIII, ramus acusticus anterior; *r.post.*N. VIII, ramus acusticus posterior; *rec. utr.*, recessus utriculi; *sac.*, sacculus; *si. post. utr.*, sinus posterior utriculi; *si. sup.utr.*, sinus superior utriculi; *utr.*, utriculus.

of the saccus endolymphaticus are filled with a milky fluid containing many lime crystals which refract light and, according to Sterzi ('99), exhibit brownian movement.

My first task was the investigation of the development of the saccus endolymphaticus, especially in regard to its histological

conditions and the first appearance of the calcareous contents. My paper begins with the stage at which Krause's investigation ('01) closes, in which the auditory vesicle is divided into utriculus and sacculus. At this time the ductus endolymphaticus is a small canal leading up from the sacculus, and the saccus is indicated by a small expansion at the distal end of the ductus.

Before starting a report of my own investigation, I shall give a short account of previous observations on the development of the endolymphatic organs in the common frog, beginning with Krause ('01), who describes the formation of the ductus as follows. The auditory plate is formed from the inner layer of ectoderm on either side of the hind-brain. It consists of a single layer of long cylindrical cells which are longest in the middle of the plate, decrease in length toward its sides, and eventually pass into the very low cells of the outer layer of the ectoderm. First the dorsal side of this plate bends downwards and grows ventrally, losing its connection with the outer layer of the ectoderm. Thus the dorsal part of the auditory vesicle becomes marked off. This first formed part is the rudiment of the ductus endolymphaticus. Then the ventral side follows, bulging outward at the same time. In this way the ductus is still more distinctly separated from the rest of the auditory vesicle and forced to the latter's median surface.

There are, to my knowledge, only three other papers on this subject, namely, those of Villy ('90), Poli ('97), and Corning ('99). All three investigators agree that the organ is formed in the above described manner.

In all vertebrates the saccus endolymphaticus originates as an expansion of the distal part of the ductus. This structure is therefore a part of the inner ear and has no connection with the lymphatic spaces lying within the skull. Most authors confine themselves to this statement, and I know of only two detailed reports on the further development of this organ, the one by Röthig and Brugsch ('02), on the chick, the other by Streeter ('16), on the human embryo.

As regards the frog in particular, the papers of Coggi ('90) and Villy ('90) are to be mentioned. Coggi gives a few details

which I shall quote in the course of my paper, while all that Villy writes on the subject is the following paragraph:

> Until the semicircular canals are formed, little is to be noticed regarding the ductus endolymphaticus except a general growth in size, accompanied by a movement towards the brain, so that it comes to lie in close contact with this organ. As the distal part comes close to the brain, it begins to expand and its duct narrows; at the same time the upper lip of the duct elongates, so as to carry the vestibular opening downwards. The distal enlarged part grows, and, as the tadpole loses its tail, assumes the permanent proportions, becoming at the same time thin walled and vascular, while the organs of the two sides meet both above and below the brain. Whether actual communication is set up is difficult to determine by means of sections alone. The growth of cartilage between the expanded end of the organ and the rest of the vestibule does not take place until late and even then a foramen is left, through which the duct passes from the vestibule to the skull-cavity. It would seem to have some function of importance in the adult, as it steadily increases in size during the growth of the tadpole and it is after the tadpole stage is passed that this increase in size becomes most rapid and the blood supply most copious.

DESCRIPTION OF DEVELOPMENT

1. Material and methods of preparation

The material consisted of larvae of Rana temporaria Linné. The investigation was conducted chiefly by means of series of sections, of which thirty were made, six representing the first stage and four each of the following stages. The larvae were first narcotized with ether and the gut extracted. The larvae were then fixed in sublimate (twenty-four hours). As I wished to pay special attention to the lime contents of the saccus, it was not possible to decalcify. This rendered section cutting very difficult. In order to make the preparations more fit for sectioning, they were left a long time in the various media. They remained in 70 per cent alcohol for twenty-four hours, in 95 per cent for thirty-six hours, and in 100 per cent for twelve hours. Thereupon a little cedar oil was added to the 100 per cent alcohol, and the quantity of oil was gradually increased. After four hours, the larvae were placed in pure cedar oil, in which they remained four weeks. At the end of this time they were brought once

more into 100 per cent alcohol (two hours) and then into xylol (one hour). They were then placed in a bath of xylol-paraffin (two hours), in paraffin 40° (eighteen hours), and finally in paraffin 58° (six hours). The best stains were obtained by a combination of haemalum with picric acid. Many larvae were also prepared macrosopically.

Stage I (fig. 2)

The youngest larva that I examined was 4 mm. long (measured from the tip of the head to the aperture of the anus). The mouth-opening and internal gills had appeared.

The invagination and constricting off of the auditory sac had also taken place. The latter organ has now a somewhat spherical form, and, as in this stage there is no cartilaginous capsule, it lies close to the side of the hind-brain, being separated from this by a thin layer of mesoderm cells (fig. 2). On its medioventral side the ganglion acusticus (*g.a.*) lies between it and the brain. It is divided into utriculus (*utr.*) and sacculus (*sac.*) by a septum and already possesses the rudiments of the semicircular canals, lagena, pars neglecta, and pars basilaris. Within the auditory sac numerous lime otoliths can be seen.

The ductus endolymphaticus (*d.e.*) is a well-differentiated canal, situated at the median side of the auditory sac. This duct starts from the upper median part of the sacculus and runs dorsally at the median side of the utriculus, extending in a dorsal direction even further than the latter organ. Its long axis thus runs in a dorsoventral direction. In contrast to later stages, it is large in comparison with the auditory sac.

At its upper end the ductus expands into a small vesicle, whose diameter is about twice as large as that of the ductus proper. This vesicle extends somewhat in a cranial direction. It represents the rudiment of the saccus endolymphaticus (*s.e.*).

The histological structure of the ductus and saccus endolymphaticus at this stage, in contrast to later ones, is exactly alike. This corresponds to the fact, established by Alexander ('90) and confirmed by Fleissig ('08), that in the development of the labyrinth the histological differentiation sets in much later than the

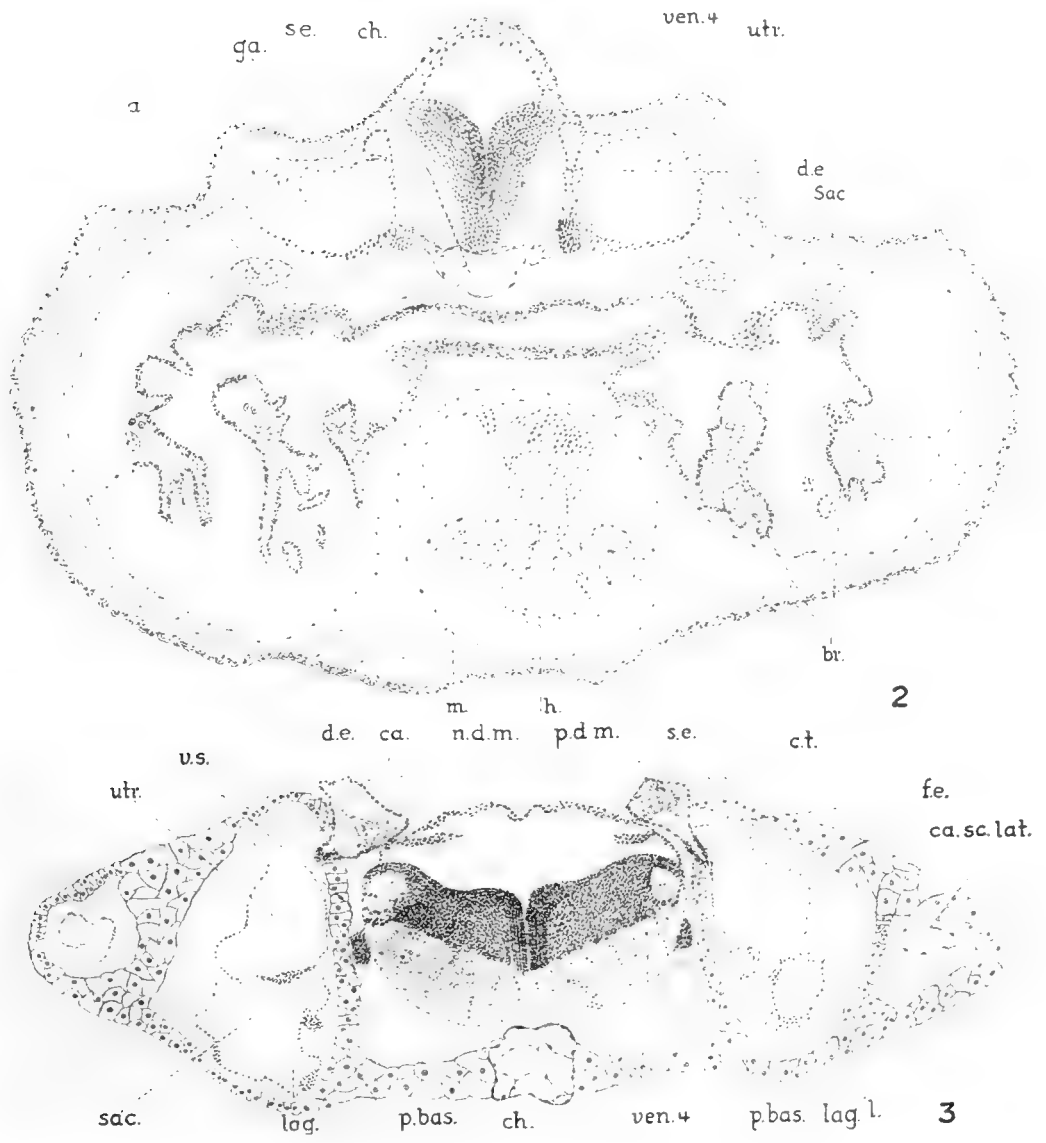


Fig. 2 Transverse section through the head of a larva. (Stage I.) Labyrinth region. *a*, *aorta br.*, gill; *ch.*, chorda; *d.e.*, ductus endolymphaticus; *g.a.*, ganglion acusticum; *h*, heart; *m*, mouth; *sac.*, sacculus; *s.e.*, saccus endolymphaticus; *utr.*, utriculus; ven. 4, fourth ventricle. Figures 2–9 drawn by means of Edinger's projecting apparatus. Leitz. obj. 3, oc. 3.

Fig. 3 Transverse section through the head of a larva. (Stage II.) Labyrinth region. *c.t.*, connective tissue; *ca.*, calcareous matter; *f.e.*, foramen endolymphaticum; *l*, labyrinth; *n.d.m.*, neural lamella of dura mater; *p.d.m.*, periosteal lamella of dura mater; *v.s.*, junction between neural and periosteal lamella of dura mater.

morphological. At the start the whole structure is lined with a one-layered epithelium of cylindrical cells.

For comparison, it can be noticed that Norris ('92) found the first indication of the saccus endolymphaticus in a larva of Amblystoma of 9 mm. length, whose auditory organ, similar to the above-described frog larva, already possessed the rudiments of the semicircular canals and the lagena. Fleissig ('08) mentions the first appearance of the saccus endolymphaticus of Phyllodactylus in an embryo 4 mm. long, at a time when the development of the labyrinth proper is far advanced. In reference to the origin of the saccus in man, Streeter ('16) writes that this organ appears at about the time of the closing off of the semicircular canals.

Stage II (figs. 3 and 11)

Stage II is based on a larva of 10 mm. length. No indications of extremities are visible.

The development of the labyrinth is far advanced and all its morphological parts have appeared (fig. 3). A cartilaginous ear capsule is completely formed, in whose median surface the foramen endolymphaticum (*f.e.*) is situated. In this stage as well as in the two following ones, this opening lies lateral to the anterior end of the plexus choroideus of the fourth ventricle.

The ductus endolymphaticus has now attained its definitive form. Its long axis has changed its dorsoventral direction and inclines somewhat in a craniocaudal direction, so that its entry into the cranial cavity lies a little more caudad than its exit from the sacculus. In consequence of this rotation, only its upper part is to be seen in figure 3. The ductus at this stage begins with a slightly broadened piece at the upper median part of the sacculus, and runs dorsally, median to the utriculus as far as the foramen endolymphaticum. Here it turns inward and leads through this aperture into the cranial cavity, where it immediately expands into the saccus endolymphaticus. As a comparison of figures 2 and 3 shows, the ductus endolymphaticus now appears smaller in proportion to the rest of the labyrinth, which has grown considerably more than the ductus.

The saccus endolymphaticus, though larger than in stage I, still remains, in contrast to the labyrinth and ductus, in a rudimentary stage, and does not arrive at the complicated form which it has in the adult animal until much later. It lies on the roof of the fourth ventricle (fig. 3, *vent. 4*) extending a little cranially and caudally beyond the foramen endolymphaticum. Its lumen is largest in the region of this opening and tapers slightly cranially and caudally.

The position of the saccus endolymphaticus in relation to the spinal meninges has been much discussed. In the first place, the descriptions of these membranes have not been uniform. Hasse ('73) and Rex ('93) mention three meninges: 1) the dura mater, which closely invests the bones; 2) the arachnoidea, separated from the dura by the subdural space, and, 3) the pia, closely enveloping the brain and the spinal cord. According to the opinion of the above-named investigators, the saccus endolymphaticus is situated in the subdural space between the dura and the arachnoidea. Sterzi ('99) has a different view. He terms the membrane immediately investing the bone, the endorhachis, and says that ventral to this lies the dura mater, whereas the innermost membrane is the arachnoidea. According to Sterzi, the saccus endolymphaticus would lie between the endorhachis and the dura mater, separated from the endorhachis by the epicalcary space, and from the dura by the epidural space. Gaupp ('04), Coggi ('90), and O'Neil ('98) describe the membranes as follows: 1) the dura mater, which is divided dorsal and lateral to the brain and the spinal cord into two lamellae, one of which closely invests the bone, while the other lies more ventrally, and, 2) a primary vascular membrane, in which a pia and an arachnoidea are as yet not distinctly differentiated. The lymphatic space between the two lamellae of the dura is called the interdural space, and that between the inner lamella of the dura and the vascular membrane the subdural space. According to these observers, the saccus endolymphaticus lies in the interdural space. My investigations confirm this view in every respect (figs. 3, 4, *ect.p.d.m.* and *n.d.m.*). As O'Neil describes in detail and I was able to verify, the dura mater is divided in

the whole region of the saccus endolymphaticus into two lamellae, which unite ventral to that structure. The saccus endolymphaticus is closely connected with the periosteal lamella of the dura, whereas it is joined loosely, if at all, to the neural lamella. Above the plexus of the fourth ventricle a union of the saccus endolymphaticus and the neural lamella of the dura takes place with the primary vascular membrane. O'Neil proved that the division of the dura into two membranes is much less marked in the salamander in consequence of the much smaller expansion of the saccus endolymphaticus.

As can be seen in figure 3, there is at this stage a difference between the histological structures of the ductus and saccus endolymphaticus. Both organs are lined with a one-layered epithelium, but the walls of the ductus are thicker than those of the saccus. They consist of cylindrical cells whose nuclei lie in the center. The walls of the saccus are composed of low plate-epithelium cells, which, in transverse sections, show polygonal dividing lines. These cells have their large oval nuclei in the center. Many dark pigment granules are found in the protoplasmic part of the cells. The saccus is surrounded by a connective-tissue membrane.

In this stage the saccus endolymphaticus still appears as an undivided sac, and has not yet the character of a gland. In spite of this fact, I found many lime crystals within the sac. This indicates that the cells are able to produce lime from the time of their formation—a fact which will not surprise us, if we remember that these cells originally came from the labyrinth.

Stage III (figs. 4, 5, and 12)

Stage III corresponds to a larva of 11 mm. length, which does not show any visible indication of extremities.

Figure 12 shows the extension of the saccus endolymphaticus. Compared to stage II, there is only a simple increase in length in a craniocaudal direction to be noticed. The saccus now extends from the optic lobes to the end of the medulla oblongata. The course of the anterior part is as follows: Starting from the

foramen endolymphaticum, it runs dorsal to the plexus of the fourth ventricle into the region of the cerebellum. There it turns to the side and runs lateral to the brain as far as the anterior end of the lobi optici, where it ends. Its largest voluminal development is found in the region of the foramen endolymphaticum, where it is very broad, covering a large part of the roof of the fourth ventricle. It narrows cranially. There is as yet no connection between the saccus of the right side and that of the

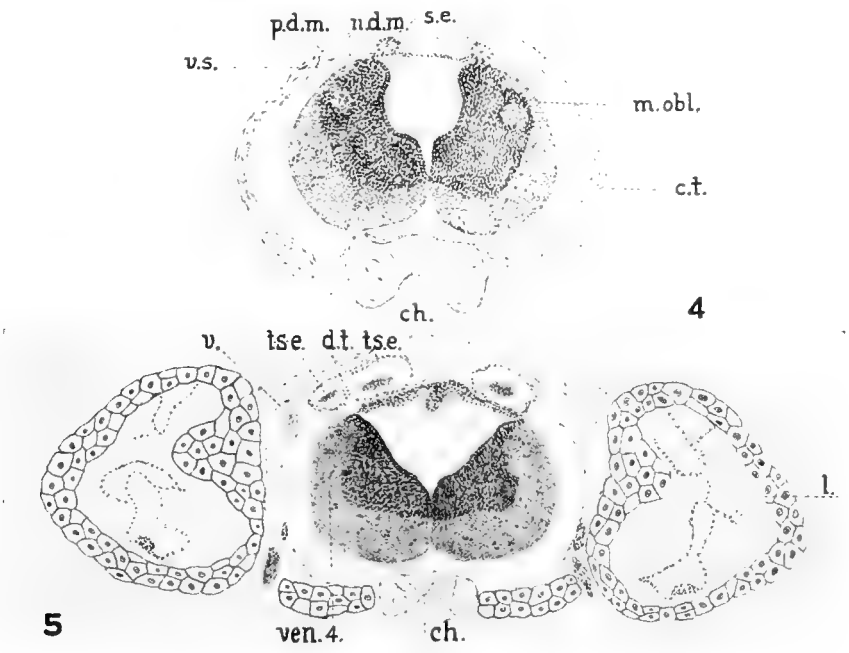


Fig. 4 Transverse section through the head of a larva. (Stage III.) Posterior part of the medulla oblongata. *m.obl.*, medulla oblongata.

Fig. 5 Transverse section through the head of a larva. (Stage III.) Labyrinth region. *d.t.*, point where saccus endolymphaticus divides; *t.s.e.*, tubuli of saccus endolymphaticus; *v*, blood vessel.

left. The part of the saccus which is situated behind the foramen endolymphaticum lies dorsal to the brain. It tapers in a caudal direction to a still larger degree than was the case in its anterior part. Here, too, the saccus of the one side is separated from that of the other (fig. 4), and there is no indication of the joining of the two sacci, such as occurs some time later in the region of the posterior end of the fourth ventricle (compare fig. 7).

The histological differentiation shows no further development than the preceding stages. The saccus is lined with the typical

epithelium. However, this organ no longer represents an undivided sac, but now consists in some parts of two parallel tubuli (fig. 5). This is brought about by division of the formerly single sac. The division does not take place in any fixed region, but may apparently occur in any part. There is great variability in this respect in the different larvae which I examined. The only thing which could be ascertained definitely in regard to the region in which the division of the saccus occurs, is the fact that the division never takes place within the region of the foramen endolymphaticum. In the specimen described here as stage III, the left saccus endolymphaticus is divided a little behind the foramen endolymphaticum into two parallel tubuli running from the front to the back (fig. 5). The median one has the greater diameter. These tubuli run a short distance toward the back and then join again. There is a similar division in the anterior part of the right saccus endolymphaticus, in the region of the lobi optici. Not only is the region in which the saccus divides not always the same, but the manner of division also varies. Sometimes the tubuli are formed by invagination and adhesion of the dorsal and ventral walls of the saccus, in other cases the invagination proceeds from the ventral wall only and continues until it reaches the dorsal side (fig. 5, *d.t.*). All parts of the saccus endolymphaticus are filled with calcareous matter.

Stage IV (figs. 6, 13, and 17)

A larva of 12 mm. length corresponds to stage IV. No trace of limbs is apparent.

The saccus endolymphaticus extends from the hemispheres into the region of the seventh vertebra (fig. 13). This stage is particularly interesting because several subdivisions of the saccus can now be distinguished. Starting from the foramen endolymphaticum, the pars anterior of the main stem runs, as in the preceding stage, dorsal to the brain as far as the lobi optici. Here it fastens itself to the lateral circumference of the middle brain, expands a little in the niche between the labyrinth and the eye and reaches the posterior part of the hemispheres. At its cranial end it sends out a process which ascends obliquely

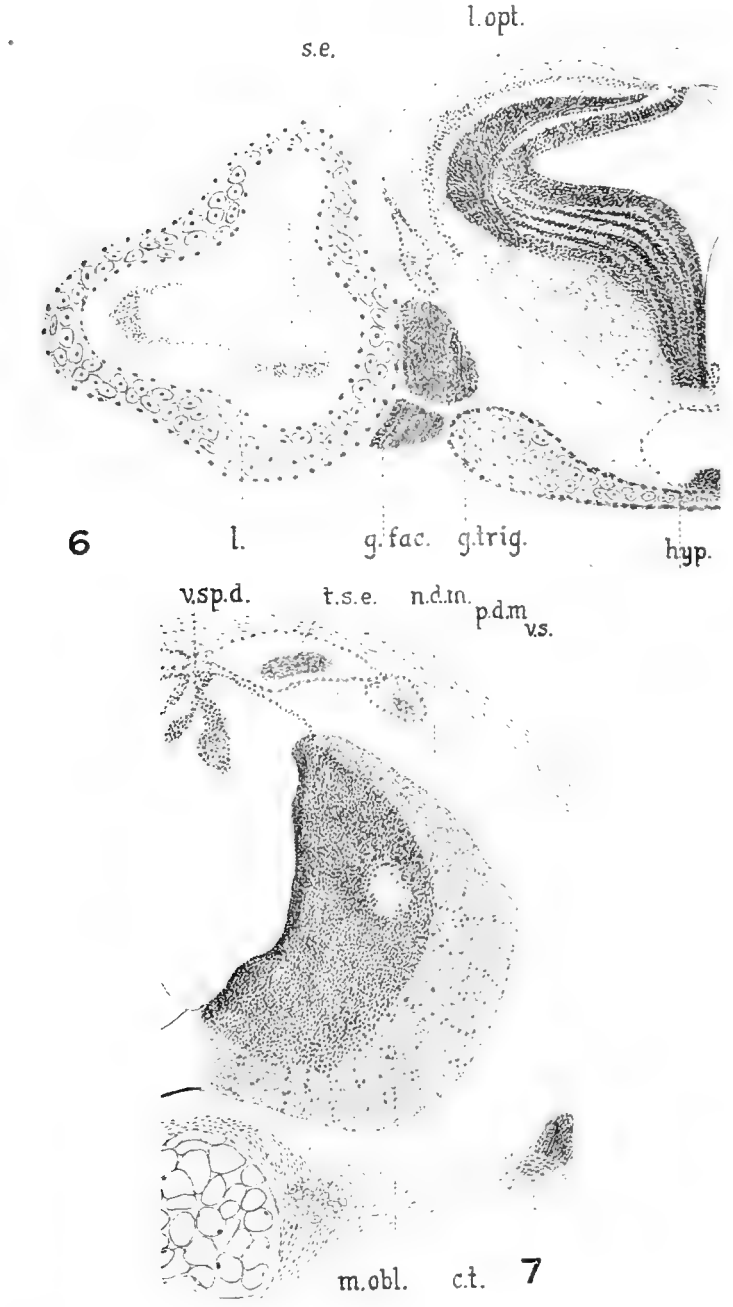


Fig. 6 Transverse section through the head of a larva. (Stage IV.) Hypophysis region. *g.fac.*, ganglion faciale; *g.trig.*, ganglion trigemini; *hyp.*, hypophysis; *l.opt.*, lobi optici; *s.e.*, saccus endolymphaticus.

Fig. 7 Transverse section through the head of the larva. (Stage V.) Posterior part of medulla oblongata. *n.d.m.*, neural lamella of dura mater; *p.d.m.*, periosteal lamella of dura mater; *t.s.e.*, tubuli of saccus endolymphaticus; *v.s.*, junction between neural and perisoteal lamellae of dura mater; *v.sp.d.*, vena spinalis dorsalis duralis.

upwards and inwards. In the middorsal line the ascending processes join and unite with the paraphysis. This connection between the two sacci, called by Gaupp the processus ascendens anterior, has now attained its definitive form.

The pars posterior of the main stem of the saccus endolymphaticus extends from the foramen endolymphaticum into the region of the seventh vertebra. The saccus of the one side is still separated from that of the other. The lumen of the saccus decreases caudally.

The lateral extension of the saccus endolymphaticus is illustrated in figure 17. As mentioned above, it lies at the side of the hemispheres, the diencephalon and the lobi optici. It is narrow in the region of the hemispheres and the diencephalon; in the region of the lobi optici, however, it widens and fastens itself onto the dorsal side of the ganglion prooticum commune (fig. 6, *g.pr.c.*). In the adult frog this ganglion represents the union of the trigeminal and facial ganglia. At the stage described here the two components are still to be recognized, the facial ganglion (*g.fsc.*) lying dorsal to that of the trigeminus (*g.trig.*).

In the larva taken to represent this stage of development, the anterior part of each saccus endolymphaticus is an undivided sac. The posterior part of each saccus is, however, divided into two tubuli directly behind the foramen endolymphaticum. These tubuli run as far as the posterior end of the fourth ventricle, where they join once more. The whole saccus is filled with lime.

Stage V (figs. 7, 14, and 18)

The sections on which the descriptions of stage V is based were made from a larva 15 mm. long, in which the anterior extremities had appeared.

The first thing to be noticed is that the position of the foramen endolymphaticum has changed in its relation to the brain. This opening no longer lies beside the plexus of the fourth ventricle, but at the side of the cerebellum. The change in position is probably due to the fact that the cerebellum has expanded backwards in the course of its rather late development.

The dorsal extension of the saccus endolymphaticus is illustrated in figure 14. In its anterior part only an increase in size can be observed. In the posterior part, however, a great change has taken place. At the end of the fourth ventricle the sacci endolymphatici of the two sides grow towards each other and meet in the median line (fig. 7). From the point of juncture the united sacci run caudally as far as the seventh vertebra.

Figure 18 shows the lateral expansion of the saccus endolymphaticus. In this stage the processus ventralis can be seen (fig. 18, *pr.vent.*). It starts from that part of the saccus stem which lies lateral to the lobi optici and dorsal to the ganglion prooticum commune. The processus extends ventrally as far as the lateral surface of this ganglion.

In the region of the foramen endolymphaticum the saccus endolymphaticus is an undivided sac. Directly in front of and behind this aperture, however, it divides into several smaller tubuli. In the cranial part four tubuli are formed, which run independently of one another into the region of the hemispheres, where they coalesce. The caudal part of the saccus remains undivided to the end of the fourth ventricle. At the place where the sacci endolymphatici of the two sides join, each saccus divides into two tubuli (fig. 7, *t.s.e.*) which run parallel to each other, and, towards their ends, split up into many small tubuli. The division sometimes takes place in a horizontal direction, sometimes in a vertical one.

Each of the tubuli is lined with the characteristic epithelium of the saccus endolymphaticus and is embedded in a delicate structure of connective tissue. The tubuli are surrounded by many blood vessels. Between the two partes spinales runs the vena spinalis dorsalis duralis (fig. 7, *v.sp.d.*). At the place where the two partes spinales separate, this vena divides into two branches, which run toward the front in the outer walls of the sacci and finally lead into the foramen trigemini. Both the stem and the two branches collect all the veins of the saccus.

The lumen of the saccus endolymphaticus is filled with calcareous matter.

Stage VI (figs. 8, 15, and 19)

The animal representing this stage was going through metamorphosis. It was 15 mm. long and possessed well-developed anterior and posterior extremities. The tail was somewhat reabsorbed.

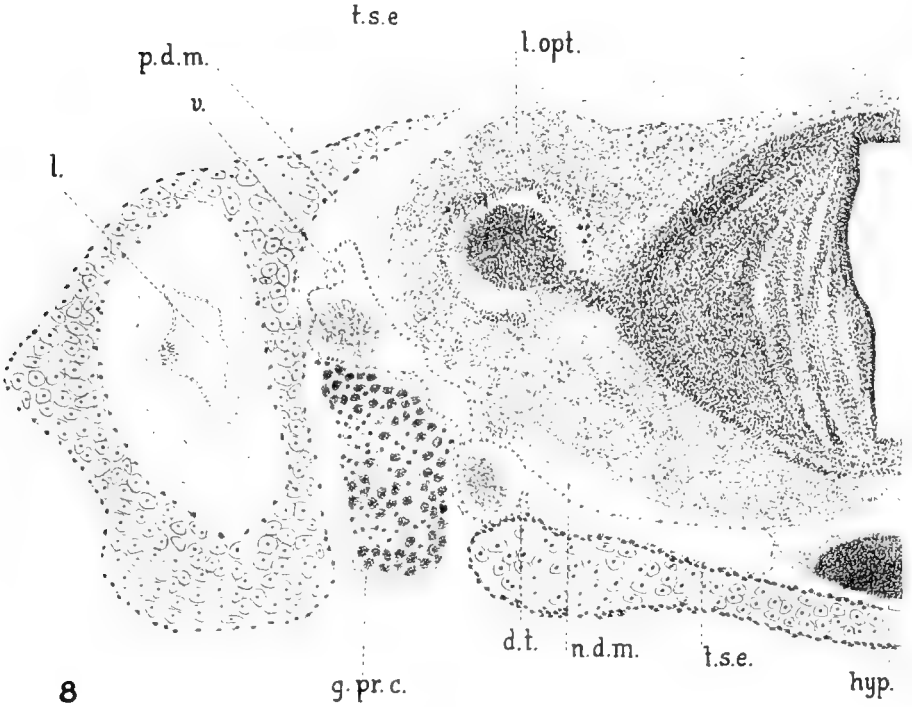
The foramen endolymphaticum now lies still farther forward in relation to the brain. It is situated lateral to the lobi optici, in which position it is also to be found in the adult frog.

In the posterior part of the saccus endolymphaticus a new communication appears between the two sacci. It is formed by the processus ascendens posterior, which, in the region of the cerebellum, runs over the brain and connects the saccus of the right side with that of the left (fig. 15, *pr.asc.post.*).

The most striking difference between this stage and the preceding one is the appearance of the calcareous sacs on the spinal ganglia. These structures are formed in the following manner: The posterior stem of the saccus, which runs backward inside of the vertebral canal, sends forth processes which extend through all the intervertebral foramina and as far as the spinal ganglia. The number and state of development of these transverse processes vary in the different larvae examined at this stage. Some specimens have many processes, others very few. The condition illustrated in figure 15 can frequently be observed. Here all the processes (*y*) have appeared, although in different degrees of development. Some of the sacs cover the entire ganglion, others only its proximal part. The most cranial processes, however, always appear first.

The processus ventralis has now completed its growth and extends over the lateral and posterior surfaces of the hypophysis (figs. 19 and 8). Behind the hypophysis it runs underneath the brain until it joins the processus of the opposite side in the mid-ventral line. In this way a connection between the two sacci endolymphatici takes place beneath the brain as well as above it.

The division of the saccus into small tubuli has progressed further, and the whole organ now consists of many such structures. This continuous division was observed in a general way by Coggi



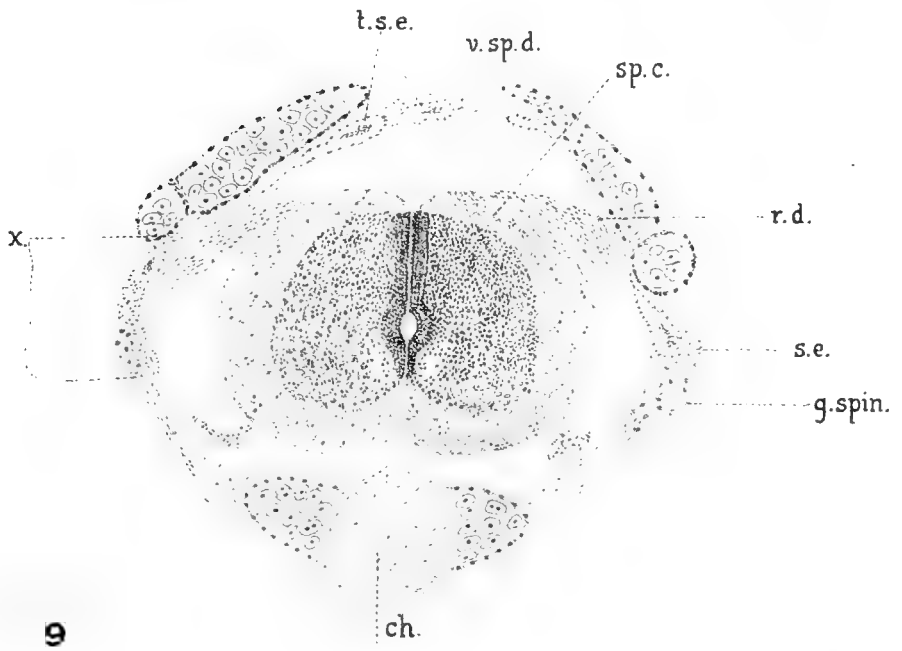


Fig. 8 Transverse section through the head of a larva. (Stage VI.) Hypophysis region. *d.t.*, point where saccus endolymphaticus divides; *g.pr.c.*, ganglion prooticum commune; *t.s.e.*, tubuli of saccus endolymphaticus; *v.*, blood vessel.

Fig. 9 Transverse section through spinal cord and 2 spinal ganglia of a young frog. (Stage VII.) *g.spin.*, spinal ganglion; *r.d.*, dorsal root of spinal nerve; *t.s.e.*, tubuli of saccus endolymphaticus; *v.sp.d.*, vena spinalis dorsalis duralis; *x.*, enlarged as fig. 10.

('90), who examined older larvae and adult frogs. He writes as follows:

> Nei girini di rana le varie cavita che formano il sacco endolinfatico sono molto meno numerose e meno suddivisi che non accada nelle rane adulte, ove la suddivisione va tant' oltra gino a formare gli otricelli microscopici che ho descritto.

Stage VII (figs. 9, 10, and 16)

This stage is based on a young frog which had just completed metamorphosis.

Compared with stage VI, the only change in the appearance of the saccus endolymphaticus is the increased size of the calcareous sacs. These structures have now assumed their definitive form. With this step the development of the saccus endolymphaticus is finished. The organ now has the following expansion.

Soon after the ductus endolymphaticus enters the cranial cavity through the foramen endolymphaticum, it expands into a large thin-walled sac, which lies in the interdural space, lateral and dorsal to the brain and the spinal cord. From the foramen endolymphaticum a part of the saccus runs cranially, lateral to the lobi optici and the diencephalon, and reaches the anterior lateral surface of the hemispheres. Two processes start from this main section of the pars cranialis anterior. From its anterior end the processus ascendens anterior runs dorsally until it meets the corresponding process of the other side. In the region of the foramen endolymphaticum the processus ventralis runs ventrally and surrounds the ganglion prooticum commune and the lateral surface of the hypophysis. Behind the latter organ it proceeds farther in a median direction until a union of the processes of the two sides takes place.

The pars posterior starts from the foramen endolymphaticum and runs caudally, lateral to the lobi optici and the cerebellum, and dorsal to the plexus choroideus of the fourth ventricle. Above the cerebellum the narrow processus ascendens posterior connects the two sacci. At the end of the fourth ventricle the pars posterior widens in a median direction and the sacci of the

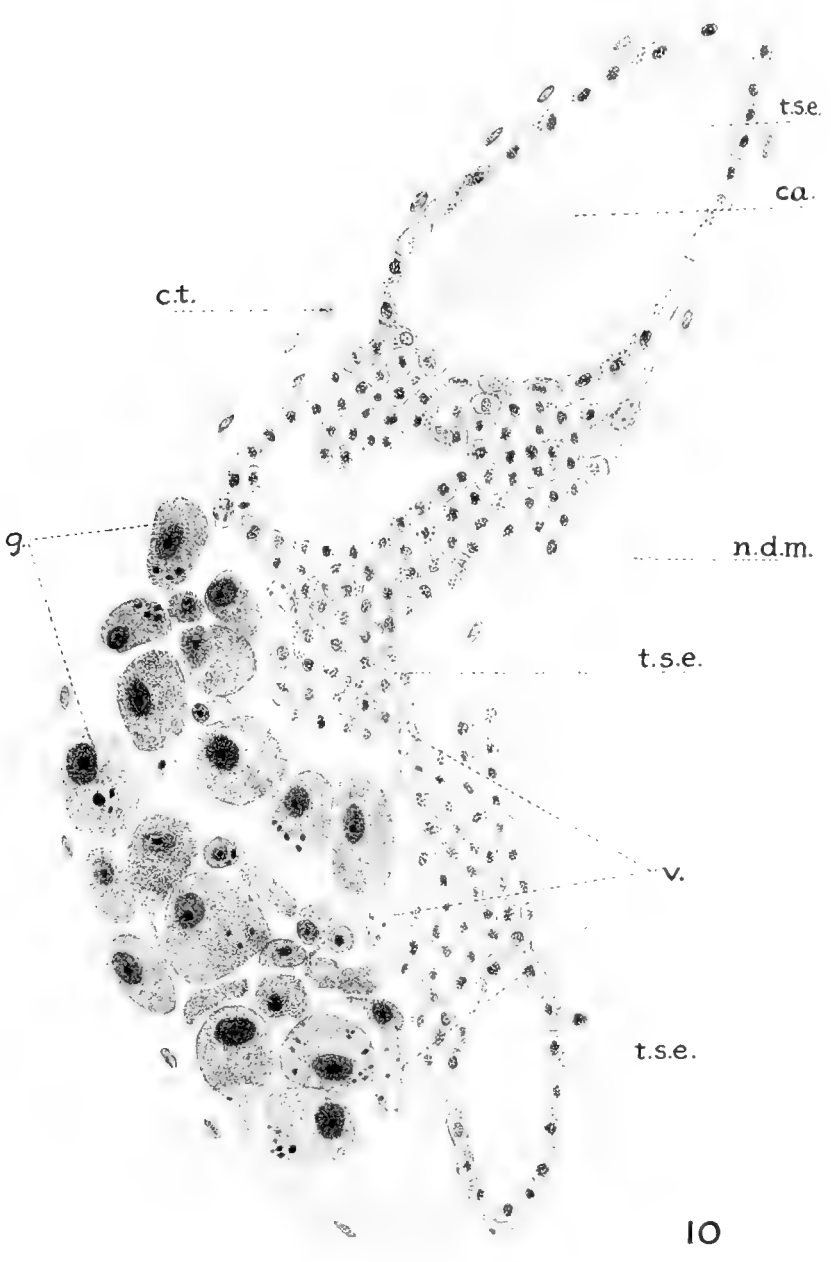


Fig. 10 Transverse section of one of the spinal ganglia and calcareous sacs shown in fig. 9. *c.t.*, connective tissue; *ca.*, calcareous matter; *g.*, ganglion cells; *n.d.m.*, neural lamella of dura mater; *t.s.e.*, tubuli of saccus endolymphaticus; *v.*, blood vessels.

Figure 10 drawn by means of Zeiss Abbe drawing apparatus. Oil immersion O, 3 mm. comp. oc. 6.

two sides unite in the middorsal line. From this point the two sacci run together in a caudal direction, giving the appearance of an unpaired structure. They leave the cranial cavity through the foramen occipitale and extend inside the canalis vertebralis to the region of the seventh vertebra, where the two halves part again. Through each intervertebral foramen processes emerge which envelop the spinal ganglia. The extreme posterior end of each saccus is formed by the calcareous sacs on the spinal ganglia IX and X.

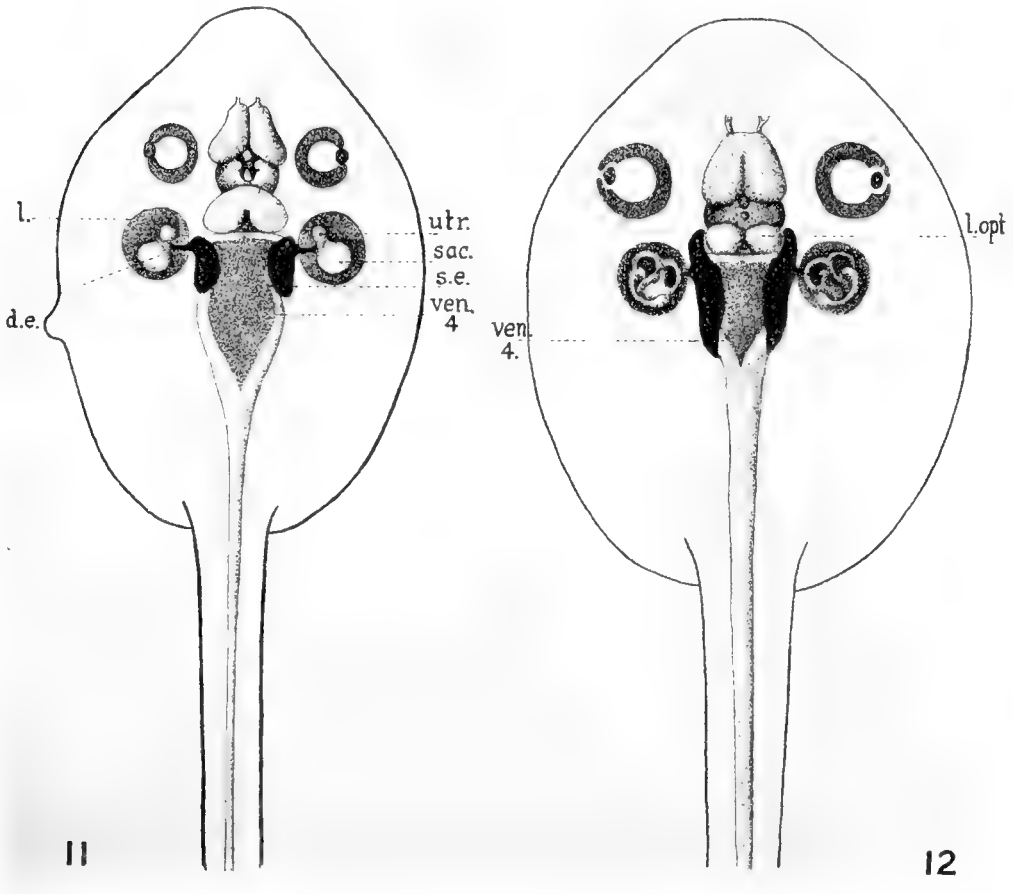
The above description corresponds with the one given by Gaupp ('07) for the adult frog. Other investigators have maintained that the expansion of the saccus endolymphaticus varies in different specimens. Gaupp explains this as follows:

> Die einzelnen Teile des Saccus endolymphaticus sind nur dann gut zu erkennen, wenn sie mit der charakteristischen milchweissen Fluessigkeit gefuellt sind. Der Fuellungszustand wechselt aber, und so kann es leicht kommen, dass einzelne Teile nicht sichtbar sind. Dies mag wohl der Grund sein, wenn der Saccus gelegentlich eine geringere Ausdehnung zu besitzen scheint.

I should like to emphasize the fact that, in the larvae and very young frogs, some parts of the saccus are invariably quite full of calcareous matter, whereas other parts contain only a very small amount. The greatest accumulation is always to be found in that part of the saccus which surrounds the ganglion prooticum commune and the hypophysis and in the part which lies on the roof of the fourth ventricle. The partes spinales and the calcareous sacs contain a fair amount, while I could observe only very few crystals in the anterior cranial part and in the processus ascendens anterior and posterior.

The differentiation of the saccus endolymphaticus into small tubuli has progressed so far that each part of that very extensive organ consists of many such structures. They are so numerous that a description of each one would lead us too far. Some facts, however, must be noticed. In the region of the foramen endolymphaticum, the saccus is an undivided sac, which, however, immediately in front and in back of this aperture, divides into many

tubuli. These again split up into still smaller ones. In the main part of the saccus all the tubuli run in a craniocaudal direction. The processus ventralis consists of four or five comparatively large tubuli running in a dorsoventral direction and coalescing in several places (fig. 8). The partes spinales and the



Figs. 11 to 19 Diagrams of the central nervous system and labyrinth (Saccus endolymphaticus black). Spinal ganglia not drawn in figs. 11 to 14.

Fig. 11 Dorsal view of larva. (Stage II.) *d.e.*, ductus endolymphaticus; *l*, labyrinth; *sac.*, sacculus; *s.e.*, saccus endolymphaticus; *utr.*, utriculus; *ven. 4*, fourth ventricle.

Fig. 12 Dorsal view of larva (Stage III.) *l.opt.*, lobi optici.

calcareous sacs are composed of a large number of tubuli. In each pars spinalis there are three of these structures. Figure 9 represents a transverse section in the region of an intervertebral foramen. I found that the tubuli of one-half of the partes spinales never join with those of the other half. The connection between the partes posteriores of the two sacci endolym-

phatici is thus only an external one. Between the two lies the vena spinalis dorsalis duralis.

The calcareous sacs (fig. 10) have been described by Lenhossék ('86). My results confirm his statements, except for his description of the position of these structures. According

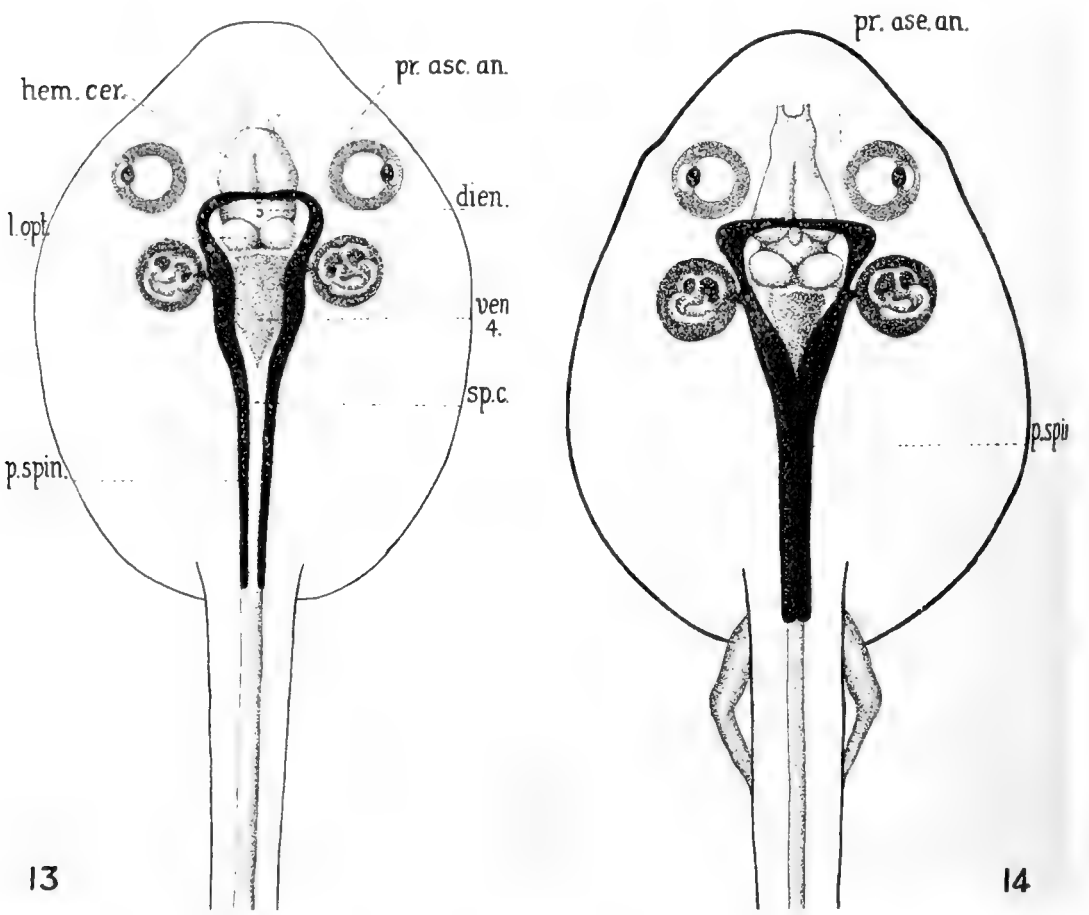


Fig. 13 Dorsal view of larva (Stage IV.) *dien.*, diencephalon; *hem. cer.*, hemisphaerae cerebri; *pr. asc. an.*, proscessus ascendens anterior; *p. spin.*, partes spinales of saccus endolymphaticus; *sp.c.*, spinal cord.

Fig. 14 Dorsal view of larva. (Stage V.)

to him, they cover only the proximal part of the ganglion, whereas my sections show that they envelop the whole ganglion. Lenhossék writes that the sacs lie inside the fibrous capsule belonging to the ganglia. This capsule sends septa of connective tissue into the interior of the sacs. There is also a thin continua-

tion of the capsule, which runs between the sacs and the ganglia. I can mention one more important fact, namely, that no nerves or nerve-endings are to be found either in the calcareous sacs or in any part of the saccus endolymphaticus. The whole organ consists merely of glandular tubuli embedded in connective tissue.

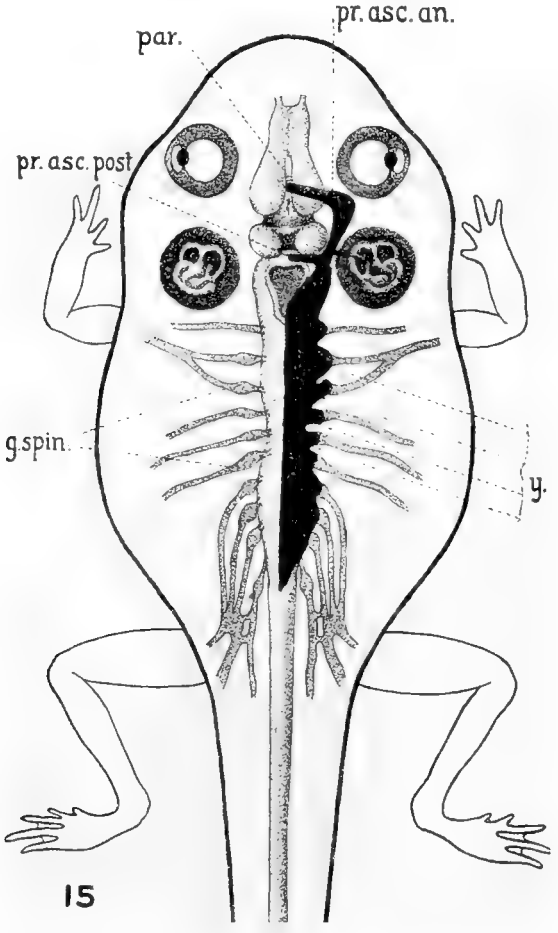


Fig. 15 Dorsal view of larva. (Stage VI.) *g. spin.*, spinal ganglia; *par.*, paraphysis; *pr.asc.post.*, processus ascendens posterior; *y.*, transverse processes in different stages of development.

The above-mentioned investigator also gives a very proper account of the finer structure of the tubuli of the calcareous sacs, which I found to hold good for the tubuli lying within the other parts of the saccus endolymphaticus. He states that these tubuli are lined with a single-layered epithelium, whose cells, in transverse section, are almost square and measure about 14 or 15μ in diameter. Some of the cells, however, are cylindri-

cal in form, while others are very flattened. He thinks this difference in shape is due to the fact that some of the tubuli are quite full of calcareous matter, whereas others contain very little. The cells have sharp, distinct outlines and oval nuclei. To this description I may add that the nucleus of the cells is very large and contains no nucleolus. The chromatin appears in the form

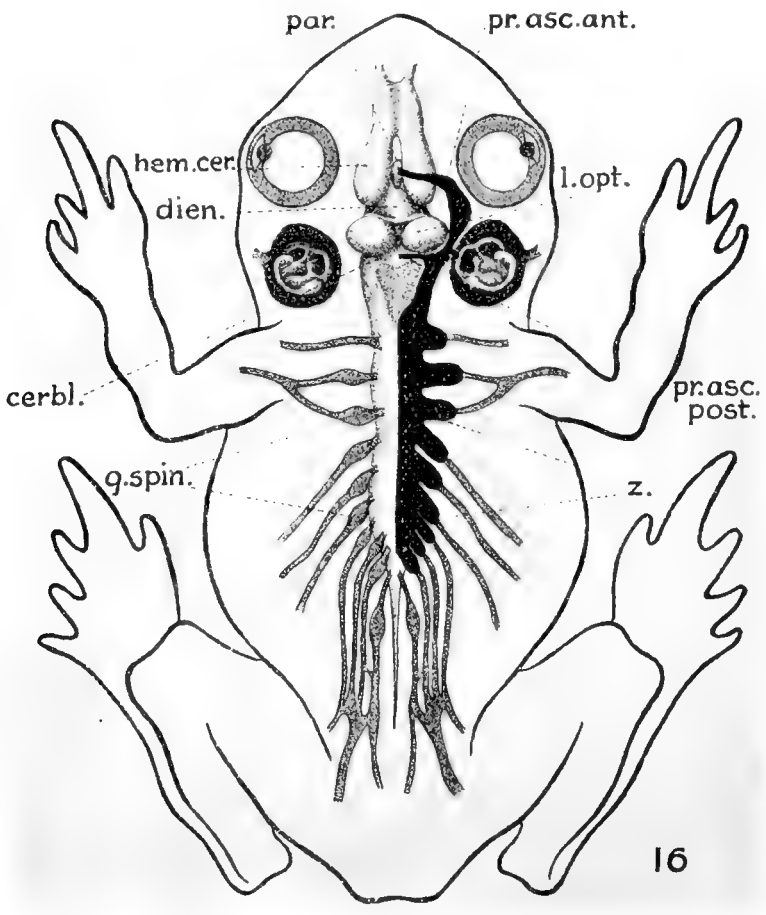


Fig. 16 Dorsal view of young frog. (Stage VII.) (Only right saccus endolymphaticus drawn.) *z.*, transverse processes in definitive form.

of large lumps—a condition which reminds one of that in the nuclei of glandular cells. The protoplasm also recalls that found in such organs, showing very fine granulation and containing some pigment globules. Lenhossék was not able to prove the existence of a membrana propria around the individual tubuli. This structure was found by Coggi ('90).

The lumen of the tubuli is filled with a liquid in which many lime crystals are suspended. Carus ('41) was the first to notice the similarity of these crystals to those in the labyrinth proper. He writes as follows:

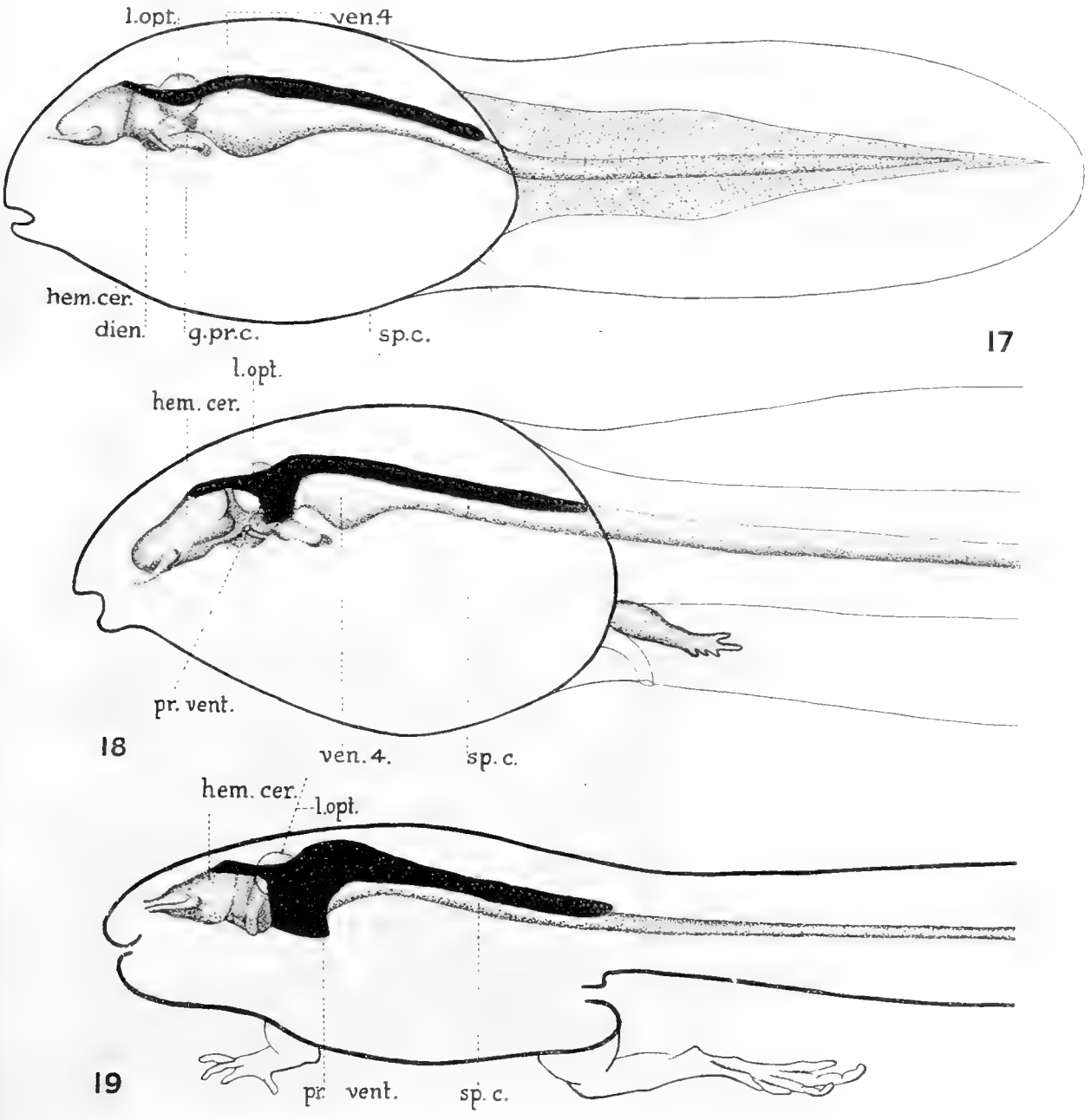


Fig. 17 Lateral view of larva. (Stage IV.) *g. pr. c.*, ganglion prooticum commune.

Fig. 18 Lateral view of larva. (Stage V.) *pr. vent.*, processus ventralis.

Fig. 19 Lateral view of larva. (Stage VI.)

Si l'on ouvre de bas en haut le labyrinthe d'une Grenouille, on est surpris de trouver le petit sac plein d'une masse crétacée presque entièrement de même nature que les singuliers corps laiteux ou crayeux qui garnissent les trous intervertébraux destinés au passage de nerfs rachidiènes. Les deux masses quand on les examine au microscope, paraissent consister en plusieurs millions de cristaux de carbonate calcaire, arrondis et ovalaires, dont les plus gros ont environ un centième de ligne de long, et dont la forme est celle d'un prisme à six pans terminé par des sommets a six faces.

The entire saccus endolymphaticus may, as Lenhossék suggests in regard to the calcareous sacs, be compared to a tubular gland without an outlet. The contents of the saccus would then represent the secretion of the glandular cells. In any case, we must not forget that these cells are derived from the labyrinth, whose epithelium has the same ability to produce lime.

Summary

The first fact to be noticed in the course of development of the saccus endolymphaticus is the comparatively late differentiation of this organ. In a larva of 4 mm. length, whose auditory organ is in an advanced state of development, the saccus endolymphaticus is present only as a slight expansion of the distal end of the ductus. At a time when all the morphological parts of the labyrinth are recognizable, this structure is still a small sac, adhering to the roof of the fourth ventricle. According to Norris ('92) and Fleissig ('08), the first indication of the saccus appears also very late in Amblystoma and Ascalabotes.

The further development of the saccus proceeds very slowly. First an increase in length takes place in a craniocaudal direction, until the saccus reaches from the hemispheres into the region of the seventh vertebra, the saccus of one side remaining separated, however, from that of the opposite side. Next there develops, in a larva 12 mm. long, the processus ascendens anterior. This is very soon followed by the joining of the partes spinales and the first indication of the processus ventralis. The processus ascendens posterior appears at the beginning of the metamorphosis. About this time the calcareous sacs also are to be seen.

These last-mentioned structures attain their definitive form at the end of the metamorphosis.

At the time of its first appearance, the saccus endolymphaticus is an undivided sac lined by a single layer of epithelium. It soon becomes partitioned into two tubuli which later subdivide into smaller ones. During the course of development, the saccus is divided more and more into small tubuli until it finally has the appearance of a glandular structure.

The histological structure of the cells lining the saccus endolymphaticus remains practically the same during the whole period of development. The cells are first cylindrical in shape, later they are cuboidal. Blood vessels are present at first only in small numbers, but later become very numerous. The calcareous contents of the saccus exist almost from the very beginning.

Throughout the entire course of development, no part of the saccus endolymphaticus suffers retrogression, its development is a continuous and direct one. Its development also proceeds very slowly. For these reasons, it is certain that the saccus endolymphaticus of the frog does not represent a larval organ.

REMARKS ON THE STRUCTURE OF THE DUCTUS AND SACCUS ENDOLYMPHATICUS IN THE VERTEBRATA

Since the investigations of Hasse ('73) and Retzius ('81), no comparative-anatomical study of the ductus and saccus endolymphaticus has been made, although some very interesting papers on this organ in individual Vertebrata have been published. Unfortunately, not all vertebrate types have been examined in regard to this particular structure. It is, however, possible to draw some conclusions from the facts hitherto ascertained. Therefore, I shall give a short description of the anatomical structure of these organs, based on the above-mentioned works and the more recent reports.

Hasse writes:

> Saemtliche Wirbeltiere besitzen eine aus dem Vestibulum sich erhebende Roehre, die, mit Ausnahme der Plagiostoma, wo dieselbe auf die Schaedeloberflaeche fuehrt, bei allen Tieren in die Schaedelhoehle

sich begibt. Es ist dies der Ductus endolymphaticus oder Aquaeductus vestibuli mit dem Saccus endolymphaticus von dem wir wissen, dass es eine blind geschlossene Ausstuelpung des Labyrinthblaeschens gegen das Cavum cranii hin darstellt.

Hasse found this structure in Myxine and Petromyzon. In these animals the organ starts from the inner ear, or saccus communis, goes through the apertura aquaeductus vestibuli, and ends in the cavum cranii with a slight enlargement. The dilated end is filled with calcareous matter.

The Elasmobranchii have been investigated by Hasse and Retzius. In these animals the ductus endolymphaticus is a very noticeable structure opening to the exterior. In Chimaera it runs from the sacculus almost straight to the top of the head. In the sharks it expands just beneath the opening, into a saccus, either a small one as in Scyllium or a somewhat larger one as in Acanthias and Aquatina. This last-named animal is, in this respect, a transition form leading to Raja, in which the ductus dilates and forms a large sac lying almost horizontally under the skin. Trygon and Torpedo have a formation similar to that in the sharks. In all cases the ductus as well as the saccus is filled with lime crystals which run out when the skin is pressed in the neighborhood of the opening. This is also the case in living animals.

Retzius examined five Ganoidei, namely, Acipenser, Lepidosteus, Amia, Polypterus, and Calamoichthys. In Acipenser the ductus endolymphaticus runs upward from the sacculus and expands beside the upper end of the utriculus into an oblong vesicle which adheres to the dura mater. In Lepidosteus and Amia the relations are similar. Polypterus and Calamoichthys, which are particularly interesting on account of their relationship to the Crossopterygii, do not deviate either.

The question whether a small duct, which in the Teleostei starts from the sacculus and leads upward a short distance only to end blindly without an enlargement, is to be looked upon as the ductus endolymphaticus has been much discussed. Krause ('01) denies this and founds his opinion on the manner of development. Wiedersheim ('09) agrees with him. On the other hand,

Fleissig ('08), Wenig ('11), and Keibel ('15) think it probable that this duct is rightly called the ductus endolymphaticus. This structure is not found in the Siluridae, Cobidae, Cyprinidae, Percidae, and Clupeidae.

The anatomical structure of the recessus labyrinthi in the Dipnoi is very interesting. Retzius was unable to find this organ, as he examined badly preserved specimens. It is now known that the ductus and saccus endolymphaticus of Neoceratodus resemble the same structures in the Ganoidei. Burne ('13) writes as follows:

> The saccus endolymphaticus is a capacious pear-shaped vesicle with bluntly rounded apex, situated to the mesial side of the space enclosed by the anterior semicircular canal, with its apex inclined somewhat forward. It is supported by a sheet of membrane (? dura mater) within which wedged in between the apices of the two sacci endolymphatici is a large vessel, probably a vein. The lower end of the saccus endolymphaticus bends slightly forward along the sinus anterior utriculi and gradually narrows to form the ductus endolymphaticus which crosses the utricle near its anterior end and opens by a funnel shaped mouth into the anterior extremity of the sacculus. These organs show a great resemblance to the same organs figured by Retzius in the sturgeon.

Protopterus, which was examined by Burckhardt ('93), differs greatly from the above-described animal. The saccus endolymphaticus is here an inflated bag lying in the cavum cranii and giving rise to many tube-like diverticulæ which are filled with calcareous matter. The organ covers nearly the whole of the sinus rhomboidalis and extends caudally as far as the root of the first pair of spinal nerves. The saccus of the one side does not communicate with that of the other.

The first description of the conditions in the Urodela was published by Calori ('50). He found between the bulbae auditoriae of the axolotl a sac containing calcareous matter, extending from the lobi optici over the corpora quadrigemina and the medulla oblongata. He connected this structure with the labyrinth, but did not explain the relations in detail. Hasse proved that this organ represents the two sacci endolymphatici, which are here amalgamated. In Triton, Hasse found condi-

tions similar to those in the axolotl, the sacci, however, being separate. In salamander, he found that the sacci extend also below the brain.

In Rana the relations are especially complicated, and accordingly were explained much later. Here the ductus leads from the sacculus into the cranial cavity, where it expands into a large saccus. In contrast to the condition in the Urodela, this organ is not confined to the cranial cavity, but extends in the vertebral canal as far as the seventh vertebra, and sends out processes which come to lie on the spinal ganglia. I have given a detailed account of the whole organ in the first part of this paper.

The different parts of the saccus endolymphaticus in Rana were discovered separately. The calcareous sacs on the spinal ganglia were necessarily the first to attract attention on account of their conspicuous position. They were found by Blasius in 1681. Hasse ('73) discovered a chalky mass in the cavum cranii, which he identified as the sacci endolymphatici. Finally Coggi ('90) proved that the sacs on the spinal ganglia are outgrowths from the main stem of the saccus.

In most of the Anura, examined in regard to this organ, the relations are similar. Sterzi ('99) described the saccus in Rana temporaria, Rana esculenta, Bufo vulgaris, Bufo viridis, and Hyla arborea. In both species of Rana he found the above-described expansion of the organ. In Bufo and Hyla there is only a slight deviation, in so far as the processes of the spinal part merely penetrate into the intervertebral foramina, but do not extend beyond these apertures. Coggi ('90) states that Discoglossus pictus and Bombinator igneus do not possess a spinal part of the saccus endolymphaticus. He does not describe the cranial part. Rex ('93) also examined Bombinator igneus. In this animal he found a thin vascular membrane lying on the roof of the fourth ventricle. This structure he takes for the degenerated saccus. In Pelobates, this author found the same expansion of the saccus as is known in Bufo.

In the Reptilia the ductus endolymphaticus is found in its typical development. The saccus, however, is very small, and contains lime in the embryo only. Carus ('45), who was the

first to examine the saccus endolymphaticus in this class, writes that in Coluber natrix the saccus is represented by a small vesicle lying directly under the suture between the parietale and occipitale. The sacci of the two sides lie close to one another, but do not join. Hasse investigated Anguis fragilis, Lacerta viridis, Chelonia midas, Testudo graeca, and Crocodilus. In all of these animals he found the expansion of the saccus similar to that in Coluber.

The only reptiles whose saccus endolymphaticus does not conform to the above-given description are the Ascalabotae. According to Wiedersheim's ('76) investigations on Phyllodactylus, the saccus does not only run upwards to the roof of the brain, but also extends backwards. It leaves the cranial cavity through a small aperture in the parietale, passes back between the muscles of the neck, and ends in the region of the pectoral girdle with a large closed sac. All the parts of the saccus are filled with calcareous matter, in the adult as well as in the embryo. In Phyllodactylus the two sacci do not join; in Ascalabotes, however, they coalesce a little behind the posterior part of the parietal suture. They separate again when leaving the cranial cavity. Many species of Platydactylus have a similar extension of the saccus. Gray ('08) did not find this formation in Tarentola mauretanica. He thinks that perhaps it was destroyed by the manner of preparation.

Hasse writes that the structure of the recessus labyrinthi in Aves is very similar to that in the Reptilia. He was not able to state whether lime crystals are present at any period of development. According to Wiedersheim ('06), the saccus is filled with lime in the embryo stage.

The Mammalia show a slight deviation in the anatomical structure of the ductus endolymphaticus. In these animals the duct arises from the labyrinth with two branches, one from the lower median side of the utriculus, the other from the upper median part of the sacculus. The two branches soon join and the ductus leads upward at the median side of the utriculus, as far as the foramen endolymphaticum. Here it runs into the cranial cavity and ends inside the dura mater with a small dila-

tation. Here, too, according to Wiedersheim, the saccus contains lime in the embryonic stage.

If we compare the anatomical structure and the relative size of the saccus endolymphaticus in the various classes of Vertebrata, we see the following interesting course of development. The saccus is present in all Vertebrata, at first as a slight dilatation at the end of the ductus, then as a large vesicle, and finally in the Anura and Ascalabotae as an enormous sac lying in the cavum cranii and the vertebral canal (respectively, the shoulder-region). From this maximum of development, the size of the saccus begins to decrease and at last returns in the Mammalia to a hardly perceptible enlargement at the end of the ductus.

The above-given summary is incomplete because the number of animals investigated in regard to the saccus endolymphaticus is small. Furthermore, the groups that have been compared do not represent a natural phylogenetic line of evolution. There are, however, some interesting facts which may perhaps be connected with some systematic problems.

Let us first examine the group of the Dipnoi. Here we find that Neoceratodus possesses a ductus and saccus endolymphaticus which are very similar to the corresponding structures in Polypterus. Thus we can add a new fact to the many points of similarity mentioned by Huxley ('76) and Dollo ('95) between the Dipnoi and the Crossopterygii. This is particularly interesting because many investigators, as Huxley, Parker ('92), Dean ('92), etc., who took their facts from Retzius, described the auditory organ of the Dipnoi as similar to that of the Selachii.

If, on the other hand, we wish to consider the relationship between the Dipnoi and the Amphibia, we find that the recessus labyrinthi of Protopterus is very similar in structure to that of Rana.

The large saccus in Protopterus has probably developed from the more simply constructed one of Neoceratodus through specialization, due, one might assume, to adaptation to terrestrial life. Protopterus is generally considered to be a much more specialized form than Neoceratodus. Thus Parker ('92) pointed out that in Protopterus the reduction of the gills has proceeded

much further than in Neoceratodus. We also find in Protopterus two pulmonary sacs, whereas Neoceratodus possesses only one. Parker writes of Protopterus:

"Although the lungs are usually said to be paired in their origin, the outgrowth which forms them, though bifurcating close to its origin, is in fact so far as I can gather unpaired at the first."

This fact might also support the view that Neoceratodus is the more primitive animal. The uniserial fin of Protopterus has been regarded as a form developed from the biserial fin of Neoceratodus. Thus it seems as if the relationship between Neoceratodus and Protopterus indicated by the structure of the recessus labyrinthi is in reality a true one.

As may be expected from their systematic relationship, all the higher vertebrates, reptiles, birds, and mammals, have a similarly constructed saccus endolymphaticus. The exceptional position of the Ascalabotae appears therefore very strange. In these animals, as we have seen, the saccus leaves the cranial cavity and runs between the muscles of the neck into the shoulder region, where it ends as a large closed vesicle. As there can be no thought of a close relationship between Ascalabotae and Anura, this similarity (at least as far as size is concerned) of the extension of the saccus is probably due to convergence. There is no question here of a convergence caused by a similar habitus, as the Ascalabotae are entirely terrestrial animals, many of them even living in deserts. Such a convergence could only be explained as having arisen, in spite of a different mode of life, through the fact that the stimulus which causes the great extension of the saccus is the same from a mechanical point of view. This question can only be answered by a knowledge of the function of the organ. As far as I know, no experiments to ascertain the function of this structure have been made.

Several theories as to its probable function have been advanced. Hasse ('73) thought this organ served to regulate the pressure in the labyrinth by sucking up the endolymph out of the sacculus, when the pressure there was too high. Wiedersheim ('76), Keibel ('15) and Streeter ('16) have adopted this theory. Hasse

added that in the case of animals possessing a very large saccus this organ might transmit the sound waves from the skull into the ear. Wiedersheim thought this also highly probable. Sterzi maintained that the saccus in Rana had the same function as the other spinal meninges, namely, that of protecting the spinal cord. Carus ('41), who observed the calcareous matter in the saccus of snake embryos and the subsequent disappearance of the same, concluded that the lime was used for the growth of bone and was thus absorbed. Gaupp ('96) has accepted this theory in regard to Rana. In this case, the growth of bone took place in the adult frog as well as in the larva. Therefore, the saccus secreted lime throughout the life of the frog.

Scott and Euclid Avenues,
St. Louis, Mo.

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Resumen por el autor, Herbert G. Willson.

Las terminaciones del bronquiolo humano.

El autor ha hecho dos reconstrucciones negativas en cera, con un aumento de 100 diámetros, una de un pulmón de un adulto y la otra del pulmón de un niño. Ambos modelos representan un bronquiolo respiratorio y sus ramas, habiéndose trazado algunas de estas hasta su terminación. Algunas de las conclusiones más importantes son las siguientes: 1) En las ramificaciones existe mayor complejidad, irregularidad y mayor entrelazamiento que lo que se ha supuesto generalmente. 2) La ramificación es dicotómica hasta que llega a las terminaciones, transformándose después en irregular, si bien con tendencia a la economía de espacio. 3) No existe espacio esférico o atrio, conforme ha descrito Miller. 4) No existen comunicaciones interalveolares directas. 5) El pulmón del niño es tan complicado en estructura como el del adulto. 6) En el pulmón adulto, durante la inspiración profunda oridinaria e área total de epitelio respiratorio y no respiratoro no es mayor de 70 metros.

Translation by José F. Nonidez
Cornell Medical College, New York

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THE TERMINALS OF THE HUMAN BRONCHIOLE

HERBERT G. WILLSON

University of Toronto

NINE FIGURES

In the hope of throwing some light on certain questions about which there has been controversy, the construction of a wax model of a respiratory bronchiole was begun at the University of Toronto in October, 1919. The work was carried out in collaboration with Prof. J. Playfair McMurrich, by whom the problem had been suggested and to whom the writer is very greatly indebted for advice and assistance.

The extreme complexity of the terminal branches of the bronchial tree is not generally appreciated. The maze of channels which occur even in a minute piece of lung tissue cannot be visualized accurately from a mere comparison of serial sections. The larger passages of the lung may be injected with wax or metal and a cast obtained by corroding away the lung tissue, but one cannot be certain of obtaining in this way a complete cast of the smaller tubes. The method of wax reconstruction of serial sections is the only plan by which one may hope to get clear ideas regarding the finer tubes, and even this method is especially difficult to apply to the bronchioles. So complicated are the branchings and so carefully has nature economized space in the lung, that if all the air passages in a piece of lung tissue are reconstructed in wax on a magnified scale, the result is practically a solid, and the model has to be dissected in order to show the relationships of tubes and air-cells.

Malpighi in 1661 demonstrated the vesicular nature of lung tissue and showed how the trachea terminates in bronchial filaments, but after his time there was no important contribution to the knowledge of the histology of the lung until the early part

of the nineteenth century, when Soemmering, Rossignol, Reisseisen, and others published the results of their researches, and Henle, by his discoveries in general histology, laid the foundation for many special investigations. Controversy now arose in regard to such questions as the exact shape of the terminal bronchioles, their method of branching, and as to whether or not there were direct communications between alveoli.

Rossignol, writing in 1847, refers to the most distal divisions of the bronchial tree as 'infundibula,' and these he describes as being thickly beset with alveoli. He notes that the alveoli of the infundibulum are of an unusually great depth, and that while the alveoli are scattered and few in the proximal part of the respiratory bronchiole, they are soon arranged close together, covering the whole surface of the last bronchial divisions. As to the method of branching, he concludes that there are both dichotomous and trichotomous divisions. In his investigations Rossignol inflated and dried the lung, after having injected the blood vessels.

In 1860 Waters described monopodial, dichotomous and trichotomous branching. His conclusions were based on the study of single sections. In man he found no alveoli in the terminal bronchiole, but only in the infundibulum. He states that at a certain place the terminal bronchiole widens into a cavity into which open six, eight, or ten canals, beset with alveoli. These canals he terms air-sacs, these being again identical with Rossignol's infundibula.

F. E. Schulze in 1871 used the term 'Alveolengang' to denote all the parts of the tubular system on which there are alveoli, excepting, however, the terminal sacs, for which he employed the term infundibula.

In 1892 W. S. Miller announced the discovery of a new element in the series of pulmonary air-spaces, terming it the 'atrium' and locating it between the air-sacs (infundibula) and the terminal bronchiole (alveolengang). This space seems to be identical with the enlargement of the terminal bronchiole described by Waters as giving origin to the air-sacs, but Miller describes it as something more than a mere enlargement, having a more

or less spherical form with numerous alveoli on its walls and giving origin to from two to five air-sacs. The lung of the dog was used in Miller's first investigation; but in 1900 and again in 1913 he published accounts of further researches, and maintained that his description held good for lung of cat, ox, child, and adult man.

In his article of 1900 he gives the following table of nomenclature for the air-spaces of the lung:

W. S. MILLER	B. N. A.	SCHÄFER	SCHULZE	KÖLLIKER
Bronchus	Bronchiolus respiratorius	Bronchial tube	Alveolengang	Alveolengang
Terminal bronchiole	Ductuli alveolares	Lobular bronchus		
Atrium				
Air-sac		Air-sac	Infundibulum	Infundibulum
Air-cell	Alveolus pulmonis	Air-cell	Alveolus	Alveolus

But at the same time he revised his own earlier terminology, substituting the B. N. A. terms for 'Bronchus' and 'Terminal bronchiole.' In his investigations Miller used the method of wax reconstruction. His results have found wide acceptance by the authors of text-books, in spite of several dissenting voices.

In 1900 Justesen gave an account of his investigations of the structure of the lung in oxen. He used corrosion preparations and also serial sections, drawings of which were made on transparent paper, so that by superposing the drawings, successive sections might be compared. He finds that each 'bronchiolus simplex' forms dichotomously two respiratory bronchioles, each of which again divides dichotomously, each of the branches so formed ending in a large cavity which he identifies with the atrium of Miller. His atria are variable in size, sometimes quite distinct, and sometimes only slight enlargements of the bronchioles, and while Miller finds two to five air-sacs on each atrium, and Waters six to ten, Justesen believes that there are normally four. Two first bud out and these then divide, so that each of the four occupies a position corresponding to one of the angles

at the base of a four-sided pyramid, the apical angle of which is occupied by the atrium. In other words, the air-sacs do not arise as accidental growths from the atria, but are formed by two successive dichotomies in planes at right angles. In the adult ox he found occasionally but three air-sacs on an atrium—a condition which he explains by supposing that in the case of the primary air-sacs the secondary dichotomy had failed owing to space exigency.

Justesen holds that the bronchial branchings occur in a definite mathematical plan and are fundamentally dichotomies, but that in the majority of the branches the dichotomy becomes modified into a sympodial arrangement, the terminal branches still retaining the dichotomous plan. If this be so, and the mathematical regularity of the dichotomies persist, the lateral branches of each sympodial stem might be expected to show a decreasing number of air-sacs as they were traced peripherally, one arising from an earlier dichotomy having twice as many air-sacs as that which arose from the succeeding dichotomy. Justesen believed that he obtained evidence in favor of this arrangement in his observation on the pig where the eparterial bronchus gave rise to as many lateral branches as did the stem branches for the rest of the lung.

F. E. Schulze, writing in 1906, takes the view that Miller's atria are not new spaces, but only those parts of the ductuli alveolares into which the sacculi open. He states:

> So wenig, wie man an einem sich unregelmässig verzweigenden Baumast diejenigen Stellen, wo sich ein Ast in zwei oder auch mehrere Endäste teilt, als besondere typische Stellen charakterisieren und mit einem eigenen Namen, sondern einfach als Teilungsstellen zu bezeichnen pflegt, so wenig scheint mir in dem respiratorischen Gangsystem der Lunge die Auszeichnung dieser Stellen durch eine besondere Benennung ('Atrium') erforderlich oder auch nur zweckmässig zu sein.

Schulze claims that normally in man and in many mammals there are direct communications between alveoli—'alveolar pores'—and in this view he is supported by Hansemann, Hassall, Zimmerman, Nicolas, and Merkel, but is opposed by Piersol, W. S. Miller, Laguesse, and Oppel.

In 1907 J. Müller investigated the lungs of most of the domestic animals, using metal corrosions as well as sections. His conclusions regarding the occurrence of atria he states as follows:

> Hinsichtlich des neuen Luftraumes, des Atriums, war es mir nun weder an den Korrosionspräparaten noch an den Schnitten bei irgend einem unserer Haussäugetiere möglich, ihn als einen Luftraum sui generis bestätigen. Wenn auch da und dort einmal ein Alveolengang vor seiner Auflösung in die Infundibula eine buchtige Erweiterung zeigte, welche etwa dem 'Atrium' Justesens entsprechen könnte, so habe ich doch niemals zwischen jedem Infundibulum und dem Alveolargang, noch auch zwischen mehreren Infundibeln und einem solchen einen oder mehrere kugelige Hohlräume eingeschaltet gesehen, welche für das konstante Vorkommen der Millerschen Atrien sprechen könnten.

Müller found alveolar pores in various animals, but not in young animals. He thinks these are pathological.

Just as my first model was completed, I received the number of The American Journal of Anatomy that contained two articles by the Japanese investigator Ogawa, who by an interesting coincidence had been working in the University of Kyoto at exactly the same problem as myself and by similar methods, but had evidently begun the construction of his model some months before I started with mine. Ogawa worked with human material, and constructed both a negative and a positive model of the terminal branchings of the lung, the former being at a magnification of 100 diameters and measuring 8 x 12 x 8 cm., while the latter was enlarged 80 diameters and measured 11.3 x 24 x 20 cm. Like Schulze and Müller, he reaches the conclusion that "Miller's atrium is an unnecessary term, at least for the human lung." In his second paper he states that "alveolar pores are normally found in many mammals, and only seldom cannot be seen." Further reference to Ogawa's work will be made when his results are compared with my own.

MATERIAL

The material used was exclusively human and consisted of portions of the lungs of two individuals obtained at autopsies performed soon after death. One of the individuals was a woman of thirty years who had died of heart disease (mitral stenosis),

while the other was a child whose age could not be definitely ascertained, but was certainly less than thirteen years. In the case of the adult, portions of suitable size were taken from the lungs and immersed in Bouin's fluid, but in the case of the child's lung the entire organ was first injected through the bronchus under gentle pressure with Bouin's fluid, and then immersed in the same fluid, portions suitable for sectioning being taken only after the tissue had been fixed in this manner. The portions selected were carried through the various grades of alcohol and imbedded in paraffin, and to secure satisfactory penetration of the paraffin they were, while in 70 per cent alcohol, placed under the bell-jar of an air-pump and the air exhausted till bubbles ceased to rise from the cut surface of the tissue.

By this method a perfect infiltration of the paraffin was obtained, and the tissue was cut into serial sections, 20 μ thick in the case of the adult lung and 30 μ in that of the child. Both series were stained with Weigert's elastic tissue stain, this being chosen with the intention of studying later the distribution of the elastic fibers in the human lung. Wax reconstructions of the air spaces, i.e., negative reconstructions of portions of each lung were made at a magnification of 100. To ensure accuracy in the superposition of the wax plates in the model of the adult, numerous bridges were left in cutting out the air-spaces, but in the second model the necessary accuracy was obtained by the use of a duplicate series of drawings of the sections made upon transparent paper. A duplicate drawing of a section about the middle of the series was covered by a sheet of glass, and on this the pieces of wax representing the corresponding air-spaces were placed one after the other, as they were cut from the wax plate. The next succeeding drawing was then carefully oriented upon that first chosen, so that the position of the air-spaces shown in the one could be accurately determined with reference to those of the other, and from the information thus obtained the pieces of wax representing the air-spaces of the second section could be accurately adjusted on those cut from the first plate. Dealing in this way with successive drawings and wax plates, half the model was built up. This completed portion was then detached

from the sheet of glass, turned upside down, and the other portion of the model was then built up in the same way. This method of orientation was found to be much more economical of time than was the use of bridges and entailed no sacrifice of accuracy.

To follow a respiratory bronchiole from its beginning to its terminals, it was necessary to make drawings from 128 sections of the adult lung. As each section was 20 μ thick, it is evident that the piece of lung containing all these branches had a thickness of 2560 μ, i.e., 2.56 mm. or a little over 1/10 of an inch. It will be evident also that the height of the completed model would be 256 mm., or a little over 10 inches. In the case of the child's lung, drawings of sixty-three sections were required, and as each section was 30 μ thick, the thickness of the piece of lung reconstructed was 1890 μ, or 1.89 mm., and the height of the completed model approximately 7.5 inches.

RESULTS

The first impression received from inspection of the completed models is that the branchings of a respiratory bronchiole are far more complicated than is revealed in the text-books, and one feels also that there is difficulty in 'labeling' the various parts according to the terms commonly used. In some places a number of alveoli are represented in the reconstruction as opening into a cavity which seems too small to deserve the name of an air-sac, while in another place one finds an alveolus which is several times as large as the ordinary alveolus. The models indicate that the minute passages in the lung are not formed in strict accordance with the usual descriptions. The two models when placed side by side suggest at once that the child's lung is a miniature of the adult lung, just as the child's hand is a miniature of the adult hand, there being no apparent difference in complexity of structure. More air-sacs occur in the volume of child's lung represented than in the greater volume of adult lung represented in the first model.

A photograph of the model from the adult lung is shown in figure 1. It starts with a non-respiratory bronchiole which is

marked *3a*, and is so designated because in tracing it back through the serial sections it was found to represent the third dichotomy from a bronchus which contained cartilage in its wall. The *3a* dichotomizes into branchings marked *4a* and *4b*, of which *4b* has not been followed any further, but *4a* again divides dichotomously into *5a* and *5b*, whose walls show alveolar outbranchings, so that they are to be regarded as respiratory bronchioles. The *5b* is followed only a short distance, but *5a* again divides into two stems, one of which was followed for some distance, but its reconstruction is omitted in the photograph for the sake of simplicity. The other stem, which may be designated *6a*, is completely reconstructed, and gives rise to all that portion of the model which is colored. It can be followed into a further dichotomy, one branch of which gives rise to the portions colored orange and green, while from the other all the remaining portions originate. The orange and green portions have been separated from the rest of the model in order that its parts might be more completely shown.

A photograph of the model on this scale, though useful for a general orientation of its parts, does not sufficiently reveal the details. These are more clearly shown in figure 2, which represents a part of the portion colored orange in figure 1 at a greater magnification. It shows a number of infundibula or air-sacs with their alveolar outbranchings, and it shows also how difficult it is to determine exactly what shall be termed an air-sac and what an air-cell. Thus the lower of the two portions colored yellow might equally well be regarded as a single air-sac with a number of complicated air-cells, or as at least two air-sacs with a common basal portion. Similarly, the upper yellow portion might be regarded as a single large air-sac or as three, according to the point of view of the observer. The terms infundibulum or air-sac (ductulus alveolaris B. N. A.) and alveolus or air-cell are all useful in conveying an idea as to the arrangement of the terminal air-spaces of the lung, but it must be remembered that in the human lung, at least, transitions exist between them; particular cases may be found where it is difficult to say whether one is dealing with an air-sac or an air-cell.

To obtain a clearer picture of the terminal branchings, those represented in both models were projected upon a single plane, the projections being based partly on tracings of various sections used in the construction of the models, and partly on sketches of the smaller parts. The result obtained in the case of the model of the child's lung is shown in figure 3. The stem marked *1* is a non-respiratory bronchiole which was traced through seventy-four sections (2.22 mm.) to reach its origin from a bronchiole with cartilage in its wall. Four dichotomies occurred in this distance. In the model of the adult lung three dichotomies occurred between the first respiratory bronchioles and the bronchiole with cartilage in its wall. The stem (*1*) divides into two branches, only one of which (*2*) was followed; this was a respiratory bronchiole, alveoli occurring on its wall. It in turn undergoes a dichotomy, only one limb of which (*3*) was followed, and then two additional dichotomies succeed in rapid succession, only one branch of each being followed. That followed from the last of these dichotomies (*6*) again divides into *7* and *8*, these again into *9* and *10* and *11* and *12*, respectively, but beyond this the branchings become irregular, and while it would be possible to interpret some of these divisions as dichotomies, there are others where the branching could be more accurately termed a trichotomy. In fact, the branching in some parts is so irregular that almost any 'method' might be read into it. The truth seems to be that, as the terminals are approached, no one system of branching is followed, but one edict is obeyed, i.e., that there must be no waste of space.

Embryological investigation has shown that in the early development the branching is dichotomous, and apparently this is continued, with some modification in certain of the branches, until there comes a time when the small bronchioles are competing with one another for space, and then they branch or send out processes in any possible direction. This competition for space of the infundibula and alveoli, seen in the complicated interdigitation of these elements from different respiratory bronchioles and in their varying form and size, is the most striking impression that one receives from a study of the models. An idea of the

manner in which the infundibula fit in with one another may be obtained from figure 4, which is a photograph of the pleural surface of the model of child's lung. The numbers on the infundibula correspond with those on the branchings shown in figure 3.

From the diagram and the accompanying photographs it will be evident that the models reveal no definite space which corresponds to Miller's atrium. Careful study of the models shows, it is true, enlargements of the respiratory bronchioles where several infundibula communicate with them, but these enlargements exhibit no definite delimitation from the remaining portions of the bronchioles, and they never assume a spherical form. Schulze was probably correct in his contention that a special name is not needed for that part of a branch from which a number of subordinate branches arise.

It is interesting to note that Justesen's description of the branchings of a respiratory bronchiole applies very closely to the branchings revealed by the models, except in regard to the atrium. Both Waters and Justesen held very decided views as to the planes in which successive branchings occur. Waters believed the plane of two diverging branches to be always at right angles to the plane of the two branches preceding. Justesen claims that "there is a strong tendency of the dichotomous divisions to lie in alternating planes cutting one another at right angles," but this was not universal. Examination of the models indicates that Waters' rule is by no means constantly true. Four angles which, according to Waters' rule, would be 90°, were found to be approximately 85°, 90°, 10°, and 45°.

The number of branchings that intervene between a non-respiratory bronchiole and an air-sac was determined in seven cases, and in three the air-sacs were reached at the fifth division, in three at the sixth, and in one case at the seventh. Ogawa found from two to nine ramifications, with an average from fourteen cases of 5.57. Laguesse found six or seven branchings.

The alveoli or air-cells on seven different air-sacs were counted, the numbers being as follows: 22, 14, 16, 18, 12, 16, and 20, giving an average of 16.8. Ogawa's average is 11. However, many of the air-sacs, as Justesen says, are bifurcated or deeply

indented, and a good deal depends on whether the subdivisions are considered as separate air-sacs or not.

Calculating from the lengths of the tubes in the model the following are the actual lengths of these tubes in the adult lung.

	mm.
No. 1	1.6
No. 2a	0.8
No. 3a	0.5
No. 4a	0.5
No. 5a	0.5
No. 6a	0.4
No. 6b	0.2

The greatest and least diameters of the non-respiratory bronchioles represented in the same model were estimated as follows:

	mm.
No. 3a	0.3 x 0.40
No. 4a	0.3 x 0.25
No. 4b	0.4 x 0.22

Similar estimates in the case of the respiratory bronchioles were:

	mm.
No. 5a	0.4 x 0.30
No. 5b	0.3 x 0.35
No. 6a	0.3 x 0.30
No. 6b	0.4 x 0.30

Measurements of the greatest and least diameters of those tubes into which the air-sacs open gave the following results in three cases,—the figures indicating the actual dimensions in the lung:

0.3 mm. x 0.2 mm.
0.5 mm. x 0.3 mm.
0.4 mm. x 0.3 mm.

This gives an average of 0.4 mm. $\times$ 0.27 mm.

Ogawa found the average diameter of an alveolar duct to be 0.24 mm., and he quotes Kölliker's estimate as 0.27 mm. and that of Schulze as from 0.4 to 0.2 mm.

It will be seen from these measurements and from the illustrations that the bronchial tree in its finer ramifications by no means shows a decrease in the diameter of successive branches

towards the periphery. There is, indeed, sometimes an increase even before the air-sacs are reached.

The air-sacs themselves show a great diversity of shape and size and frequently they are recurrent. Figure 2 shows plainly the tendency of the air-sacs to widen out to a greater diameter than the bronchiole from which they arise. It was, no doubt, this widening-out tendency which caused the early investigators to use the term 'infundibulum,' though the air-sacs are not funnel-shaped. Calculations of the actual size of eight air-sacs gave the following results, the measurements being taken in three dimensions:

0.4 x 0.8 x 0.4 mm.
0.3 x 0.6 x 0.3 mm.
0.7 x 0.4 x 0.3 mm.
0.6 x 0.3 x 0.4 mm.
1.0 x 0.4 x 0.5 mm.
0.3 x 0.4 x 0.3 mm.
0.5 x 0.3 x 0.2 mm.
0.4 x 0.6 x 0.2 mm.

The air-sacs or alveoli, as shown in the model, vary greatly in size and shape. The following estimates were made of the actual diameters in three directions of the alveoli of the lung: (All the measurements given above have reference to the adult lung.)

0.05 x 0.06 x 0.07 mm.
0.08 x 0.08 x 0.12 mm.
0.08 x 0.10 x 0.13 mm.
0.06 x 0.08 x 0.10 mm.
0.12 x 0.15 x 0.20 mm.
0.08 x 0.10 x 0.13 mm.
0.08 x 0.05 x 0.15 mm.
0.08 x 0.10 x 0.10 mm.
Average: 0.075 x 0.09 x 0.125 mm.
Extremes: 0.05 and 0.20 mm.

Ogawa in the case of a man of thirty-one years found an average of 0.1 mm. for depth and breadth of an alveolus, and in the case of a man of fifty-six years, his estimates are 0.15 mm. depth and 0.19 mm. breadth. Ogawa's extreme estimates, counting both his cases, are 0.04 and 0.21. It will be seen that many of the

alveoli represented in the model are elongated to a greater extent than is usually described, though, as has been mentioned, Rossignol noted that certain alveoli had unusual depth. The models confirm the observations of Rossignol regarding alveoli.

In the construction of the models and in the examination of the sections, careful search was made for evidence of interalveolar communications, but no evidence of their existence was found. The air-sacs interlock with wonderful closeness, so that there is absolutely no waste of space, and because of this close interlocking it sometimes requires great care to satisfy oneself of the absence of interalveolar communications, but in no case could such communications be demonstrated.

THE AREA OF PULMONARY AIR-SPACES

During the course of this work the interesting question of the total area of the respiratory air-spaces naturally suggested itself, and an attempt was made to answer it by estimating the total area of the respiratory epithelium in a cubic millimeter of lung tissue and then multiplying this by the total volume of the lung, expressed in cubic millimeters. It is evident that such a method can give only approximately the actual respiratory surface in the lung, since it takes no account of the variations that may occur in the size and number of the air-spaces in various cubic millimeters of the lung, and it fails to make allowance for the larger non-respiratory bronchioles and bronchi. Yet the calculation seemed worth carrying out, as it promised, at least, a maximum figure beyond which the total respiratory surface could not possibly extend.

In order to estimate the area represented in one cubic millimeter of lung tissue, a square of 100 mm. side was marked out on each of fifty successive drawings in the series of adult lung, the squares being oriented so that the series of squares represented successive sections of tissue. Since the sections were 20 μ thick, the fifty squares together represented sections totaling 1 cu. mm. in volume. The total perimeter of the various air-passages in each of these squares was measured. This was done by transferring the drawings within each square to millimeter paper, and

counting the number of millimeters in the perimeter of each air-space in that square, and totalling the amount. The grand total for the fifty squares amounted to 69346 mm. Since the magnification was 100 diameters, the corresponding perimeter in the actual lung would be $\frac{69346}{100}$ mm. and if this be multiplied by the thickness of the sections $\frac{20}{(1000)}$ the result will be nearly 14 sq. mm. of respiratory surface in 1 cu. mm. of lung tissue.

The lung tissue used in this estimation was obtained after the lungs had collapsed—the pleura having been opened. Vierordt estimates the volume of the lungs in this condition to be from 3005 to 3975 ccm. Taking the volume as the average of these, 3400 ccm. or 3400000 cmm., the area of the walls of the air-passages (respiratory and non-respiratory) is approximately $\frac{69346}{100} \times \frac{20}{1000} \times 3400000$ sq. mm., or about 47 square meters.

Now, the volume varies as the cube of like dimensions, while the area varies as the square of like dimensions, so that the area would not be doubled if the volume of the lung were doubled by expansion of air-passages. According to Arnold, the volume of the lung when fully inflated is 6805 ccm., and Vierordt states that the volume is 9521 ccm. 'bei stärkster Füllung.' For the areas corresponding to these estimates the extreme limits might fairly be placed at 70 and 90 square meters, respectively. Vierordt's estimate possibly refers to artificial inflation of the lungs after death. For the volume of the lungs on deep inspiration, Arnold's estimate seems a reasonable one, since 5500 ccm., in the case of the adult lung, is the approximate total volume of complemental, tidal, supplemental, and residual air. We are thus led to the conclusion that on ordinary deep inspiration the total area of the respiratory and non-respiratory epithelium is approximately 70 square meters, and the respiratory area alone must be considerably less than this. In order to estimate how much less, one would have to know the proportionate amount of respiratory to non-respiratory epithelium in the air-passages, and this is not known.

It might be pointed out that the method of multiplying the perimeter by the thickness of the section is exact only in the case of a tube of uniform diameter. To illustrate, it is plain that the cylinder formed by a pile of coppers has an area on its curved surface equal to the sum of the areas of the edges of the coppers, while the area of the curved surface of a cone is really greater than the total area of the edges of a number of discs of gradually diminishing diameter piled up to represent a cone. Here, then, is a source of error which tends towards making the result too low, while errors of omission in the counting or tracing would tend in the same direction. On the other hand, the cubic millimeter of lung tissue on which our calculation is based contained only the finer branchings of air-passages, so that the result would be accurate only if the whole lung were made up of such fine branchings. This source of error, tending to make a too high result, can hardly be canceled by the factors referred to above. The figures given probably represent the extreme upper limits of area corresponding to the respective degrees of expansion.

In Hermann's Handbuch der Physiologie there is given a calculation by Zuntz of the area of the respiratory surface. Zuntz assumes the average diameter of an alveolus to be 0.2 mm. when the lung is moderately inflated, and the total air-space of the lung to be 3400 to 3700 ccm. He considers that at least 3000 ccm. of this space is occupied by alveoli and infundibula. He calculates the volume of an alveolus as though it were a sphere, and arrives at the following result for volume and area of a single alveolus:

Volume, 0.00414 ccm.
Area, 0.125+ sq.mm.

Reducing the 3000 ccm. to cubic millimeters, he divides this volume by the volume of a single alveolus, and reaches the conclusion that the number of alveoli in the lung is 725 million. On the basis of this result and the estimated area of a single alveolus, he concludes that the area of the respiratory surface is 90 square meters, when the lung is inflated to a moderate extent.

Aeby used the same figures as Zuntz for volume and area of a single alveolus, assuming the average alveolus to be of spherical

form and to have a diameter of 0.2 mm. Since the volume of such a sphere is 0.004+ cmm., Aeby concludes that in a cubic millimeter of lung tissue there would be 250 such alveoli, each with an area of 0.125+ sq. mm., so that in a cubic millimeter of lung tissue the total area would be 250 × .125 or 31.25 sq. mm.

Nicolas gives estimates of the different areas of respiratory epithelium corresponding to the various degrees of expansion. He considers the maximum volume of air which the lungs will hold to be 4970 ccm. in the average man. His statements are based on calculations by Aeby:

> Le nombre total des alvéoles est immense. Huschke l'avait évalué à 1700 ou 1800 millions. Selon Aeby ce chiffre est beaucoup trop élevé. D'aprés ses calculs chaque millimétre cube de poumon comprendrait 250 alvéoles représentant une surface de 31.2 millimétres carrés. En estimant le volume du poumon à 1617 centimétres cubes chez l'homme et à 1290 chez la femme, on obtiendrait chez le premier une somme totale de 404 millions d'alvéoles et chez la seconde de 322 millions, (en chiffres ronds). Cette quantité correspondrait à une surface de 50 à 40 métres carrés pendant l'expiration forcèe, de 79 (homme) à 63 métres carrés (femme) pendant l'état moyen de repos, et enfin de 129 (homme) à 103 métres carrès (femme) lors d'une dilation compléte.

The result here given of 129 square meters is greatly in excess of my maximum result, in spite of the fact that in the calculations of Aeby and Nicolas the figure representing the volume of the lungs is smaller. The difference arises from the difference in the estimate of the number of square millimeters of area per cubic millimeter of lung tissue. Nicolas uses Aeby's estimate of 31.2 sq. mm., while my estimate is 14 sq. mm. To obtain a result of 31.2 sq. mm., according to my method of calculation, the air-spaces cut in an area of 1 sq.mm. would have to be much more numerous than those of any of my sections of adult lung.

F. E. Schulze also refers to Aeby's calculations. After explaining that Aeby assumes the average diameter of an alveolus to be 200 μ, he states that he cannot agree with Aeby's estimate of the number of alveoli and corresponding total respiratory surface. Schulze used his own estimate of the volume, 1500 ccm., for the lungs of the average man. To get the volume of the respiratory parenchyma he deducts 20 per cent, leaving 1200

ccm. He takes the volume of an alveolus to be 200^3 cubic microns. Reducing the 1200 ccm. to cubic microns, he divides the volume of a single alveolus into this total volume and arrives at the conclusion that there are 150 million alveoli in the lung, thus:

$$\frac{12 \times 10^{14}}{2^3 \times 10^6} = 150{,}000{,}000$$

He then calculates the area of an alveolus as 5×200^2 square microns, and estimates the total respiratory surface as $5 \times 200^2 \times 150{,}000{,}000$ square microns, or 30 square meters.

The estimates of Schulze and Zuntz are much higher than mine in proportion to the figures which they use for the volume of the lung.

In our calculation it was found that in the adult, a cubic millimeter of lung tissue represented the following area of lining of air passages:

$$\frac{69346}{100} \times \frac{20}{1000} = 13.869 \text{ sq. mm., or nearly 14 sq.mm.}$$

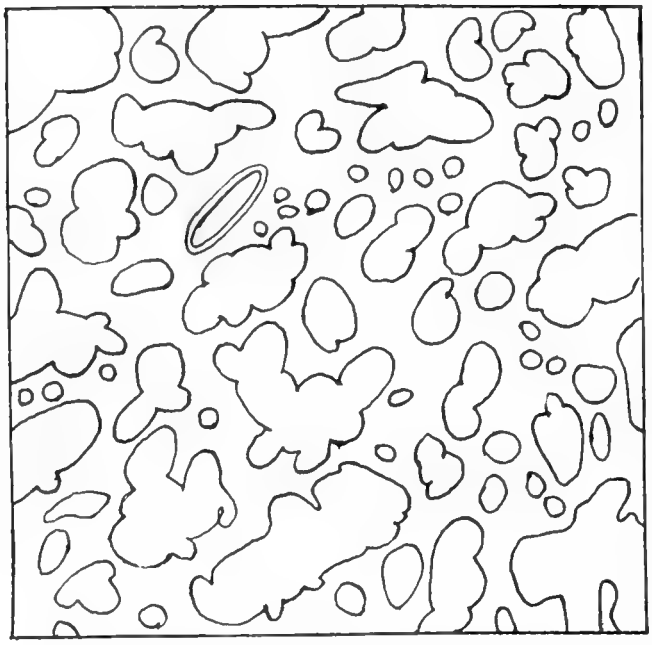
Similar calculations were made of the corresponding area in the child's lung, and estimates were made also from sections of emphysematous human lung and from the lung of an opossum. The results are given below.

ADULT NORMAL	CHILD	MAN OF 61, EMPHYSEMATOUS	OPOSSUM
14 sq.mm. (nearly)	19 sq.mm.	6 sq.mm. to 8.713 sq.mm.	27 sq.mm.

Some of the tracings on which these calculations are based are reproduced in figures 5 to 9.

In the child's lung only a few typical sections were counted, and only one reading was taken of the opossum lung.

In the emphysematous lung, the total perimeters of twenty-five consecutive sections were counted, the sections being of tissue near the pleura, though there were other parts, also near the pleura, where the emphysema was much more marked. The readings of the twenty-five squares gave a total of 21784 mm., an average of 871.3 mm., which corresponds in the actual



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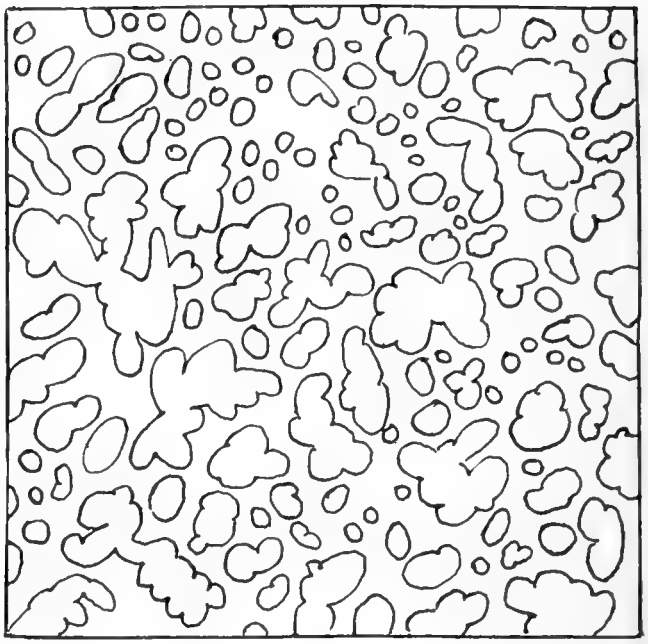

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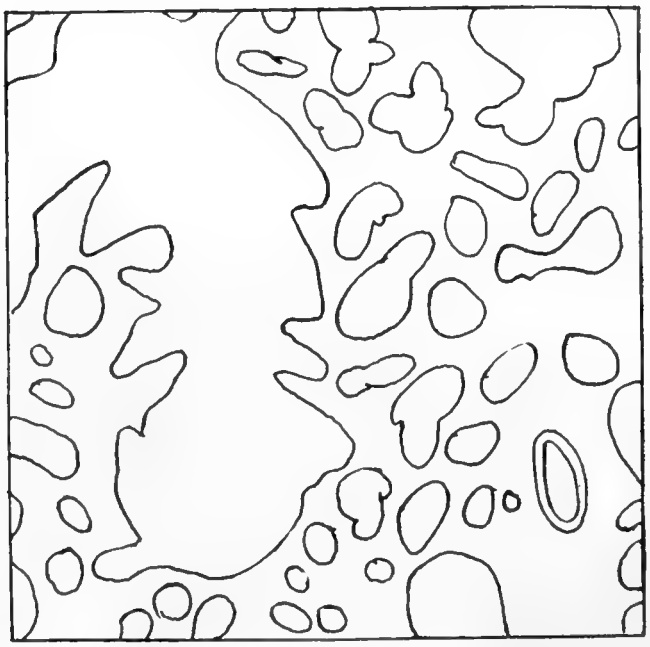

7

Figs. 5 to 7 Each figure represents a square millimeter of a section of lung tissue, traced with a projection apparatus. The figures were traced at the same magnification (× 100) and reduced in reproduction to × 60. The double lines represent blood vessels. Fig. 5, section of a child's lung. Fig. 6, section of an opossum lung. Fig. 7, section of an adult human lung.

8

9

Figs. 8 and 9 Each figure represents a square millimeter of a section of emphysematous (human) lung tissue, traced with projection apparatus. Both figures were traced at a magnification of 100 and reduced in reproduction to × 60.

lung tissue to an average perimeter of 8.713 mm. Using a more direct method of calculation than previously, this average perimeter of 8.713 mm. multiplied by 1 (millimeter) gives 8.713 sq. mm., the approximate area of lining epithelium in each cubic millimeter of emphysematous lung. The readings of eight other sections of emphysematous lung, taken from near the pleura, gave a total of 4728 mm., or an average of 591 mm., corresponding in the actual lung to 5.91 mm., or nearly 6 mm. This average perimeter in 1 sq.mm. corresponds to an area of 6 sq.mm. per cubic millimeter of lung tissue.

It will be seen that the average for all the readings of the emphysematous lung indicates that a man with emphysema might possibly have only half the normal amount of respiratory epithelium per unit of lung volume.

CONCLUSIONS REGARDING THE HUMAN LUNG

1. In the branching of the respiratory bronchioles there is far greater complexity, irregularity, and a greater degree of interlocking than is usually described.

2. There is no spherical space, or 'atrium,' such as has been described by Miller.

3. The method of branching of the bronchioles is dichotomous until the terminals are approached, and then the branching becomes irregular.

4. Counting as the first branch, a respiratory bronchiole arising from a non-respiratory one, the air-sac is usually reached at the fifth to seventh branch.

5. There are normally no direct communications between adjacent alveoli.

6. The bronchioles do not decrease in diameter as the periphery is approached, but remain of fairly uniform size until the air-sacs are reached, and the air-sacs are, as a rule, of greater diameter than the tubes from which they arise.

7. Waters' rule, that the planes of successive dichotomies cut one another at right angles, is only exceptionally confirmed.

8. The lung of the child is just as complex in structure as that of the adult.

9. It is calculated that during ordinary deep inspiration the total area of respiratory and non-respiratory epithelium in the adult lung is not greater than 70 square meters.

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PLATE 1

EXPLANATION OF FIGURE

1 Reconstruction from an adult lung. This model was made at a magnification of 100 diameters. The distance in the model from the top of the part colored orange to the bottom of the part colored green is 7 inches.

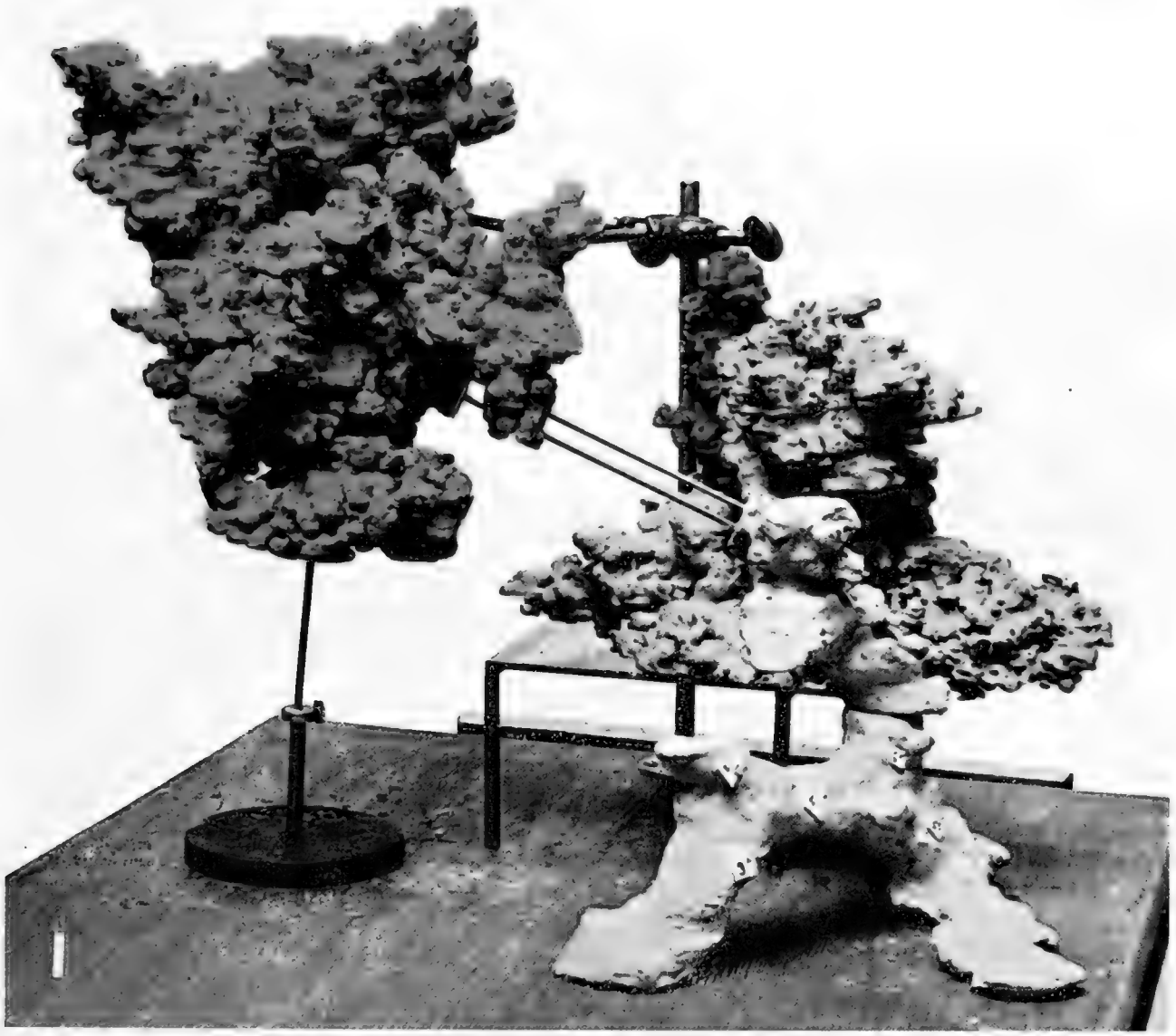

PLATE 2

EXPLANATION OF FIGURE

2 Part of a reconstruction of an adult lung, more highly magnified. This part is the right half of the portion colored orange in plate 1, seen from above. The illustration shows the part at its actual size in the model, which was constructed at magnification of 100 diameters.

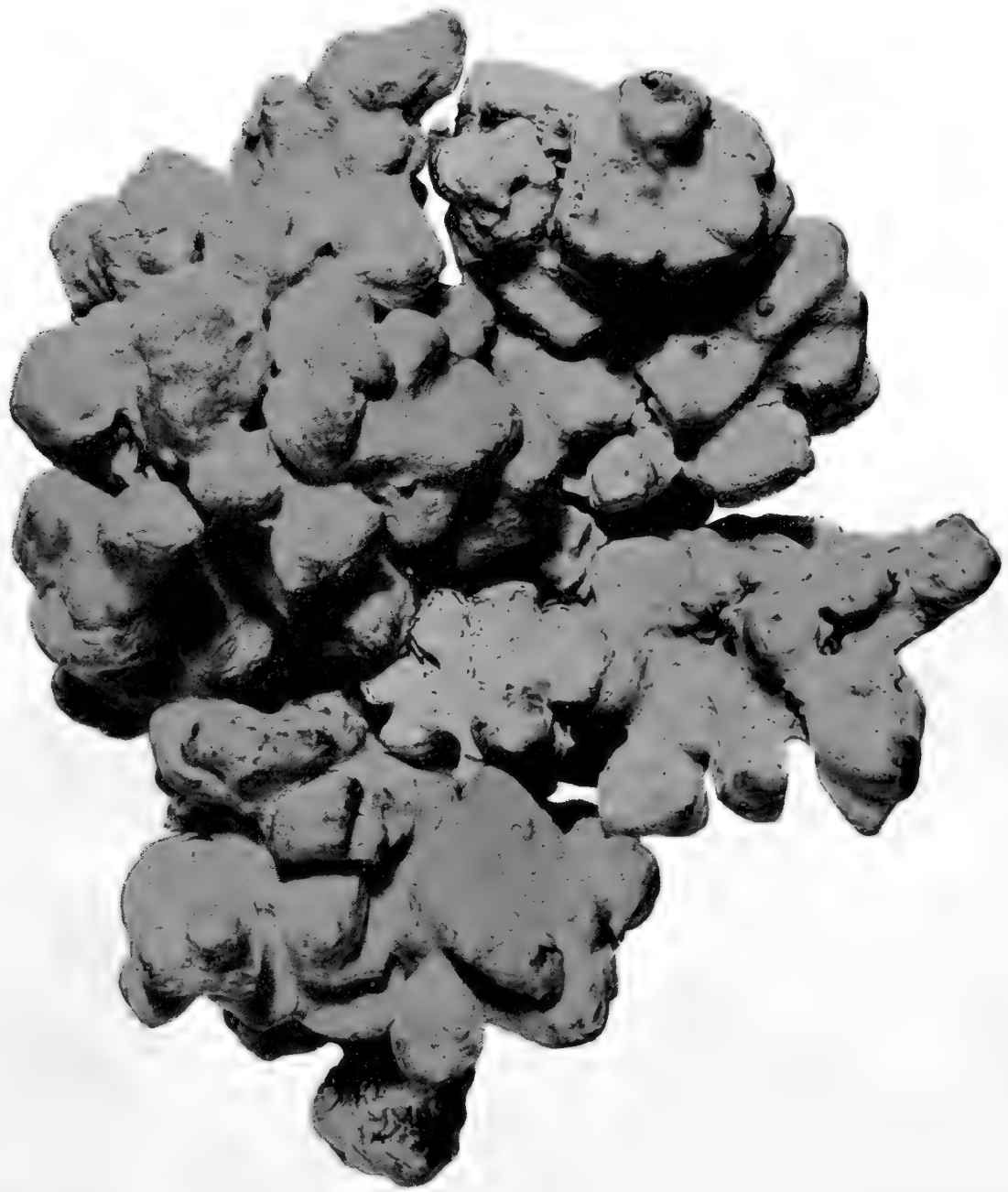

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PLATE 3

EXPLANATION OF FIGURE

3 Diagram of a reconstruction from a child's lung, showing the terminal branchings of a respiratory bronchiole, as projected upon a single plane. The numbers on the diagram correspond to those shown in plate 4.

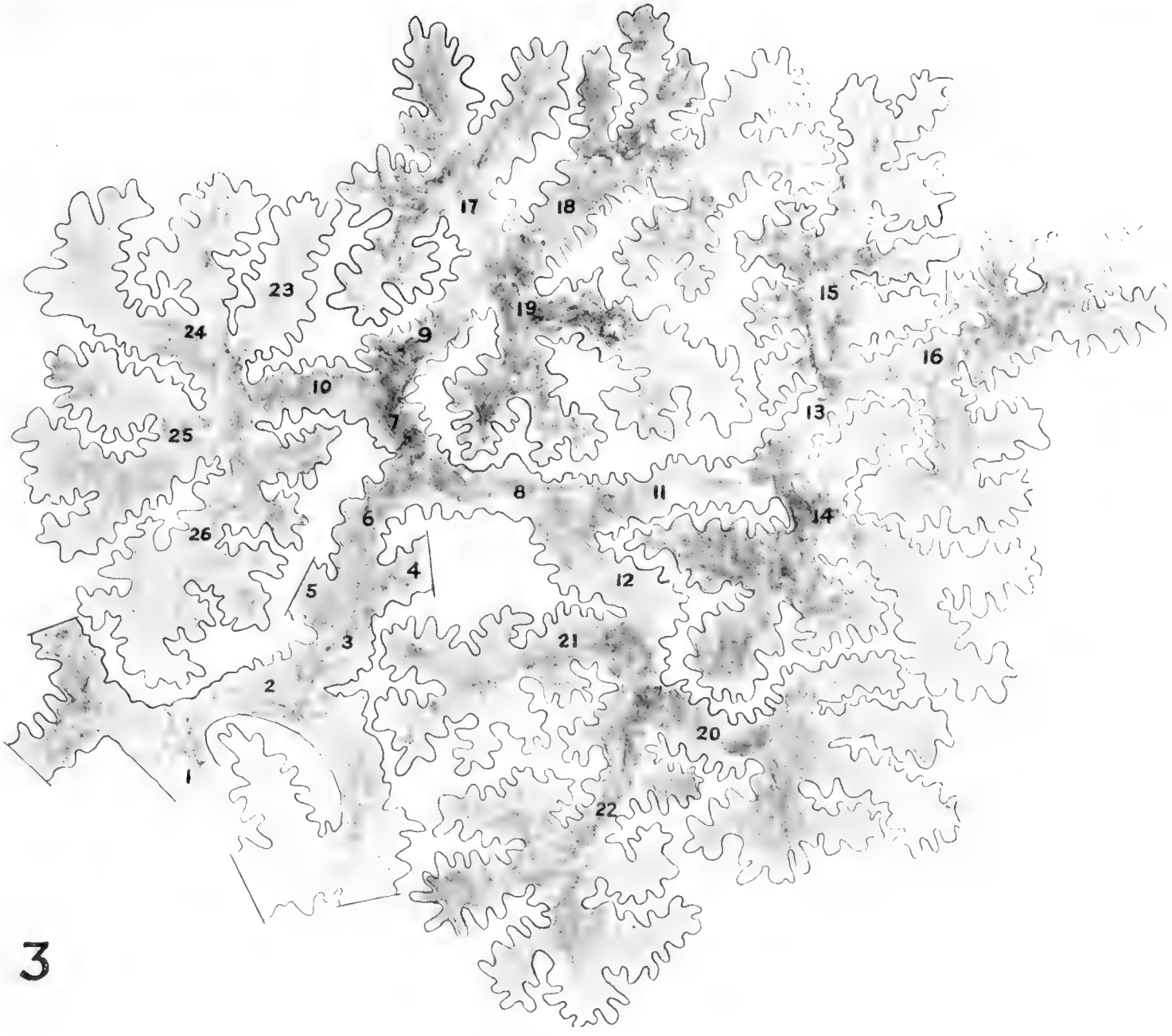

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PLATE 4

EXPLANATION OF FIGURE

4 Reconstruction from a child's lung, showing the air-sacs as they abut against the pleura. The model was made at magnification of 100 diameters. The part of the model shown in the illustration has a vertical height of $6\frac{1}{2}$ inches. The numbers correspond to those in the diagram, plate 3.


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Resumen por el autor, Edgar Allen

El ciclo éstrico del ratón.

El autor compara las observaciones llevadas a cabo en animales vivos con los cambios histológicos en el tracto genital y los ovarios. Estos cambios son de naturaleza cíclica y requieren un periodo medio de cuatro a seis dias. Los estados están representados por quiescencia, crecimiento, el climax éstrico y degeneración. Se manifiestan en la vagina por la formación cíclica y la degeneración de una capa córnea. Los cambos degenerativos en el epitelio uterino no siguen a su extirpación; como consecuencia de esto la hemorragia uterina es rara. La extrusión de los núcleos en el epitelio ciliado del oviducto es paralela a las fases degenerativas del útero y la vagina.

En le ovario existen grandes folículos durante la fase anabólica y son reemplazados por cuerpos amarillos en la fase catabólica. Por consiguiente, la ovulación separa a estas fases y tiene lugar durante el estro. La clasificación del ratón entre las especies de ovulación espontánea es errónea. Algunos ovulan con regularidad, otros tan solo de un modo esporádico, y otros tan solo ovulan con un estímulo sexual adicional. Diferentes modos en la longitud del ciclo son peculiares de diversas razas de ratones. Puesto que el ciclo es corto y la ovulacón puede ser espontánea o no serlo, los ovarios pueden contener tres o cuatro series de cuerpos amarillos voluminosos o no contener ninguno. En ambos casos se presentan ciclos normales. A consecuencia de esto parece justificada la conclusión de que los cuerpos amarillos del estro no poseen función causativa en los procesos anabólicos o catabólicos del cico éstrico. Las pruebas acumuladas indican la presencia de óvulos en los folículos grandes como causa del crecimento del tracto gental y el estro, y su ausencia o atresia como la causa de la fase degenerativa.

Translation by José F. Nonidez
Cornell Medical College, New York

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THE OESTROUS CYCLE IN THE MOUSE

EDGAR ALLEN

Department of Anatomy, Washington University School of Medicine

TWENTY-FOUR FIGURES

CONTENTS

1. INTRODUCTION AND LITERATURE

Although the mouse, Mus musculus, has been used for embryological purposes for nearly a century, no exact knowledge of its oestrous cycle is available. One reason for this is that the mouse, like other rodents, comes into 'heat' and receives the male within from six to twenty-four hours after parturition, and most investigators have timed their collection of embryological material from this 'heat' moment. Another reason for our lack of knowledge of the oestrous cycle in this form is the fact that external signs of 'heat' have been relied upon for diagnosis. Concerning this, Heape states: "It is difficult to determine the length of the prooestrum and oestrus in rodents, since the external signs which characterize these conditions are comparatively

slight." In many animals I have found them to be either entirely absent or so slight as to make an accurate diagnosis impossible.

Discussing the sexual phenomena of rodents, Marshall says: "Mus decumanus and M. musculus are known to experience a recurrence of the dioestrous cycle for more than nine months of the year in the absence of the male." The other three months are probably winter. No observations on the length of this cycle are given.

Sobotta ('95) is the only embryologist supposedly timing his stages from an oestrous period other than that following parturition, and he believed the duration of the cycle to be equal to the gestation period, i.e., 20+ days. Apparently all writers up until as late as 1916–17 have followed Sobotta in this particular.

Danforth in unpublished observations in 1914–15 attempted to check the length of the cycle by tabulating the number of days between two successive litters and computing the greatest common divisor of these intervals. In his records of sixty-six animals, twenty-three were excluded because of the possible complication due to the mother lactating while pregnant. The greatest common divisor of the modes of his curve was found to be 4 to 6 days. This evidence is inconclusive, but it at least casts doubt on the existence of a uniform oestrous cycle of 20+ days.

H. P. Smith ('17) attempted to approach the problem from a consideration of the ovarian cycle. This method permits of only one reliable observation, other than that of parturition, on one animal, namely, that made on histological examination of the ovaries after death. Conclusions must be drawn from a series of animals on each of which only one observation is made. The individual variation among even litter mates is so great as to make this method very inaccurate although a large number of animals be used. By this method Smith was led to conclude that the oestrous cycle is one of great variability, averaging seventeen and one-half days. He began the collection of his series at parturition, and his results are therefore really the recovery time of the ovaries—the time required for a resumption

of ovulation after the interference of pregnancy. The variation in this period is known to be great even within a single species, therefore the conclusions drawn can scarcely be applied to the normal ovarian cycle.

Estimates of previous oestrous periods arrived at from histological comparisons of the corpora lutea are not reliable since, as will be shown in the present paper, many mice when isolated from males do not ovulate spontaneously during oestrus.

Daniels ('10) and King ('13) have shown, in the mouse and rat, respectively, that if an animal becomes pregnant at the oestrous period following parturition and suckles her litter as well, the gestation period of her second litter may be lengthened, in some cases to from twenty-four to thirty days, an increase of 20 to 50 per cent. Kirkham ('16–'17) showed this delay to be due to a failure of the embryos to implant in the uterine mucosa. The embryos apparently remain free in the uterus in a state of inhibited growth for a time equal to the extension of the gestation period. No histological differences have been reported between the uterine mucosa of pregnant mice and those pregnant and also lactating. Long and Evans ('20), working on the rat, have reported histological difference between corpora lutea under these two conditions and ('21) a limiting influence of combined pregnancy and lactation on the growth of the vaginal epithelium. For several reasons, therefore, it seems better to time embryological material from a mating during a heat period not immediately preceded by parturition.

Further literature on the oestrous cycle in the mouse is limited to isolated observations made incidentally during investigations of other problems.

Concerning the literature on other rodents Marshall ('10) accepts Heape's statement that "dioestrous cycles recur for five or six months in the domestic rabbit, and that if oestrus is experienced in winter it may occur independently of the possibility of pregnancy. While some animals exhibit oestrus every three weeks fairly regularly, others do so every ten days; on the whole, I think 10–15 days is the usual length of their dioestrous cycle."

This is the sort of observation on which so much of the literature of oestrus is based.

Lataste has done considerable careful work on the vaginal plug of rodents and in this connection notes that the dioestrous cycle is usually about ten days.

Loeb ('11 a) reported a sexual cycle in the guinea-pig recurring every twenty to twenty-five days and described the histological changes in the uterus and ovaries at intervals. In a later paper ('11 b) he concludes the duration of the cycle to be from fifteen to twenty days.

In 1917, Stockard and Papanicolaou described a method of diagnosing the stages of oestrus in animals showing only slight external signs of their condition by a histological examination of the cell contents of the vaginal fluid. The cellular content of this fluid changes characteristically as the cycle progresses. This method offers the advantage of providing a complete history obtained from the repeated observation of reliable criteria upon the same living animal. It thus permits the study of individual variations and gives a record of the events taking place before killing the animal to study histologically the internal genital organs. By this method they obtained an oestrous cycle in the guinea-pig of remarkable regularity averaging 16+ days. This method also permitted Stockard and Papanicolaou to locate very exactly the moment of ovulation in a living guinea-pig. The rupture of the follicle occurs when the vaginal smear shows a definite cellular picture.

In the rat, Heape placed the duration of the oestrous cycle at ten days. Long and Evans ('20), using the above-mentioned vaginal fluid examination method, have shown this to be from four to six days, or actually only one-half as long as Heape supposed.

Of course much valuable information concerning certain phases of the sexual cycle in the mouse is already at hand in the work of Sobotta, Kirkham, Long and Mark, Smith, and others; this information, however, may be added to when approached from another viewpoint.

2. MATERIAL AND METHOD

Variation in the duration of the oestrous cycle has been reported in nearly all mammals studied, not only in closely allied forms, but within the limits of the same species. It seemed desirable, therefore, to include some individuals from each strain of mice in our colony. Several variations in other phenomena peculiar to certain strains had formerly been noticed, the most striking being the difference of reaction to ether anaesthesia: Albinos in our stock are very susceptible, while brown and black are quite resistant. It was thought important to find if there was any variation in the oestrous cycle typical of different strains. The stock chosen included brown, black, albino, dominant white with black eyes, gray, agouti, and yellow, as well as hybrids of these strains. More than ninety animals have been used in this work, the majority being young virgin mice. A few who had born litters were included for comparison, but in all cases records were not begun until the animals had been separated from males for at least a month after the removal of their young.

It has long been known that to maintain the oestrous rhythm the animals must be healthy, well fed, and under uniform environmental conditions. To eliminate crowding, no more than four animals were placed in one cage. Cages had a floor space of 850 sq. cm. and a capacity of 17,000 cc.

A constant supply of water was accessible to the mice through small holes in the ends of test tube containers. The food consisted of oats, cracked corn, sunflower seed, and dog biscuits.

Usually a little hay was added as nest-building material. During the winter the animals were kept in a heated room.

Methods

Stockard and Papanicolaou ('17) have shown the most reliable criterion of the condition of heat in the guinea-pig to be the cell content of the vaginal fluid. Their technique, slightly modified because of the smaller size of the mouse, was followed. Some animals were examined three times daily to get the exact time relations of the various phases of the cell changes, but for the

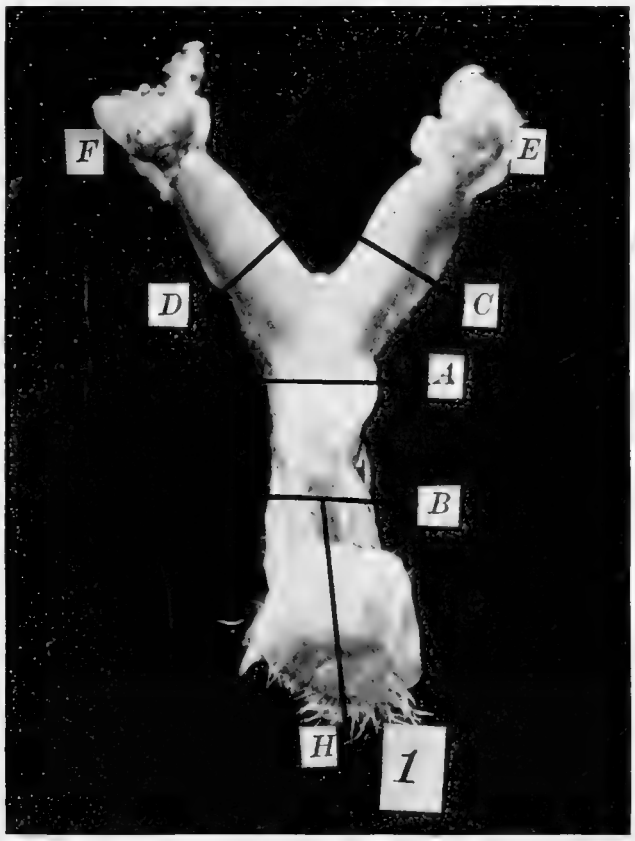
accumulation of data as to cycle length, examination once daily was found to be adequate. The condition of the vulva, the degree of opening of the vagina, and the nature of the vaginal contents were noted. A histological examination of the smears was always the deciding factor in the diagnosis. Smears were made by the usual bacteriological technique. Haematoxylin and aqueous eosin were used as stains. A block with a converging trough which could be quickly covered was devised for holding the animal while smears were being taken.

Animals used for correlating the conditions of different parts of the genital tracks with each other and with the cellular changes in the smears were examined and smears collected for several cycles before they were killed. These repeated examinations gave some indication of their 'degree of sexuality.' Smears were invariably taken immediately before killing. The animals were killed instantly by a sharp rap at the base of the skull, the abdominal cavity opened, and the conditions of the uterine cornua noted as to size, transparency, blood supply, and contractility. Shortly after the body cavity is opened, the uterus contracts to such an extent that the marked differences present at various stages in the cycle cannot be detected; therefore, an immediate inspection is necessary. After examining the fresh uterus the bladder was clipped off, the symphysis pubis sectioned, the skin was cut around the vulva, and the phallus, vagina, uterus, oviducts, and ovaries were dissected out and placed on a glass slide. After a minute's drying they adhere to the slide and can be fixed without contortion. Bouin's fluid was used as a fixing reagent and proved satisfactory for both uteri and ovaries. After hardening in the lower alcohols, the organs were cut to facilitate orientation and imbedding. Serial sections 10 μ thick were made of the ovaries and oviducts. In trimming these organs it is important that the peritoneal ovarian sac be left intact to protect the surface of the ovary during dehydration and imbedding. Several transverse serial sections of the middle of each uterine cornu, the body of the uterus, the vagina, and the cervix, as well as several serial sagittal sections through the phallus and lower vagina, were cut at a thickness of 6 to 8 μ.

Cut surfaces were oriented so that the same regions in different animals could be compared (text fig. 1).

3. POINTS OF IMPORTANCE IN THE ANATOMY OF THE GENITAL ORGANS OF THE MOUSE

There are some points in the anatomy of the genital organs of the mouse which need emphasis since they have an important bearing on certain aspects of this work.



Text fig. 1 Genital organs before fixation, showing the planes of histologica sections. *A*, body of uterus; *B*, vagina; *C*, middle of left cornu of uterus; *D* middle of right cornu; *E*, left ovary and oviduct; *F*, right ovary and oviduct *H*, sagittal section of phallus and lower vagina.

The vagina in the mouse opens directly on the vulva without the protection of any structures homologous to the labia minora. Its stratified epithelium is devoid of glands. The phallus is large and prominent and is traversed in its entire length by the urethra. The cervix is relatively short, the division into the lumina of the uterine cornua occurring 1 to 3 mm. above the opening of the external os. A stratified epithelium, similar,

but considerably lower than that of the vagina, is continued up into the cervix in virgins as well as in multiparae, but beginning at the branching of the cervical canal, it becomes simple, non-ciliated, cuboidal or columnar. Glands are uniformly distributed throughout the uterine mucosa except along the line of attachment of its broad 'mesentery-like' ligament. The entrance of the oviduct into the uterine cornu is surrounded by valve-like folds of the mucosa, which make it very difficult to inject the oviducts from the uterus. These valves are in a position to guard against a back flow of fluid from the distended uterus into the oviducts, which might be fatal to the passage of ova down the tubes. The oviducts are ciliated only at the ovarian end where the mucosa is thrown into high folds. Throughout the rest of their extent the epithelium is simple columnar. The uterine end can be distinguished from the middle segment by the low folds of its mucosa, those in the middle segment being relatively high.

The ovaries are situated just caudad to the kidneys and are completely surrounded by closed sacs of peritoneum from which the openings of the oviducts lead. Therefore, the number of ova in the tubes and periovarian sacs is always the number ovulated, since none can escape into the peritoneal cavity.

4. EXTERNAL MORPHOLOGICAL CHANGES DURING THE OESTROUS CYCLE

a. Evidence from the external genitalia and cell contents of the vagina. Among many rodents there is very little or no discharge during 'heat.' The degree to which congestion and reddening occur in the vulva is also variable, often being totally absent or so slight as to be a very poor criterion of oestrus. In a total of 355 cycles chosen at random to decide this point in the mouse, only 190, or 53.5 per cent, showed well-marked external signs. And seventy-three, or 23.3 per cent, showed the vulva and vagina in an apparently resting condition, although by the cell contents of the vaginal fluid oestrus was shown to be present. A few animals evidenced continued external signs of 'heat' during the metoestrum and the dioestrous interval, so

that here again external signs as criteria of a condition of oestrus are not reliable. That such signs are due primarily to congestion and consequent poor tonus of the muscle layers of the vagina is indicated by their rapid disappearance coincident with the relaxation of the urethral sphincter following death. However, to settle the question as to their value in diagnosis, the external signs were always noted when taking smears, and as they were typical in 53.5 per cent of cases, a description is desirable. Heape's terminology is followed in describing the phases of the cycle, with emphasis on the following points: 1) that growth and congestion continue through oestrus; 2) that the metoestrum may be further subdivided into two periods.

To eliminate a too frequent use of 'oestrum' in its various forms, the stage will be designated as shown below:

D, dioestrum, period of relative quiescence.
P, prooestrum, period of augmented growth and congestion.
O, oestrum, period of sexual excitement, the climax of prooestrous conditions.
M_1, M_2 metoestrum, period of return to the dioestrous condition.

Stage D. In the mouse during the dioestrous interval the vulva is very inconspicuous and the orifice of the vagina is usually tightly closed. There is a little fluid which is viscous and stringy. The smear shows epithelial cells, usually only a few, in various stages of nuclear degeneration and cytoplasmic shrinkage, and always some polymorphonuclear leucocytes.

Stage P. During the prooestrum, if external signs are marked, the vulva is pink or red and swollen, the vagina gapes open, the fluid in its lumen is serous, and the smear shows only nucleated epithelial cells (fig. 2).

Stage O. During the oestrous period, the vulva may still be swollen, the orifice of the vagina open and a dull white in color, the vaginal mucosa almost dry, and the smear shows only cornified, non-nucleated, red eosin-staining cell remains (fig. 3). In animals where external signs are absent or slight, the finding of only cornified cells in the smear makes a diagnosis of 'heat' possible.

Stage M_1. In the first half of the metoestrum, the vulva has usually lost most of its swelling, the orifice of the vagina still gapes open and is whitish, occasionally showing small granulations, the lumen is still dry, and the smear shows red cornified elements of the previous periods, always very numerous, and now bunched or caked (fig. 4).

Stage M_2. This stage is evidenced by a normal vulva and a tightly or partly closed vaginal opening. The vaginal contents change as the period progresses from a pasty or viscous to a fluid consistency, and the smear shows polymorphonuclear leucocytes among the red horny elements. A few polymorphs and many cornified cells is an early M_2 stage, and a heavy leucocytic infiltration and decrease in number of the cornified cells is a late one (fig. 5).

This stage then merges into D with a decrease in leucocytosis and the appearance in the smear of small numbers of nucleated epithelial cells. By standardizing the technique of smear preparation, stages O and M_1 are easily differentiated, although the basis for this division is the number and clumping rather than any change in the character of the cells themselves.

The separate phases as well as the duration of the whole cycle show great variation under the most uniform conditions of food and environment. Further discussion may be postponed until the histological condition of the various genital organs can be described.

5. ANIMALS STUDIED HISTOLOGICALLY

A series of twenty-seven carefully timed animals was prepared for histological study. They consisted chiefly of four strains: albino, black, brown, and gray. Observations were made daily for a period of from three to five weeks before killing, in order to obtain an accurate record of the number and duration of oestrous periods as an aid to the interpretation of ovarian conditions. The smear made just before killing usually followed the routine smear for that day by three to six hours, so that two observations at short intervals were available for diagnosis.

The criteria used in placing the animals are the following:

1. The cell contents of the vaginal smear at death.

2. The number of layers of vaginal epithelium unaffected by cornification or leucocytic infiltration.[1]

3. The position, with regard to the free surface of the epithelium, of the granular and horny layers when present.

4. If ova are present in the uterine tubes, the segment in which they lie indicates the time of ovulation with regard to oestrus.

5. The stage of development of the corpora lutea when present aids in a correct arrangement of the series. Their age is estimated by the position of the ova in the tubes, and compared with corpora lutea of pregnancy (recorded in column 7, table 1).

The following table of animals killed for histological examination represents a complete, closely spaced series. It seems convenient to begin at the middle of the dioestrous interval and return to the same point in the next cycle.

The results of the histological examination of this series of organs can be most satisfactorily presented by means of a detailed description of typical animals (marked ⁂ in table 1) representing the midpoints of the five stages of the cycle.

The dioestrous interval

Stage D is best represented in this series by animal ⁂2, a virgin mouse seven months old. She was killed after 53 days, observation, during which time nine complete cycles were recorded, making the average duration 5.9 days. In six of these nine cycles pronounced external signs were apparent. The last four oestrous periods occurred 3 to 5, 11, 14 to 15, and 19 to 20 days before her death. The record shows her to have been in an early day of the dioestrous interval after a cycle of from 7 to 8 days' duration marked by external signs and containing a three-day oestrous period.

A gross examination of the internal genital organs was made immediately after opening the body cavity before there was any

[1] These underlying layers are very difficult to count in some of the phases, so that two numbers are used to express a limit of error.

TABLE 1

Animals examined histologically arranged in order of progressive phases of the oestrous cycle as diagnosed by vaginal smears and microscopical examination of the genital organs. (For key to table, see footnote.)[2]

CYCLE SEQUENCE	COLLECTION NUMBER	NUMBER OF LAYERS OF VAGINAL EPITHELIUM	POSITION OF GRANULAR AND HORNY LAYERS	SEGMENT OF OVIDUCT CONTAINING OVA	AGE OF MOST RECENT CORPORA LUTEA IN DAYS	AGE EQUIVALENT OF CORPORA LUTEA OF PREGNANCY	SMEAR	STAGE
1	26	5–6	Gone	2nd–3rd	2+	30–50 hrs.	0NE, 0P	D
✱2	*24*	5–6	Gone	3rd	3	3+ days	1NE, 3P	D
3	8	5–7	Gone	3rd+	4	3+ days	1NE, 2P	D
4	11	5–9	Gone	None	?		0NE, 1P	D
5	13	3–5	Gone	None	5		0NE, 1P	D
6	15	5–6	Gone	None			0NE, 0P	D
7	6		Gone	None	?		0NE, 0P	D
8	3	7–10	Before formation	3rd	3		0C, 1NE	P
✱9	*10*	12–13	Granular, no horny	None	3–4	3+ days	1NE, 0P	P
10	12	11–13	Under 2–4	None	3–4	3+ days	0NE, 0P	P
11	14	11–13	Under 2–4	None	?		0C, 1NE	P
12	9	8–10	Under 3–5	None	5		0C, 1NE	P
13	23	9–10	Under 3–4	*None	None	*	1C, 1NE	P
14	1	10–11	Superficial	None	5		1C	O
✱15	*4*	8–12	Superficial	None	5		1C	O
16	2			None	5+		1C	O
17	16	12–13	Superficial	None	7–8		1C	O
18	21	9–12	Superficial	1st–2nd	1	3– 7 hrs.	3C, Bu	M_1
✱19	*19*	11–12	Superficial	2nd	2	30–35 hrs.	3C, Bu	M_1
20	18	10–12	Superficial	*None	None	*	2C, Bu	M_1
21	7	4–6	In lumen	2nd–3rd	2	20–40 hrs.	2C, Bu	M_1
22	20	6–7	In lumen	2nd–3rd	2	40–50 hrs.	3C, Bu	M_1
23	25	4–7	In lumen	3rd	3	50–60 hrs.	2C, 1P	M_2
24	17	7–9	In lumen	3rd	3	60–70 hrs.	1C, 1P	M_2
25	22	7–8	In lumen	3rd	3	50–60 hrs.	2C, 2P	M_2
✱26	*27*	6–8	In lumen	2nd–3rd	2 & 3	30–50 hrs.	1C, 3P	M_2
27	5	5–6	In lumen	3rd	3	72+ hrs.	1C, 2P	M_2

[2] *Key to table 1.* Column 1 represents the progressive order of the series beginning with an early phase of the dioestrous stage. ✱, Animals described in detail as typical of the five stages of the cycle; * (in columns 5 and 7), ovulation not spontaneous; *0, 1, 2, 3* (in column 8), represent relative numbers of cell types; *C*, cornified epithelial cells; *NE*, nucleated epithelial cells; *P*, polymorphonuclear leucocytes.

obliteration through contraction of the characteristics which differentiate between the O and D uterus. The anastomosing uterine and ovarian vessels were small, the uterus was of medium size and anemic.

Histological examination of cross-sections of the vagina midway between the cervix and the vulva shows the lumen to be small in diameter, with the walls thin, folded, and collapsed. Free nucleated epithelial cells and leucocytes are present in considerable numbers. There is no basement membrane under the epithelium and the deepest layer of the stratum germinativum shows no distinct cell membrane on the side adjacent to the stroma. The epithelium not extensively infiltrated with leucocytes is only five or six layers deep and shows no signs of cornification (fig. 6). Very few mitoses are to be found. Scattered leucocytes are abundant in the one or two superficial layers, and in several places small clumps are gathered in clear lacunae.

In general the description of the vaginal epithelium holds true for that of the lower cervical canal; there is no basement membrane beneath the epithelium, which is five or six layers high, and no sign of cornification.

Cross-sections through the uterine cornua midway between their junction and the attachments of the oviducts show narrow, slit-shaped lumina indicative of a lack of distention. There are very few cells of any sort free in the lumina. The epithelium has no basement membrane, the lower parts of its cells stain lightly, and cell walls are not readily distinguishable. In many places the cells are piled up four to eight layers deep in poorly staining syncytial masses. Mitoses are only occasional, being present in a few instances in the syncytial masses. The epithelium is intact everywhere, although it is quite heavily infiltrated with leucocytes. Some of the glands show functional activity, as evidenced by slight distention.

The oviducts were adherent to the periovarian sacs and were sectioned serially with the ovaries.

The oviducts are moderately distended throughout, and are entirely free from leucocytic infiltration. The non-ciliated epithelium, lining the second and third portions, shows few signs

of degeneration, but the ciliated cells covering the highly folded mucosa of the part leading from the ovisac show all stages of the extrusion of their nuclei. Some of these extruded nuclei still adhere to the free surface of the cells and are similar to nuclei of normal cells. Others show varying degrees of pycnosis. Many small regions are to be found devoid of cilia, apparently marking cells from which the nuclei have been extruded. A further description of this process will be undertaken later.

The two oviducts in this animal contained eight ova, still in good condition, although in none was the zona pellucida prominent. (This may be due to the fixing reagent as stated by Sansom, '20.) In most of the ova the second maturation spindles were still intact, but rarely were polar bodies recognizable. The ova were bunched in the last segment of the tubes. Therefore, ovulation occurred three days previously (H. P. Smith, '17).

The ovaries contain eight medium-sized follicles, all superficially located. The primary liquor folliculi is beginning to form, but has not yet reached a stage far enough advanced to make possible the distinction of a cumulus. The nuclei of the ova are in the resting stage. In this pair of ovaries, there are more than thirty atretic follicles in all stages of degeneration, most of them deeply situated in the stroma. There are at least three sets of corpora lutea present.[3] The most recent corpora lutea are easily distinguishable from the older ones by their blue color, the latter staining more heavily with eosin. There are eight of this last set. They are similar in degree of development, so that a description of one will suffice for all. The central lake is almost obliterated, chiefly by the hypertrophy of the granulosa cells which do not at this stage take eosin readily. The theca interna has entirely disappeared and the ingrowth of connective tissue has reached the edges of the lake and woven a fine reticulum about the inner walls of the luteal cells, at the same time beginning an arrangement of these cells into cords. Slight vascularization is evident, but there are erythrocytes in

[3] It should be understood here that all corpora lutea referred to in this paper are corpora lutea of oestrus, as no pregnant nor lactating animals have been used.

the central cavity in only one of the eight corpora lutea of this set. From the position of the ova in the tubes, it was concluded that ovulation has occurred three days previous to killing. This set of corpora lutea of oestrus is, therefore, three days old. They are surely the equivalent of the seventy-two-hour corpora lutea of pregnancy.

The next older corpora lutea are greatly degenerated. They are small, deeply placed, irregular in outline, and poorly demarcated from the surrounding stroma. The proportion of connective-tissue cells to luteal cells is about equal. As stated above, the cytoplasm of the luteal cells stains red with eosin, but it is a faded or blotchy red. No distinct large blood vessels are present as in the first set described. A third set consisting of only a few still more poorly defined corpora lutea is present. These are very small, staining a light pink, and are almost completely obliterated by a connective-tissue ingrowth.

Of the three sets of corpora lutea distinguishable, the age of the youngest can be placed at three days, with a limit of error of about twenty hours. From the results of the examination of other ovaries of this series, the second set is at least ten days old, and the third still older.

There are a few cells present in these ovaries which could be defined as interstitial.

The prooestrum

Animal ♯9 was chosen as typical of the stage P condition. A routine examination was made at 1 P.M. and another just before killing at 4.30 P.M., an interval of three and one-half hours, in which time many of the leucocytes had disappeared from the vaginal contents, indicating an early stage P. Previous to killing she had been observed for sixteen days, during which time four complete cycles had been recorded, making the average duration four days. External signs were marked during all four of the oestrous periods. These occurred 4, 7 to 8, 11 to 12, 15 to 16 days before death. Her record is one of minimum cycle duration and perfect regularity.

The uterine and ovarian vessels were congested (two or three times larger than during the D period), and the uterine cornua were much distended. When the animal was killed the uterus did not expel this fluid (as the bladder expels the urine) and when held up between the observer and the light, the uterine cornua are so transparent that the folds of the mucosa are easily visible. The ovarian capsules were not distended.

Cross-sections of the vagina show a fairly large lumen containing some nucleated epithelial cells and a very few apparently degenerate polymorphonuclear leucocytes. The epithelium shows a basement membrane in a few restricted regions. There are twelve to thirteen layers of epithelial cells, of which the outer four to five layers stain very lightly with eosin. This demarcation is made still clearer by a well-formed granular layer which serves as a line of division. The layers superficial to this are not so flattened as those immediately underlying the stratum granulosum. There are as yet no other signs of cornification (fig. 8). Mitoses are abundant in the germinativum. The epithelium is free from leucocytes except for a very few in the most superficial layer of cells. The nuclei of the stroma are loosely packed and intercellular spaces are everywhere evident. The sagittal section shows the same conditions, except that a thin cornified layer still remains on the ventral side of the vagina at its opening on to the vulva.

The cervical epithelium just before it merges into the simple epithelium of the uterus, although at this point being only from three to five layers high, is yet divided into two regions, a deep, darkly staining, and a superficial, lightly staining one. It is similar in most respects to that of the vagina except as regards height.

The lumina of the uterine cornua are large. The fluid with which they are distended is not coagulated by Bouin's reagent. There are no cells of any sort free in the lumina. The epithelium is low columnar and has a distinct basement membrane in all but a few regions. Mitoses are frequent. No leucocytes are to be found in the epithelium and only an occasional one is in the subepithelial zone which is most heavily infested by them

during the D stage. The gland ducts are distended and their cell outlines are clearly defined. Mitoses are absent, which indicates little growth activity in the glands during hyperfunction. Glands are distributed evenly throughout the mucosa except opposite the line of the attachment of the broad ligament. The nuclei in the stroma are densely packed, possibly because of distention of the lumen.

The oviducts are slightly distended. Their epithelium is in good condition in the non-ciliated portions. In the ciliated segment, all stages in the process of extrusion of nuclei are apparent, but to a less degree than in the D stage animal previously described. No leucocytes are present.

There are no ova to be found in either oviduct. Therefore, if ovulation occurred at the last O period, that should have been at least four days before death.

Sections of the ovaries show a marked hyperemia. There are two sets of follicles containing liquor, the first one composed of ten follicles, which are large and distended with considerable amounts of liquor. They are all superficially located. The cumuli are intact and still solid masses of cells. The nuclei of the ova are in a resting condition. The second set of follicles is less mature, being medium sized, with the liquor still confined to small pools at the poles of the follicles.

There are several medium-sized atretic follicles in which the cells of the cumuli have entirely degenerated, leaving the ova free in the liquor. The granulosa cells also show marked signs of atresia.

The ovaries of this animal are unusually large, which may be partly accounted for by their hyperemia, but the unusual number of corpora lutea is probably the main reason. At first glance the ovaries appear to be just large masses of corpora lutea. Three sets are easily distinguishable. Those of the first set are superficial, medium sized, and stain dark blue. Their central lakes are completely ingrown, but two of them show small patches of red blood corpuscles at their centers. They are at least four days old.

The corpora of the second set are larger than those of the first, are superficially located, and deep red in staining reaction. The cells are arranged in 'cords,' but their walls are not clear-cut and the cytoplasm has a blotchy appearance. The edges of these corpora lutea in section are clearly defined from the surrounding stroma. Theca interna cells as a distinct layer no longer exist. This set probably corresponds to the second ovulation before death, occurring at some time during the O period; therefore, seven to eight days previously.

A third set of corpora lutea, less clearly defined from the stroma, red staining, and about the size of the first set, is present. The stroma has begun to invade their surfaces and the proportion of connective-tissue cells to luteal cells throughout has increased. The record of this mouse shows an O period eleven to twelve days before death, so that this third set must be at least that old. There are a few cells clearly definable as interstitial in these ovaries. They are restricted in distribution to limited areas near the periphery.

The oestrous period

Animal ✻15 shows a typical oestrous condition. She had been examined for twenty-eight days previous to killing, in which time only three complete cycles had been recorded, making the average duration $9\frac{1}{3}$ days. Her oestrous periods were 6, 16 to 17, and 28 days previous to death. Her last cycle was of five days' duration. Her first and second cycles had long dioestrous intervals, seven to eight days in each case, while the other four stages required only three to four days.

Upon opening the body cavity, the uterine and ovarian vessels showed some congestion, though less than that reported during the prooestrous period. The uterus was moderately distended, but less transparent. Gross examination shows the same conditions as found in the prooestrum to prevail, although to a less degree.

The lumen of the vagina in cross-section is much folded and collapsed, as would be expected from the record of the examina-

tion before death. It contains a small number of detached cornified epithelial cells which are very thin in cross-section. A distinct basement membrane clearly marks off the epithelium from the stroma. The epithelium is eight to twelve cells deep under the granular and horny layers, which are now superficially placed in all regions. A few shrunken blue staining cells with pycnotic nuclei still remain in the deeper parts of the crypts between folds in the mucosa. They represent the last stages of degeneration of the cells of the prooestrous smear. Mitoses are frequent. There are no leucocytes present in the epithelium (fig. 10).

The cervical epithelium is six to eight layers high, but shows no cornification as yet. Except for these two points, the description of the vaginal epithelium holds good for that of the cervix; i.e., it has a clear-cut basement membrane, contains frequent mitoses, and is free from leucocytes.

The lumina of the uterine cornua are moderately distended, but contain no cells of any sort. The basement membrane of the epithelium is distinct and heavy and the cells are columnar (fig. 15). Mitoses are moderately frequent. No leucocytes are to be found in the epithelium. Gland lumina show a slight distention pointing to moderate functional activity, but the scarcity of mitotic figures indicates little growth. Cell borders are distinct and the cells show no degenerative changes. Although a few leucocytes are distributed through the stroma, only an occasional one is found in the gland epithelium. In section, the uterus shows slight hyperemia.

The oviducts are moderately distended. In the ciliated portions nuclear extrusion is still apparent, but to a less degree than during the P stage. Consequently, larger surfaces present unbroken ciliation. There are no leucocytes present. No ova are to be found in the tubes; therefore, if ovulation occurred at the last oestrus, this must have been at least four days previous to killing.

There are two sets of normal follicles far enough matured to show liquor folliculi. The ten follicles of the first set are the largest found in this series of animals. They are all superficially

placed, and although the cumuli are still intact, the secondary liquor folliculi is present in them in small pools. The ova show resting nuclei centrally placed. The second set of follicles are medium sized and show only an early beginning of liquor formation. There are several atretic follicles present in various stages of granulosa degeneration.

There are from twenty to thirty corpora lutea in this pair of ovaries, among which at least three sets can be defined. The most recent ones, ten in number, are superficially placed and quite large. These corpora stain a light blue in contrast to the red-staining older sets. They are clearly defined from the stroma, and their constituent cells which are arranged in cords show distinct cell walls. Although the ovaries are hyperemic, there are few erythrocytes to be seen in these corpora lutea, which is in marked contrast to the small vessels crowded with erythrocytes in the four-day corpora of the stage D animal described above. They must correspond, therefore, to the O period recorded on the sixth day before death. The other two sets of corpora lutea are distinguishable chiefly by size, position in regard to the surface of the ovary, and proportionate ingrowth of stroma cells at their surfaces. The cytoplasm of the 'luteal' cells of both sets stains a blotchy red. There are a few interstitial cells in these ovaries.

The early metoestrum

Animal ♯19, representing a typical M_1 stage, was one of three females of a litter of homozygous brown mice separated from males at the time of weaning, and examined daily for the appearance of her first oestrus. An open vagina was first noted on July 12th, at the age of three and one-half months, the membrane closing the orifice having ruptured during the night of the 11th. Routine smears were started on the 13th, and continued for forty days, during which time five complete cycles were recorded, making the average duration eight days. If, however, the first two longer periods are excluded, it brings this average down to seven days. At only one of these periods did she evidence external signs of oestrus. Previous 'heat' periods are recorded

2 to 3, 9 to 10, 14 to 16, 23 to 24, and 34 days before she was killed. A gross examination showed the ovarian and uterine vessels to be relatively small and inconspicuous. The uterine cornua were small in diameter and quite opaque. The periovarian sacs were slightly distended.

Cross-sections of the vagina (fig. 11) show a semicollapsed lumen crowded with masses of non-nucleated, cornified, deeply red-staining, epithelial cells. The epithelium has no basement membrane and the deepest layer of the germinativum is in intimate relation with the connective-tissue cells of the stroma. There are eleven or twelve layers of epithelial cells under the granular and horny layers, which are everywhere superficial and intact except for the delaminated masses in the lumen. There are very few or no mitoses present in the germinative layers. Leucocytes are also absent.

The lumina of the uterine cornua are not entirely collapsed, but contain in several of the sections a few nucleated epithelial cells. The basement membrane of the uterine epithelium is entirely lacking and in its place is a broad red-staining band. The deeper edges of the epithelial cells and the most superficial stroma cells are involved in it, and their structures contained in this band become blurred and take the stain poorly. Small vacuoles are apparent in and among the epithelial cells. A very few leucocytes have already entered the degenerate band or zone under the epithelium.

The oviducts are moderately distended. Vacuoles are present in the non-ciliated epithelium of the segments adjacent to the uteri and in the ciliated regions extrusion of nuclei is general (fig. 19).

Eight ova are present in the second segments of the tubes. Membranes and maturation spindles are intact in every case, and polar bodies are still adherent. The animal was killed then during the second day after ovulation.

There are from ten to fourteen medium-sized follicles in which the liquor folliculi is beginning to form at the poles. There are none sufficiently distended to indicate the approach of an ovulation. A very few small and medium-sized atretic follicles are present.

There are over thirty corpora lutea in these two ovaries. The most recent set number eight, which corresponds with the number of ova in the tubes. They are blue staining and have large central lakes of liquor. Theca interna cells are still evident at the periphery and connective-tissue sprouts have not yet grown completely through the granulosa cells. No erythrocytes are included, indicating that vascularization is not far advanced. Judging by the position of the ova in the tubes, these corpora lutea are in their second day of development.

The other corpora lutea are divisible into at least three sets. They are all red-staining, solid masses of cells, differing chiefly in size, location, and clearness of demarcation from the surrounding stroma. The second oldest set are much larger than the recent ones, clearly defined, and peripherally situated. The third oldest set are smaller than the second, but larger than the first. They are separated from the germinal epithelium by several layers of connective-tissue cells. The second and third sets correspond to the ovulations during the oestrous periods nine to ten and fourteen to sixteen days previous to death. This mouse has apparently ovulated spontaneously at every oestrous period.

The late metoestrum

Animal ♯26 had been examined for twenty-two successive days before killing, during which time five complete cycles had been recorded, giving an average duration of $4\frac{2}{3}$ days. External signs were marked at only two of these five periods, and in one of these instances they extended into the late metoestrum. Her previous oestrous periods were 2 to 3, 6 to 7, 11, 16 to 17, and 20 to 21 days previous to her death.

The last smear shows all degrees of epithelial degeneration and cytolysis. The cornified cells present a varied staining reaction to eosin, some being bright red, others a faded pink, and a few almost colorless. Where one cell is isolated in a group of leucocytes its exoplasm is a clear colorless zone, suggesting the extraction of the eosin staining cytoplasm.

Immediately after killing, gross examination showed her uterus to be anemic, small, and opaque.

The lumen of the vagina is collapsed and the walls much folded. It is crowded with polymorphs, among which lie big fragments of delaminated cornified elements. They are very thin in section. The epithelium is without any vestige of a basement membrane, pointed shoots of the stroma penetrating into the lower layer of the germinativum. There remain only four to seven layers of healthy epithelial cells beneath a broad region which has been reduced almost to the appearance of a fine reticulum by the infiltration of enormous numbers of leucocytes which lie in its meshes. In regions less affected, small clear lacunae containing several leucocytes are distributed throughout the epithelium. No signs of a granular layer exist, and the cornified layer has been freed from its attachments in most places. This action is just as marked in the crypts of the mucosa as on the ridges. Leucocytosis is at its maximum in this animal. In spite of this extended destruction of the epithelium an occasional mitosis may be found.

The sagittal section shows that the process of leucocytosis does not extend far through the vaginal orifice, for as it approaches the vulva the epithelium still appears normal and its granular and horny layers are superficial but intact. As these layers are traced back into the lumen, the eleidin granules begin to disappear and the cells gradually to stain pink and then red until they merge into a fully formed cornified layer.

The lumen of the uterus is small in diameter and contains an occasional small mass of free cells. The epithelium in most regions is quite completely degenerated. There is no basement membrane, but in its place a broad, blurred red or pinkish band involving the lower part of the epithelial cells and the adjacent stroma. The epithelium consists for the most part of several layers of nuclei, which might lead one to classify it as pseudostratified. But cell outlines are lacking or very indistinct, so that it represents the appearance of unorganized masses of nuclei in poorly staining cytoplasm. In a very few restricted areas occasional mitoses are evident, but for the greater part no signs of growth activity are apparent. The epithelium and, to a greater extent, the subepithelial zone are heavily infiltrated with leucocytes (fig. 16).

The glands show a minimum of function, as judged by the slight distention of their ducts. A few mitoses are scattered through their epithelium. Leucocytes in small numbers are present between and beneath their cells, but seem to exert little cytolytic action.

The oviducts, especially the segments adjacent to the uterus, are distended. The epithelium is high columnar and contains a few vacuolated cells in the non-ciliated portions, while in the ciliated section the extrusion of nuclei is marked.

There are seven ova present in the tubes. Some of them show signs of degeneration. The membranes are lacking and the chromatin material has disappeared. Two ova in the right oviduct still show the second maturation spindle and one has a polar body in which separate chromosomes can be distinguished. These two ova are in the second segment of the oviducts, while all the rest from this ovulation are in the third.

Most noticeable in the examination of the left ovary was the unusually numerous follicles of moderately large size, ranging from an average diameter of 0.32 mm. to 0.4 mm. There are twelve of these in the left ovary and seven in the right. The liquor folliculi is well formed, the cumuli are intact, and the nuclei of the ova resting. One of these large normal follicles in the right ovary is sausage shaped and contains two ova, each with its separate cumulus and its resting nucleus.

Twenty to thirty corpora lutea are present in both ovaries. The most recent set are blue staining, but are not all at the same stage of development. Two in the left, and three in the right ovary have small central lakes, and two of those in the right ovary contain erythrocytes plainly indicative of "bleeding into the central cavity." The shoots from the cells of the theca interna have grown completely through the layers of the granulosa in these five corpora and have formed five connective-tissue reticula around the central lakes of liquor. In the other two recent corpora in the right ovary, the central lakes are much larger and the connective-tissue sprouts have not yet grown through the granulosa cells. There is no distinguishable histological difference in the luteal cells of the two stages. Five of this

set of corpora lutea are in their third and two in their second day of development. There are two older sets of red corpora lutea in these ovaries corresponding to the oestrous periods six to seven and eleven days previous to killing. The large number of these in the right ovary as contrasted with the few in the left would point to a hyperfunction of the former, at least during the last three oestrous periods. The unusual number of normal, moderately large follicles in the left ovary indicates the shifting of the major function for the next oestrus.

There are a few interstitial cells in these ovaries. Leucocytes do not appear in significant numbers, although leucocytosis is at its height in the vagina and uterus.

6. SUMMARY OF STAGES BY ORGANS

1. External signs

Although certain external signs may occur during the prooestrous and oestrous periods in the mouse, these periods may be present without them or the signs may continue after 'heat' has passed. When present, the external signs consist of a swelling and coloration of the vulva due to congestion and the gaping open of the vaginal orifice.

2. Vaginal content

Changes in the cells and the fluidity of the vaginal contents are a much more reliable criterion of the condition of oestrus. During the prooestrum and oestrum there are no leucocytes present in the vaginal contents. At all other times they are found there in varying numbers. During the dioestrous interval (D) epithelial cells (chiefly nucleated) and leucocytes are present; in the prooestrum (P) only light staining cells with pycnotic nuclei; during oestrus (O) single, non-nucleated, eosin-staining cornified elements constitute the smear; these are clumped in masses in the early metoestrum (M_1), and invaded by leucocytes as this period progresses until most of the cornified elements disappear and a dioestrous condition again prevails (figs. 2 to 5). In stage D the vaginal content is viscous or stringy; in P it is

serous. As the O stage begins the epithelium may become dry, and then granular in the M_1 period. As the leucocytes invade the masses of C cells during the M_2 stage, the contents of the lumen become pasty, then milky, and finally stringy again as this stage merges into the dioestrous interval.

3. Histology of the vagina

During the D interval the epithelium is low (three to seven layers), has no clear-cut basement membrane, and is freely infiltrated with polymorphonuclear leucocytes. At the end of this interval, a basement membrane begins to be evident, mitoses become more frequent, new layers of cells are added, and leucocytosis ceases. In the P stage growth processes reach a maximum which thickens the epithelium to ten to thirteen layers. A distinct basement membrane is present in most places. Then the outer three to five layers begin to degenerate, as is shown by their loss of affinity for cytoplasmic stains and the pycnosis of their nuclei. These two areas become clearly separated by the formation of a granular layer. This is converted into a cornified layer (stratum lucidum), which is not superficial as would be supposed, but underlies three to five layers of nucleated cells. As the P merges into the O stage, these superficial layers disappear (probably through autolysis or continued cornification) until the cornified layer becomes superficial, at which time oestrus is evident. By this time continued growth has piled up the epithelium to a thickness of twelve or thirteen cell layers under the stratum granulosum which first formed at about the eighth layer. The definition of the lower layer of the germinativum from the adjacent stroma is very clear-cut. As the M_1 period sets in the cornified layer begins to be delaminated and the lumen of the vagina is filled with fragments or masses of C cells. The basement membrane becomes thinner and mesenchymal papillae indent the lower layers of the epithelium. Leucocytes filter in from the stroma and collect in the superficial layers of the germinativum. After they have accumulated in considerable numbers here, they pass on into the cornified masses in the lumen, stage M_2, and in a day's time may completely dissolve them.

Their continued action in the superficial part of the germinativum reduces these layers to the appearance of a reticulum containing large clumps of leucocytes. This process gradually declines until the vaginal epithelium returns to a typical dioestrous condition (figs. 6 to 13).

Growth activity seems practically at a standstill in the early D stage. Toward the end of this interval the growth curve begins to rise until it attains its maximum in a late stage P, after which it gradually falls during O and M_1 to its minimum in the late M_2 and early D stages.

4. The uterine changes

Although the uterus goes through a cycle of changes, they are far less striking than those occurring in the vagina. Gross changes consist of a marked hyperemia and distention during the P stage, which diminishes gradually during oestrus and disappears as the M_1 period progresses. During the D interval the uterus is anemic.

Histological changes in the uterus consist of periodic growth, degeneration, and leucocytosis of the epithelial cells. These phases coincide with those in the vagina. A hyperfunction of the uterine glands (judged by the distention of their ducts) is the evident cause of the distention of the uterine cornua during the P and O stages, at which time they are very transparent. Uterine glands are distributed everywhere throughout the uterine mucosa except along the line of attachment of the broad ligament. The glandular epithelium escapes much of the degeneration and destruction common to the other epithelial tissues. Mitoses are most frequent in the glands during the late D and early P stages, becoming less in number as functional activity increases. In other words, the growth wave in the glands slightly precedes that in the uterine and vaginal epithelium.

The shape of the uterine epithelial cells varies chiefly with the degree of distention of the cornua, and is consequently a poor criterion of growth. Degenerative processes are first apparent in this tissue in the fading of the basement membrane (which is so clear-cut during the P and O stages) into a pink-staining

band which includes the basal sides of the epithelial cells and the superficial stroma. This is quite marked in the M_1 stage before leucocytosis has begun, which would indicate that the destruction of the epithelium by leucocytosis is secondary to degenerative changes in that tissue. It is in this subepithelial zone that the leucocytes collect in greatest numbers and from which they further invade the epithelium. A few places appear to be exempt, retaining their healthy appearance. Seldom is a region found entirely denuded of its epithelium, but this tissue becomes markedly degenerate (figs. 14 to 16). This material does not show greater numbers of mitoses in the uterine epithelium near the openings of the gland ducts than in other regions.

5. Changes in the oviducts

The oviducts apparently escape entirely the periodic leucocytosis, so extreme in the rest of the genital tract. They do exhibit definite cyclic changes, however. Earlier in this paper the oviducts have been divided into segments distinguishable by the presence or absence of cilia, the height of the folds of the mucosa, and the thickness of the muscle layers. The segment leading from the periovarian sac is ciliated, the remaining portion has simple non-ciliated columnar epithelium.

During the P and O stages the nuclei of the ciliated portion are ranged in a quite regular row. As the M_1 phase of the cycle advances, some of them migrate to the free ends of the cells which loose their cilia and through which these nuclei are extruded. They may retain the appearance of normal nuclei or become pycnotic before they are extruded, but when lying on the free surface of the epithelium, are shrunken and dark staining. This process reaches its height during the M_2 and early D stages, at which time the epithelium may become greatly vacuolated. It is therefore of degenerative significance (figs. 18 and 19).

The non-ciliated portions of the uterine tubes also show varying degrees of vacuolization, which seems to result from a hypersecretion of this epithelium. As yet it has not been possible to correlate this with definite phases of the oestrous cycle.

Ova may be found in the tubes during the early D interval, and if this is very short, may still be present in the succeeding P stage. They remain in good condition in the oviduct, with the second maturation spindles and sometimes the polar bodies intact, for two and even three days. During the third day, in the segments proximal to the cornua, they may begin to fragment. Their degeneration here must be by autolysis, as no leucocytes are present in the lumen of the oviducts. If occasionally they remain intact and pass into the uterine cornua on the fourth day after ovulation, phagocytosis may be their fate, for leucocytes are present there if the mouse has not passed the dioestrous interval.

6. The ovaries

In a consideration of the ovarian cycle, two main subdivisions will be made for animals that do and those that do not ovulate spontaneously (i.e., without the added stimulus of sexual contact). Those only occasionally ovulating spontaneously are obviously intergrades and will not be considered separately.

Where ovulation is spontaneous, three ovarian structures are involved: the follicles, the corpora lutea forming after their rupture, and possibly interstitial tissue. Where ovulation is not spontaneous there are, of course, no corpora lutea present, but atretic follicles take on an added significance.

Large, normal follicles are always present in the P and O stages, while none are to be found in the M_1 period. This, with the added evidence deduced from the position of the ova in the tubes, checked by standardized corpora lutea, shows ovulation to occur at the end of oestrus. The follicles usually rupture synchronously, but this is not necessarily so. In twenty-seven animals studied histologically three showed almost a day's difference between the position of the ova in the tubes, which is checked by a difference in the degree of development of the corresponding corpora lutea. It takes a period equal to at least one oestrous cycle for the maturation of the follicle from a medium sized stage with primary liquor folliculi forming at the poles to a large one greatly distended with a single lake of liquor (fig. 20).

Corpora lutea of oestrus in the mouse differ in no details distinguishable histologically from those of pregnancy during the first four days of development at least. In only a few cases is "bleeding into the central cavity" found. Three days are required for the hypertrophy of the former granulosa cells and the ingrowth of the theca interna to completely fill the central lake of tertiary liquor folliculi. For five or six days (unless another ovulation intervenes) these newly formed corpora stain blue with haematoxylin, and are therefore distinguishable until the next oestrus. After this time they have an affinity for eosin. If size is taken as a criterion of development, corpora lutea of oestrus in the mouse do not attain their maximum until an age of from ten to fourteen days is reached. This usually corresponds with the second ovulation after the one initiating their growth as corpora lutea. Even at the third ovulation following their start they may be equal in size to five-day corpora (fig. 24). Thus, an ovary containing from ten to sixteen large, clearly defined corpora lutea, the result of three ovulations between the fifth and sixteenth days preceding, may again ovulate. After twenty days, at which time they are usually not superficially located, ingrowth of cells from the stroma obliterates their outlines.

In animals of the first class, then, there are always present in the ovaries one recent set of blue-staining and two, three, or four older sets of red-staining corpora lutea, and normal follicles of medium to large sizes. In the O period follicles attain their largest size, and the youngest set of corpora are solid and blue staining. In the M_1 stage the former corpora stain red and a more recent set of blue-staining ones are developing in the ruptured follicles. At this time (one day after ovulation) they contain large central lakes of tertiary liquor folliculi (fig. 22). The largest follicles are medium sized and liquor is forming at their poles. During the M_2 stage the ovary is not subject to the general leucocytosis occurring in the uterus and vagina at this time. In the middle of the D interval the blue corpora are solid and the largest follicles contain one fairly large lake of primary liquor folliculi. During the P stage the follicles become

more distended and an increase in size is apparent in the corpora lutea.

In the second class of animals (those which do not ovulate spontaneously) the follicles attain their largest size during oestrus, which may be considerably prolonged. The follicles failing to rupture, the cumuli disintegrate and atresia begins in the granulosa cells, continuing until the follicular epithelium is entirely gone. Contained ova may fragment in the late stages of follicular atresia or they may persist with maturation spindles intact until most of the granulosa cells have disappeared. The finding of several distinct sets of abnormal follicles in progressively later stages of atresia when no corpora lutea are present in the ovaries, but several oestrous periods have been recorded for the animal, makes it possible to approximately estimate the time required for a certain degree of follicular atresia. In late stages of degeneration, former large follicles are reduced to medium and even small size. Record of previous oestrous cycles is necessary to properly evaluate the conditions of follicular atresia in the ovary of the mouse.

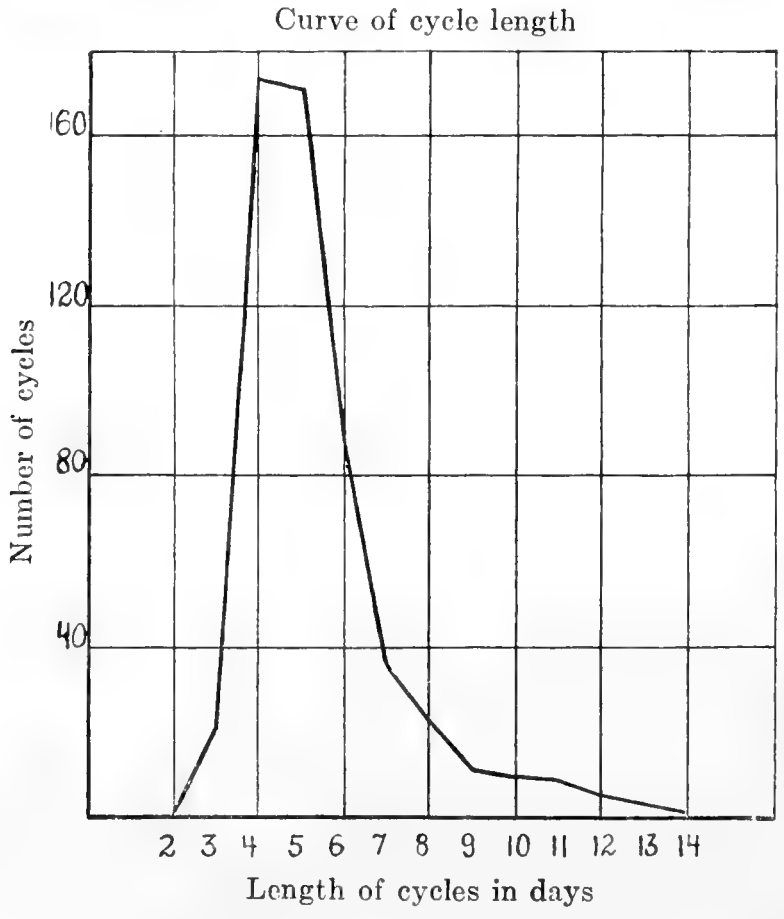
Interstitial tissue is present in the ovaries in varying but usually small amounts in the majority of the animals in this series. Its distribution is for the most part peripheral. Its presence or amount is not peculiar to any stage of the cycle.

7. TIME RELATIONS OF THE CYCLE

The duration of the cycle and of its various stages shows great variability. This is represented in the curve (graph 1) obtained by plotting the number of cycles against their duration in days. A total of 563 cycles is included. The mode of the curve falls at $4\frac{1}{2}$ days. An average duration of four to six days therefore represents the findings.

The dioestrous interval shows a greater variation than any other stage, lasting from less than a day to as long as fourteen days. Its length is usually from one to three days, however. In several cases of an extremely long D interval, the smears may at times show considerable amounts of cornified cells but the presence of leucocytes makes evident the diagnosis of the stage D.

Stage P, as diagnosed by the smear method, may be less than one day, because leucocytes may not entirely disappear from the superficial vaginal epithelium until after growth in the deep layers is well under way. Stage O usually lasts one or two days, but in several cases unbroken O smears have continued for nine days, and four days of heat are not uncommon. M_1 and M_2 stages usually last a day each and show little variation.



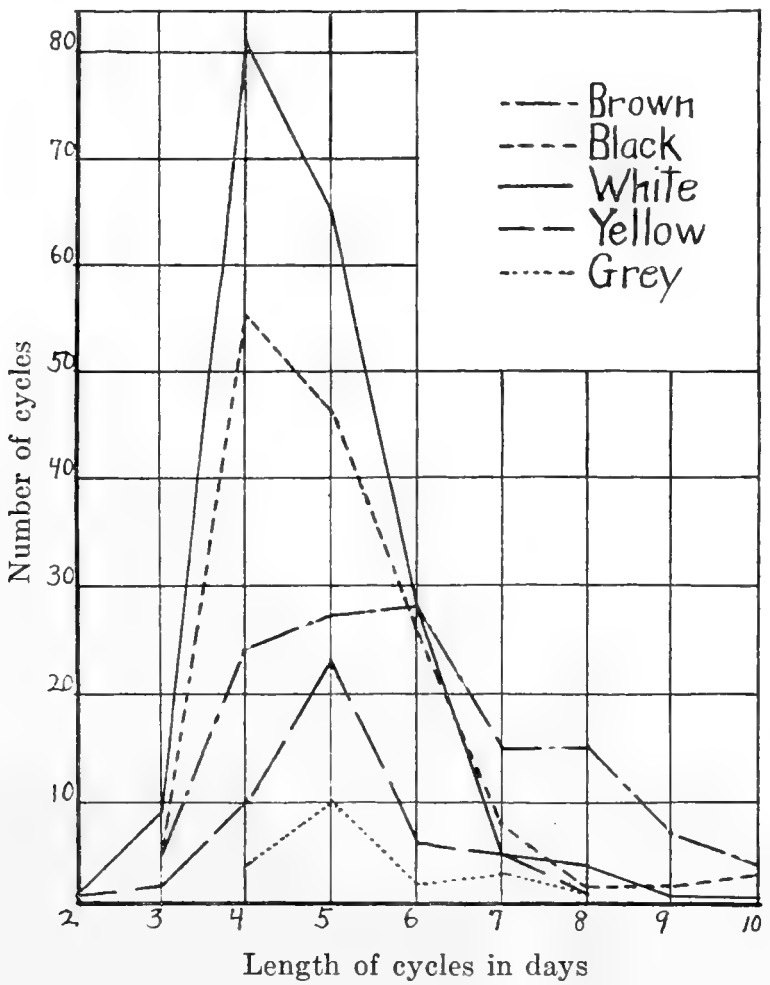
Graph 1

The data accumulated in this study indicate no correlation between the number of ova produced at an oestrus and the length of the cycle.

While variability of cycle length is great in the same animal, it is still greater in different strains. Plotting the number of cycles against their duration in mice of different coat color gave the following curves. The mode of the curve for brown coat color is six days. This strain has the greatest number of unusu-

ally long cycles. That for black and albino is four days. Albinos in our stock are browns minus the color factor. The mode for yellow and gray falls at five days. The identity of yellow and gray is more significant, since these grays are recessive derivatives of the yellow strain. The stock used included a black-eyed, dominant white mouse which has a lethal factor. Her

Variations in cycle length in different strains



Graph 2

average for seven cycles was 7.7 days, which, compared to the mode of four days for the albino strain, is a striking difference. This is only one case, but it raises the question of the relation of this lethal factor to the duration of the oestrous cycle. These findings indicate that a genetic factor is accountable for some of the variation in cycle length among different strains of mice.

As to variation in the same litter, my material is too meager to warrant any statement. However, the following data concerning four albino mice from the same litter are of interest. These animals were examined for 146 days, making from twenty-four to thirty cycles for each animal. The average cycle length is as follows:

TABLE 2

	ANIMALS			
	53 W	53 WNL	53 WNR	53½ E
Average, total.....	4.8	5.7	4.1	5.2
Number of unusually long cycles.....	0	4 (9, 11, 11, 10 days)	1 (8 days)	2 (10, 10 days)
Average minus long cycles.....	4.8	4.8	4.9	4.9

Reference to the first line shows considerable variation, but if the seven longer cycles, which are approximately twice the average length, be excluded, there is a difference of only 0.1 of a day, which is well within the limit of error.

It is difficult to generalize on the variation with age of the individual. The first and second cycles following puberty are usually longer than the average during later life.

Four mice were kept in a cold room at a temperature varying from 40° to 55°F. for five weeks during the middle of summer to see if continued low temperature would retard the cycle. It resulted only in their building an elaborate, covered 'house-nest' in which they spent most of the time. The duration of their oestrous cycles was not affected.

Tumors appeared in two mice which were undergoing routine examination, which was continued in one case until the tumor grew to an inconvenient size. The first cycle after the appearance of the tumor contained a seventeen-day dioestrous interval succeeded by a one-day oestrus. This was followed by an eight-day D interval, after which time she was mated. No pregnancy resulted. In this case sexual activity was at least greatly retarded.

8. GENERAL DISCUSSION

The literature on this subject is so extensive that it is practicable to discuss here only that bearing directly on special phases of this work.

In many particulars the oestrous changes in the mouse are similar to those described by investigators of other rodents. Important among the recent workers in this field are Heape ('05), on the rabbit; Konigstein ('07), on the rabbit, rat, and guinea-pig; Rubaschin ('05), Bouin and Ancel ('10), L. Loeb ('11), Lams ('13), Stockard and Papanicolaou ('17), on the guinea-pig, and Long and Evans ('20), on the rat.

Many of my observations merely establish for the mouse processes previously described in other rodents. It has been a pleasure to confirm in many instances the observations of earlier investigators, but in some respects conditions in the mouse throw a new light on certain of these sexual problems and a few new interpretations seem warranted.

In discussing the duration of the cycle, Heape ('00) says: "The differences in sexual periodicity in polyoestrous mammals in allied forms and even within the limits of the same species is due to variation in the quiescent period." In the mouse another possibility presents itself. The mode of the curve for the cycle duration in brown mice is six days—an interval of two days longer than that for black and white and one day longer than that for yellow and gray. Long 'heat' periods are more common to browns than to the other strains. Therefore, although the variation in the dioestrous interval accounts for much of the cycle variation, a good deal of it is also due to the variation in the duration of the oestrous stage itself. The variation in cycle length is great sometimes in the same animal and among different individuals from one litter. In the latter, however, it is less than in different strains of mice.

As stated earlier in the paper, albinos in our stock are browns minus the color factor. The mode of the curve for whites being two days shorter than that for browns suggests that with the loss of the determiner for color a shorter cycle has been effected. Again the modes of the curves of cycle duration of yellow and

gray mice coincide, and they are both derived from the same original pair of animals. These facts point to a genetic factor as partly responsible for variation in the oestrous cycle and direct attention to the ovum itself as the ultimate cause.

The present findings of a much shorter oestrous cycle in the mouse than had previously been suspected explains the apparent exceptions reported by Kirkham and Smith to an oestrous cycle of twenty or seventeen and one-half days' duration.

Kirkham ('16) reports a case in which "a set of normal eggs in the 2-cell stage was found six days post partem." Smith ('17) reports ovulation in a mouse six and one-half days after parturition. These may be explained by a failure to ovulate or of the ova to be fertilized at the oestrus following parturition, and the recurrence of another oestrous period five days later.

1. Oestrous changes in the genital tract

a. The vagina and cervix uteri. 1. There is in the mouse little discharge from the uterus into the vagina such as occurs in menstruating animals. Uterine epithelial cells are not often found in the smears. Also, although the uterine cornua during the P and O stages are greatly distended with fluid, the vaginal mucosa, especially during oestrus, is usually dry. Apparently the musculature of the cervix functions as an efficient sphincter.

2. In the mouse the massing of the cornified cells of the vaginal epithelium in clumps (when a standardized smear technique is used) indicates that ovulation has already occurred when that is to be spontaneous. It is therefore of importance in diagnosing ovarian conditions.

As to the fate of the leucocytes so abundant in the M_2 stage, many degenerate in the lumen. It was stated in the descriptive section that during the D interval the vaginal contents were viscous and stringy. These stringy masses when spread on slides show a fine web structure with polymorphs at the interstices entangling varying numbers of epithelial cells. The fine web processes seem to be made of the greatly attenuated protoplasm of the leucocytes.

3. Long and Evans ('20) have again called attention to the formation of the cornified and granular layers of the vaginal epithelium in the rat and guinea-pig as a remarkable histogenetic process because they do not form superficially. However, it should be observed that the overlying layers, although nucleated, early lôse their affinity for stain even before the appearance of granular layer which precedes cornification (described under animal 8, fig. 7).

b. The uterus. 1. Gross changes. The attention of investigators has been directed towards the uterus from the very beginnings of anatomical study, primarily because of its importance to the embryo during pregnancy. In the absence of pregnancy in the primates, its striking changes during the menstrual cycle have led to its designation as 'the organ of menstruation.' In the mouse the vagina, and also the oviducts to a less extent, share with it the typical oestrous changes.

2. Destructive histological changes. The amount of epithelial and connective-tissue destruction during the metoestrum and menstruation has been under continual controversy which seems to have compromised on "great variability even within the same species." In menstruating animals extensive removal of epithelium has been reported and denied, but nearly always bleeding occurs. Bleeding has also been reported in many of the lower mammals which periodically exhibit typical oestrus. Lataste ('87) has recorded bleeding during 'heat' in several European rodents. Stockard and Papanicolaou ('17) have reported an occasional slight bleeding in the guinea-pig. There is little denudation of the epithelium and only rarely bleeding during the metoestrum in the mouse. The process is restricted to the degeneration of the epithelium in situ (it seldom breaks free from the stroma) and heavy subsequent leucocytosis. This lack of severe destruction may be accounted for in the mouse by the rapidly ensuing prooestrum, or period of growth, which may set in three days after the metoestrum begins.

3. Is leucocytosis primary or secondary? Loeb ('11), in discussing this problem in the guinea-pig, says: "It is not very probable that the changes in the epithelium (of the uterus)

following ovulation are brought about by a disintegration of some of the epithelial cells." Stockard and Papanicolaou ('17), also working with the guinea-pig, say: "Large vacuoles are to be seen between the epithelial cells, and these are probably produced by the dissolving power of the leucocytes." Long and Evans ('20) state that destruction in the uterine epithelium in the rat is due to 'vacuolar' degeneration. A closely timed series of material in the mouse shows distinct degenerative changes evident in the substitution for the basement membrane of a light staining zone before leucocytosis begins. It seems more probable that these vacuoles in the uterine epithelium are comparable to those in the oviducts (where no leucocytosis occurs) and are evidences of degeneration which may be the cause of the leucocytosis. The evidence from the mouse indicates that epithelial degeneration is a primary, and the leucocytosis a secondary, phenomenon.

4. Regenerative changes. Many regions of the surface uterine epithelium as well as the glands escape destruction during a single metoestrum in the mouse. Also when active mitosis begins it does not appear to be restricted to regions adjacent to the openings of the gland ducts.

5. The distribution of glands. The distribution of glands throughout the uterine mucosa seems possibly to be of some significance in the consideration of the implantation of the blastocysts. Huber ('15), in his contribution to the embryology of the albino rat, calls attention to the even spacing of the implantation sites which are ranged along the sides of the cornua adjacent to the attachment of the uterine ligaments. In the mouse, in all the animals studied, glands were distributed everywhere throughout the mucosa except along this line. That this is the future site of placentae is interesting.

c. Cyclic changes in the oviducts. 1. The oviducts seem to have been overlooked by most investigators in considering degenerative changes during the oestrous cycle. So far as I have been able to find, no cyclic degenerative changes have been reported in them in the lower mammals. In summarizing the discussion of the question in man, Novak ('21) disposes of the

cases of tubal menstruation reported in the literature as for the most part occurring after hysterectomy from the stump of the oviduct. These are obviously not normal. Czyzewicz ('08) (quoted by Novak) reported after the study of six normal oviducts at different stages of the menstrual cycle that they did not share menstrual phenomena with the uterus.

The oviducts, uterus, and vagina have a common origin from the mullerian ducts, so possibly factors causing cyclic changes in the uterus and vagina may be expressed in some way in the oviducts. They are not, however, subjected to the periodic leucocytosis of the rest of the genital tract. The extrusion of nuclei in the ciliated portion, beginning in the early metoestrum and continuing in a marked degree sometimes to the middle of the following prooestrum, may be interpreted as of degenerative significance, paralleling as it does the stages of "degeneration and removal by leucocytosis" in the uterus and vagina.

2. The importance of certain structural features of the oviducts of the mouse. The careful work of Sobotta ('95), Huber ('15), and H. P. Smith ('17) had made the differentiation between the segments of the oviduct easy. The time of passage of the ova through the different segments as worked out by Smith, ovulation being calculated in the light of the observations of Long and Mark ('11), has been used in placing the time of ovulation and consequently the estimation of the age of corpora lutea.

3. The passage of the ova down the uterine tubes. The mechanism of the passage of the ova down the greater extent of the oviducts is still not understood in the mouse. Ciliary action accounts only for their entry into and passage through the segment proximal to the ovary. Peristaltic action may possibly furnish motive power for the rest of their passage. Waves of peristalsis were not, however, apparent in my material, but the oviducts were always somewhat distended. Valve-like folds of the mucosa at the entrance of the tubes into the cornua have already been mentioned as a possible check to backflow of fluid from the uterus. The arrangement of the muscle layers of the oviduct as they merge into those of the uterine cornu is

identical with that in the 'bile duct sphincter' recently emphasized by Mann ('20). It is possible that they serve such a function here. Surely, a back flow of fluid from a distended uterus would be fatal to the passage of ova down the tubes, if that be by peristalsis.

d. The ovaries. Many interesting ovarian problems have arisen in the course of this work.

a. The follicles and ovulation. 1. Spontaneous ovulation. Certain species of animals (the rabbit and ferret are examples) usually do not ovulate spontaneously at oestrus. There has been much discussion concerning this question in the mouse. Tafani ('89), Sobotta ('95), and several later investigators claim an ovulation without added sexual stimulation. Garlach ('06) and Bouin and Ancel ('09) state that in the mouse ovulation is dependent upon coition. That it usually occurs spontaneously the first day after parturition has been emphasized by many investigators, and made use of by Long and Mark ('11) in artificial insemination.

Possibly the size of the ovarian vessels immediately following parturition may be the deciding factor at this ovulation in some mice, but it is certain that in many mice not lately pregnant ovulation is not spontaneous at every oestrus and in some virgin mice it need not have occurred at all, although several oestrous cycles have been recorded, which indicates the presence of ripe follicles. It is obvious, therefore, that the classification of the mouse as a species ovulating spontaneously is not accurate. While it is not possible to diagnose the absence of spontaneous ovulation by actual cell content of the smear, unusually long oestrous periods in certain animals may indicate a failure or at least a difficulty in ovulation, for the presence of ripe follicles seems universal in all animals in the oestrous condition.

2. Is ovulation in litter-bearing animals synchronous? Long and Mark ('11), in considering the maturation of the ovum of the mouse, state that ovulation is a synchronous process, although imperfectly so. Sobotta and Burchard report similarly for the rat. Huber finds fertilized ova usually bunched in their passage down the oviduct in the rat, which would indicate a synchronous

ovulation. In three mice of this series a distinct difference in the position of the ova in the tubes and a difference in degree of development of the corresponding corpora lutea may indicate that the synchronism is less perfect where mating or artificial insemination do not occur. In these three mice the ova ovulated at one oestrus were plainly separated into two groups with a day's difference between their ovulation time.

3. The number of ova produced at one ovulation. The number of ova produced at an ovulation as an indication of the degree of fecundity of an animal is always of interest. When approached from the number in new-born litters, the failure of insemination, faulty implantation, intra-uterine death, and mortality at parturition introduce an extensive error. Therefore, the problem is most accurately solved through ovarian studies.

Counts of recent corpora lutea checked in many cases by comparison with the number of corresponding ova in the tubes, the number of the next older set of red corpora lutea, and also the number of largest-sized follicles in prooestrous and oestrous ovaries give a total of 449 ova produced at forty-nine oestrous periods.[4] This makes an average of nine ova to an oestrus. Of these 449, 230 were counted in left ovaries and 219 in right, showing the function almost equally divided between the two ovaries in virgin and non-mated animals. Long and Mark, in a large series, obtained an average of seven ova from the ovulation following parturition—a 22 per cent difference. It is possible that a supervening pregnancy reduces the number of ova produced at the oestrus following parturition.

4. The possibility of alternation of major function. The finding of a variation in the numbers of embryos in the horns of bicornuate uteri has raised the question as to alternation of major function between the two ovaries. In twenty-one animals in which data were available for the number of ova produced at two, three, and sometimes four oestrous periods, four show marked and two slight alternation of function, while in

[4] All large follicles need not rupture, which introduces a slight error into this data.

the others the function is quite evenly divided between the two ovaries.

5. The period required for the growth of follicles from medium size to ovulation size. My material points to an interval of surely not more than two cycles (eight to twelve days) and probably only one (four to six days) between the beginning of the formation of primary liquor folliculi at the poles of the medium-sized follicles and the time of their final distention and rupture (fig. 20).

6. Atretic follicles. An observation which seems almost universal, although it varies greatly in degree in different ovaries, is the presence of atretic follicles of all sizes. Some investigators have assumed that ovulation is always spontaneous and concluded that follicles atrophy without regard to the stage of the oestrous cycle. This conclusion does not necessarily follow the finding of atretic follicles of different sizes in the mouse ovary. There are two possibilities: first, follicles at any stage of development may atrophy or, second, they may attain full size, but fail to rupture at a certain oestrous period, and begin atrophy and resorption. The finding of maturation spindles in the ova in many of these atretic follicles points to their former large and mature state. Continued atrophy may decrease the size until there are few granulosa cells present and most of the liquor folliculi has disappeared. It has been possible to trace sets of atretic follicles corresponding in progressive degree of atresia to previously recorded oestrous periods through a time equivalent to three full cycles. In some of these the ova have completed their intra-ovarian maturation stages, in others the nuclei are in a resting condition. The former points to the follicular apparatus, the latter to the ovum itself as the cause of atresia, which seems to be by cytolysis of the follicular epithelium.

b. Corpora lutea. 1. Differences in those of pregnancy and oestrus. So much importance has been attributed to the corpora lutea that a study of those of oestrus has been followed with great interest. The time relations of the position of the ova in the uterine tubes has been so carefully worked out that an estimation of the age of these corpora is quite accurate.

Sobotta ('95) has standardized the development of the corpora lutea of pregnancy in the mouse, and carefully timed material has been available in the Washington University Anatomical Collection with which to check this work. Therefore, a comparison of the corpora lutea of oestrus with those of pregnancy has been possible. Sobotta states that their persistence and ultimate size is not altered by conception. Surely, there is no difference discernible histologically during the first four days, and if their later size be any criterion as to the degree of their development, they do not reach a maximum until two new sets have been added, i.e., eight to twelve days after that ovulation which resulted in their formation.

2. Do they inhibit ovulation? Beard ('98) has been followed by many investigators in advancing the idea that corpora lutea prevent ovulation. The fact that they are present in greatly hypertrophied form during pregnancy and that ovulation does not occur at this time seems conclusive enough proof.

Loeb ('18) first proved experimentally, by the removal of the corpora lutea of pregnancy in the guinea-pig after they had ceased to be essential to the tenure of the fetus, that a new ovulation could be induced earlier than would have occurred otherwise. He then extended the work to the corpora lutea of oestrus in non-pregnant animals and found the same effect of inhibition on ovulation. Papanicolaou ('20) has confirmed these experiments. The conclusion drawn was "the corpus luteum, itself the result of an ovulation, provides a mechanism preventing ovulation." However this may be in the guinea-pig, it is surely not normally applicable to the mouse during oestrus for (because of the shorter cycle) ovulation occurs when two or three sets of recent large corpora lutea are present in the ovary. In litter-bearing animals the corpora lutea constitute no inconsiderable part of the ovaries. Might not ovulation following their excision be merely an expression of the compensatory hypertrophy of the remaining ovarian tissue? Corpora lutea of oestrus most surely do not normally prevent ovulation in the mouse.

3. Do they exert a destructive influence on the mucosa of the genital tract? Frankel ('03) attempted to prove that the corpus luteum by means of an internal secretion exerted a destructive influence on the uterus. Although newly forming corpora lutea are present during the metoestrum in the ovaries of mice which ovulate spontaneously, in those which do not, none need be present. And yet typical metoestrous degenerative changes in the genital tract occur. Consequently, the corpora lutea can hardly be considered as the cause of cyclical degeneration.

4. Do the corpora lutea furnish a growth stimulus? Stockard and Papanicolaou think it probable that the corpora lutea of oestrus exert a protective influence on the uterine and vaginal mucosae, because they find well-developed corpora present during the stages in which no degenerative changes are apparent in these organs, and because corpora of oestrus begin to retrogress before the next metoestrum sets in. They write: "The facts obtained in the present investigation might not fully warrant the position that the corpus luteum really exerted an active protective influence over the uterine mucosa, but they certainly in no sense suggest, and actually speak against, any injurious action on the mucosa by the secretion of the corpus luteum." This may be taken as a constructive suggestion to combat Frankel's views. That the corpora lutea are not the principal factors in the growth phase of the genital tract during the prooestrum in the mouse is plain for two reasons: 1) In those animals where spontaneous ovulation is the rule and where two or three sets of large corpora are always present, these degenerative changes occur just the same. 2) In animals that never have ovulated spontaneously, and where consequently no corpora are present in the ovaries, the same regenerative growth processes occur. Consequently, Stockard and Papanicolaou's explanation of growth under the protective influence of a secretion from the corpora lutea is hardly applicable to the mouse.

Therefore, evidence from the mouse brings strongly into question the value of corpora lutea of oestrus, 1) as inhibitors of ovulation, 2) as sources of destructive hormonal influence, or, 3) of endocrine growth stimulus exerted on the uterus.

c. The cause of cyclic oestrous changes. Many investigators have postulated theories from histological evidence. Since 1900 the problems have been approached also by experimental methods and a complicated mechanism built up on experimental evidence to account for oestrous changes. A short review of these results seems advisable.

1. Is the cause of oestrous phenomena inherent in the genital tract itself? Heape believed that oestrus and menstruation might occur after the removal of the ovaries. Consequently, he postulated an extra-ovarian cause of these phenomena. Since 1900, however, accumulated evidence from spayed animals has proved that without the ovaries the oestrous or menstrual cycle slowly disappears and the uterus and vagina atrophy. Halban and others, and recently Stockard and Papanicolaou ('17), have called attention to a cyclic atrophy following ovariotomy. This is also evident in the mouse in a periodic appearance at intervals equivalent to the oestrous cycle of cornified cells in the smear, always in the presence of leucocytes, however. From this, cyclic changes inherent in the uterus itself might be implied. Without the presence of the ovaries, however, no growth or regenerative processes occur. Might not cyclic degeneration in the uterus and vagina after spaying be merely secondarily induced by ovarian influences?

Temporarily let us exclude from consideration as improbable any inherent cyclic nature of the uterus itself and see if the ovaries may supply the cause. Three structures are present there that might be responsible: the follicles, the corpora lutea formed after their rupture, and possibly interstitial cells.

2. The follicles or the interstitial tissue. Large follicles distended with liquor folliculi have almost invariably been reported present during the prooestrous and oestrous periods in mammals, although the work of Heape ('98) and Leopold ('94) throws some doubt on their being always present in the primates. Consequently, many investigators believe the ripening follicles to be the cause of these phenomena. They are present in all mice studied during the prooestrous and oestrous stages.

Marshall ('14) believes he has experimentally disproved the ripening follicles as a factor in the mechanism of cyclic sexual changes. For criticisms of this work the reader is referred to Stockard and Papanicolaou ('17) and Robinson ('20). It seems to the writer that Marshall's conclusions are not justified. Marshall falls back on the ovarian interstitial tissue as the cause of oestrus. Interstitial tissue in the ovary is a very ill-defined, intangible substance. Any cells not connective-tissue cells appearing between the follicles may be called interstitial. If they bear a resemblance to secreting cells they may be called typical interstitial cells. They are believed by most investigators to be epithelial in nature and to have an origin similar to that of the ova and the follicle cells. The interstitial cells in the mouse ovary are chiefly peripheral in distribution and not to be confused with those appearing in many forms in the theca of atretic follicles or originating from old corpora lutea after connective-tissue ingrowth. There seems to be no periodic hypertrophy of these cells such as would be expected if they were the cause of cyclic oestrous changes in the genital tract.

3. The corpora lutea. As has already been pointed out, the corpora lutea, have had attributed to them the following functions, the inhibition of ovulation even in non-pregnant animals and the cause of degenerative menstrual changes in the uterus of primates, and metoestrous changes in the uterus and vagina of other mammals (by analogy), and, finally, the source of a protective influence on the vaginal and uterine mucosae.

In mice in which ovulation does not occur spontaneously, and consequently in whose ovaries there are no corpora lutea, the normal oestrous cycle goes on regularly. In mice that ovulate spontaneously several healthy sets of corpora lutea are present during the whole cycle. There seems to be no escape from the conclusion that in the oestrous cycle in the mouse (unaffected by pregnancy) the corpus luteum has no primary causative function.

Since the evidence seems to discredit both interstitial tissue and corpora lutea as causative factors in the phenomena of the normal oestrous cycle, the follicles are the only remaining ovarian possibility.

4. Evidence for the follicles. In the mouse and in most mammals studied very large follicles are present in the ovaries during the prooestrous and oestrous stages and absent during the metoestrum and dioestrous interval. The histological evidence from many sources in all mammals studied excepting certain primates overwhelmingly points to the presence of large follicles as the cause of oestrous changes. However, no direct experimental proof has yet been produced.

The cause of long oestrous periods. Animal ♯20 was diagnosed by the smear method to be in stage M_1 when killed. If this diagnosis was correct, degenerative changes should have begun in the genital tract and ovulation should have occurred one or two days previously. On histological examination, however, the epithelium of the vagina and uterus was found to be in an actively growing condition and a set of large normal follicles were present in the ovaries. Four normal oestrous periods had been recorded for the animal and yet the ovaries contain no corpora lutea. Consequently, ovulation had not been spontaneous and it is probable that the large follicles present at death would not have ruptured had the animal lived. Her present oestrus was first apparent four days before her death. Would it not seem probable, then, that the continued growth phase in the genital tract might (in the absence of ovulation) be directly dependent upon the retention of mature ova in large normal follicles? If so, this is a strong suggestion as to the cause of oestrus. In mice that do not ovulate spontaneously (therefore the simplest condition), they are always present during the pro-oestrous and oestrous stages and absent or atretic during the met- and dioestrum. As the possibility of the corpora lutea and the interstitial tissue actively sharing as causes seems slight in the mouse, all the evidence points to the presence of large normal follicles as the cause of the growth and congestion of the anabolic periods and the absence of normal follicles as the primary cause of the catabolic periods in the genital tract.

In animals that ovulate spontaneously, the rupture of the follicles is the dividing line between these two phases, while in animals that require an added stimulus for ovulation, atresia

of the follicles is coincident with the beginning of the metoestrum. This indicates that the growth and congestion of the pro-oestrous and oestrous stages are caused by maturing ova in large normal follicles, and the degeneration and removal by leucocytosis of much of the uterine and vaginal epithelium results after the extrusion of the ovum from, or its atrophy in the follicle.

That ovulation is the dividing line and that mice of different strains have distinct modes of cycle length seems to point to the maturing ova themselves as the ultimate cause of growth changes and the absence of them from the follicle as the cause of degenerative phenomena of the oestrous cycle.

9. SUMMARY

1. External signs are unreliable criteria of oestrus in mice. The presence of cornified cells in the vaginal smear is a much more accurate indication. When these cells appear in masses, ovulation has usually occurred.

2. The chief changes in the vaginal epithelium are its rapid growth, the formation of a stratum corneum, and (after ovulation) its degeneration and removal by leucocytosis. The stratum germinativum is also partly destroyed. It may grow from four to six to twelve or thirteen layers in thickness in one day.

3. There is considerable degeneration and leucocytosis in the uterine epithelium which is, however, seldom removed from the stroma. Bleeding rarely occurs in the mouse, but a heavy leucocytic infiltration takes place during the metoestrum.

4. Periodic degenerative changes in the oviduct parallel those in the rest of the genital tract. They are evidenced in extrusion of nuclei from the ciliated epithelium.

5. Ovulation is the dividing line between the anabolic and catabolic phases of the oestrous cycle, maturing ova in large follicles always being present during the pro-oestrum and oestrum, while newly forming corpora lutea or large atretic follicles replace them in the metoestrum.

6. Ovulation is not always spontaneous in virgin or unmated mice. In some it is regularly spontaneous, in others sporadically so, and in a few it seldom occurs without an added stimulus.

7. The average duration of the cycle is four to six days. The mode of the curve for brown mice is six days; for yellows and grays, five days; for blacks and albinos, four days. The yellows and grays were descendants from one pair of mice, the albinos are browns minus the color factor. Therefore, a genetic factor seems partly responsible for variation in cycle duration, and this may be tied up with the determiner for coat color.

8. Ovulation need not necessarily be synchronous in unmated mice.

9. Pregnancy may reduce the number of ova produced at the ovulation following parturition.

10. There is no difference discernible histologically between corpora lutea of oestrus and pregnancy during the first four days of their development.

11. Corpora lutea of oestrus do not normally inhibit ovulation in the mouse.

12. In mice that ovulate spontaneously two or three sets of corpora lutea of oestrus may be present at all times; in mice that do not ovulate spontaneously corpora lutea may be entirely absent, and yet normal oestrous cycles are experienced in both types of animals. Therefore, the writer concludes that they have no primary causative relation to oestrous changes in the genital tract.

13. As ovulation or the beginning of atresia of follicles is the dividing line between the anabolic and catabolic phases, and as the genetic factor summarized in no. 8 points to the ova themselves, the conclusion is drawn that the presence of maturing ova in large follicles is the cause of the prooestrum and oestrus, and that the removal of the ova at ovulation (or their atresia if this fails to occur) is the primary cause of the degenerative changes of the metoestrum.

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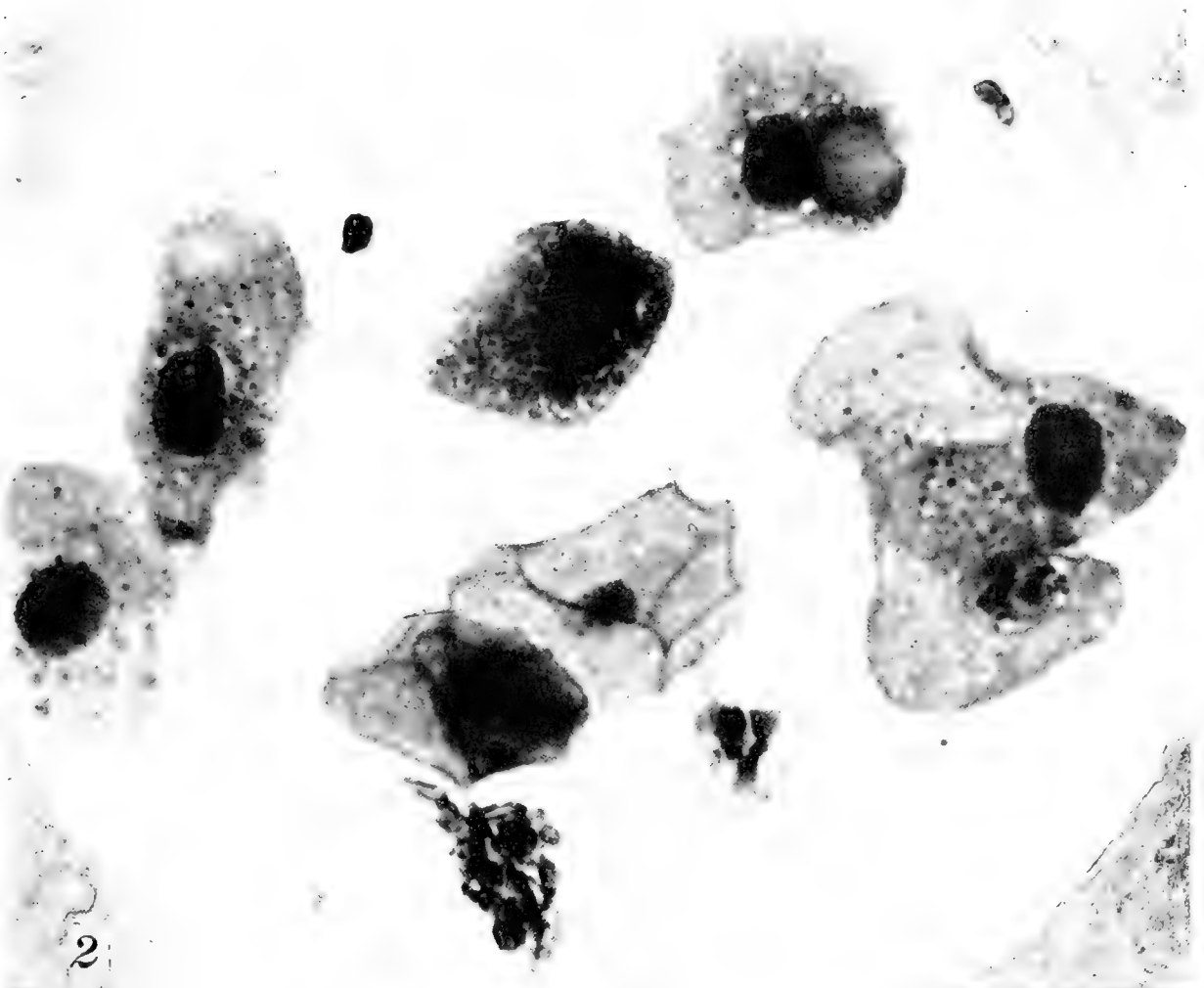
PLATES

PLATE 1

EXPLANATION OF FIGURES

2 Epithelial cells of the prooestrous vaginal smear. × 800. Cytoplasm takes a blue or purple tinge when stained with haematoxylin and eosin.

3 Epithelial cells of oestrous vaginal smear. × 800. The nuclei have lost their affinity for basic dye; the cells are cornified and stain a bright red with eosin.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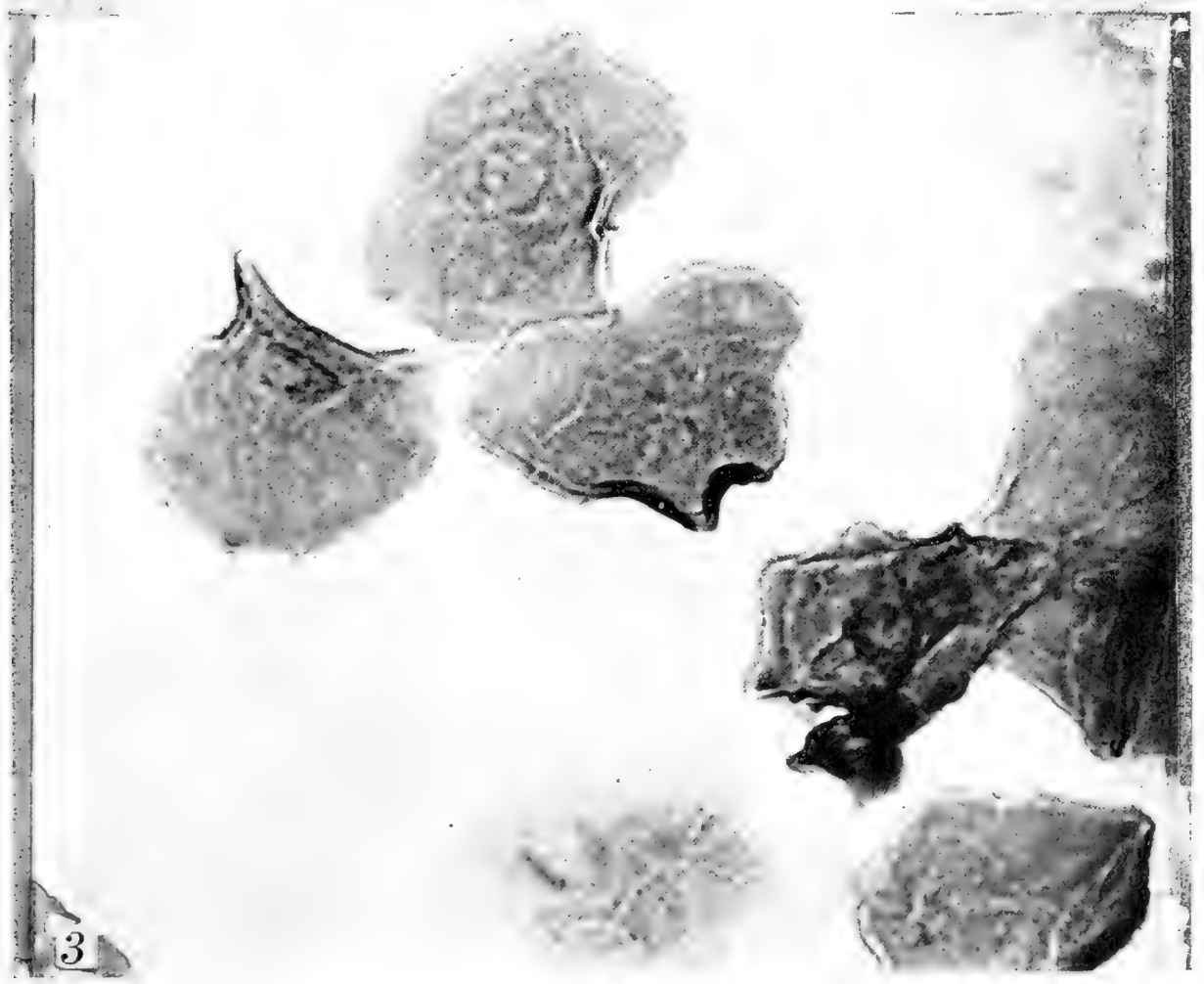
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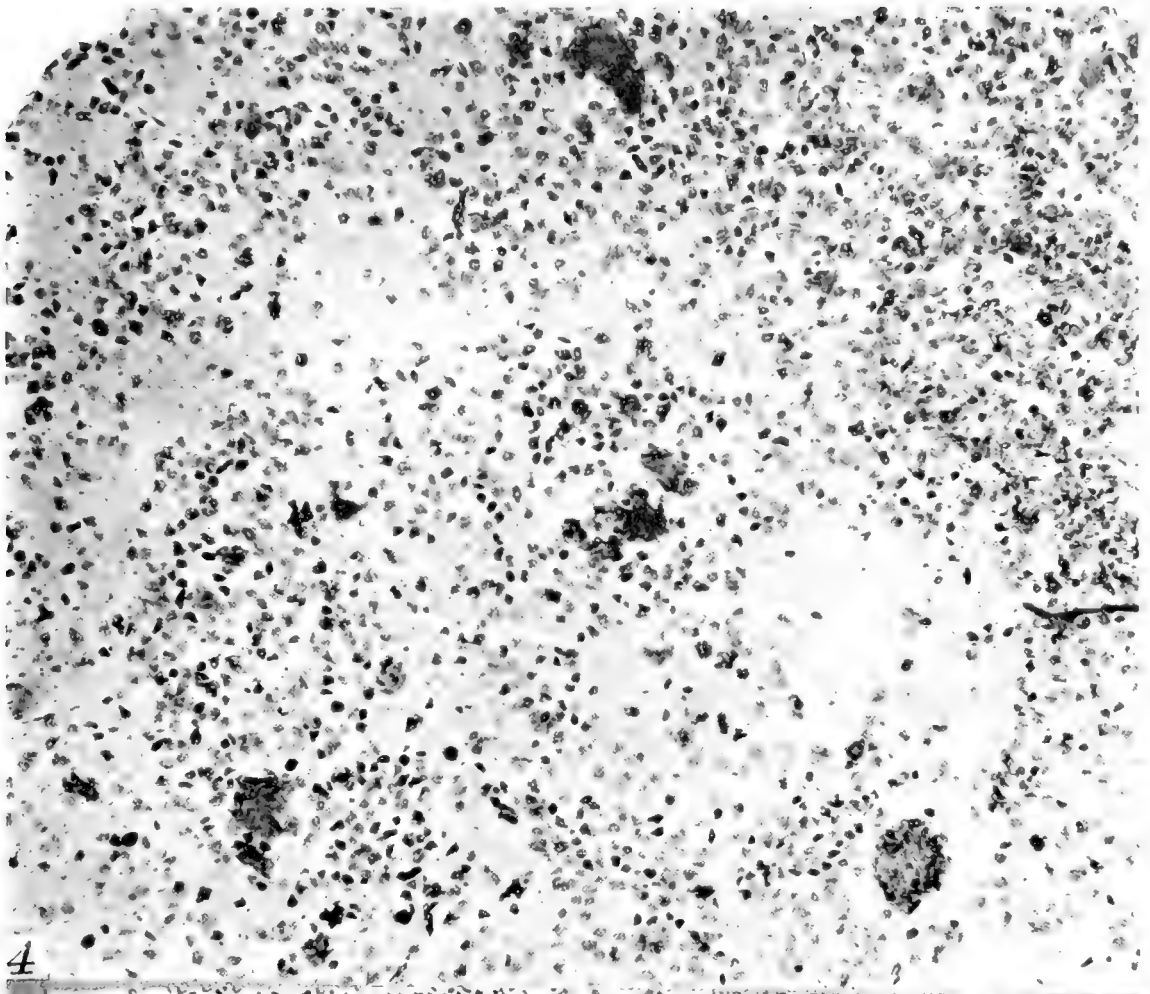
PLATE 2

EXPLANATION OF FIGURES

4 Stage M_1 smear. × 35. Cells are similar to those in figure 3, but all degrees of cornification are represented by variable staining affinity, and clumps or masses of cells are frequent.

5 A late stage M_2 smear. × 125. Non-nucleated cornified cells of the previous stage (massed in center) and nucleated epithelial cells of the deeper epithelium surrounded by great numbers of polymorphonuclear leucocytes. Some of the nucleated cells show clear exoplasmic zones indicating the extraction of eosin staining cytoplasm without the entrance of the leucocytes into the cells.



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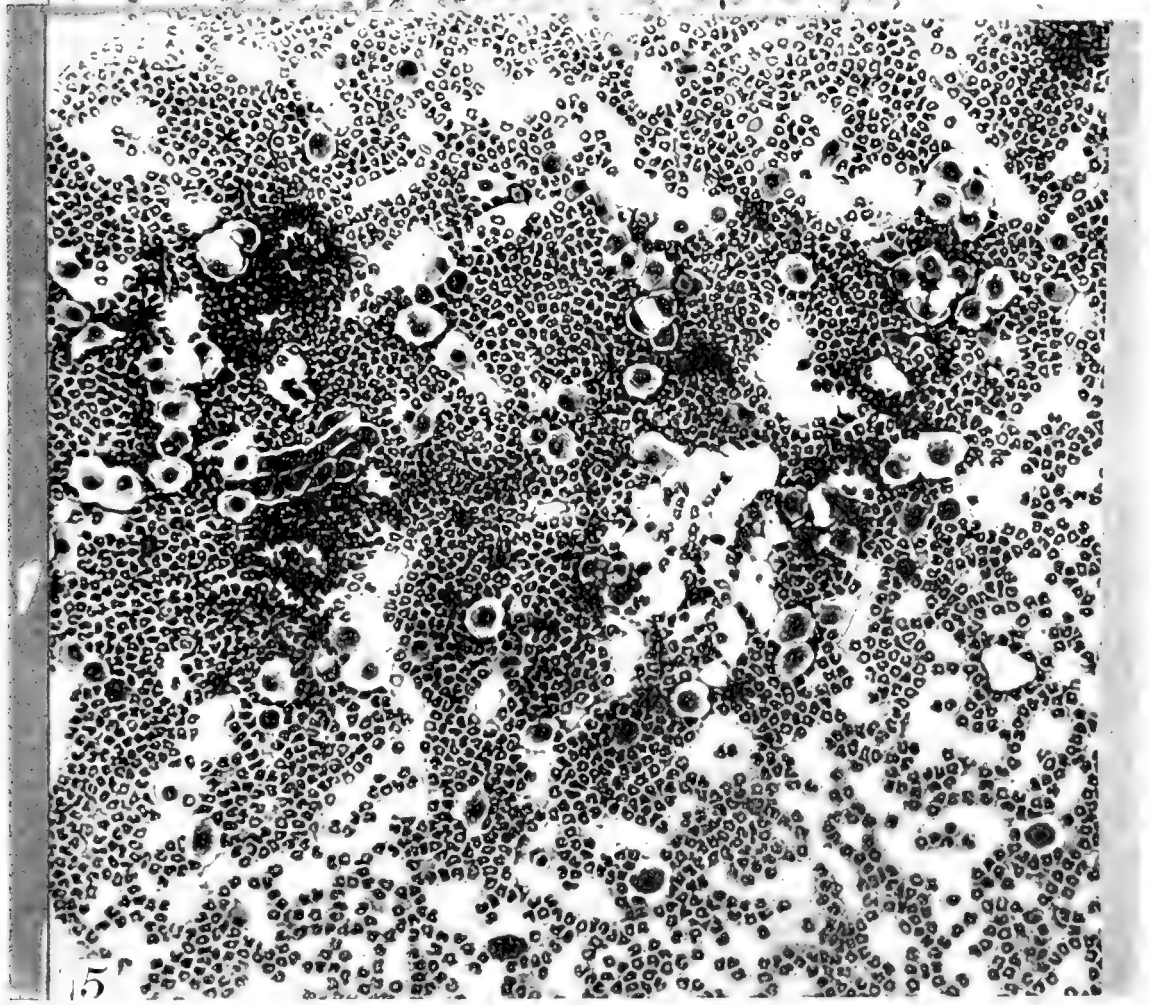

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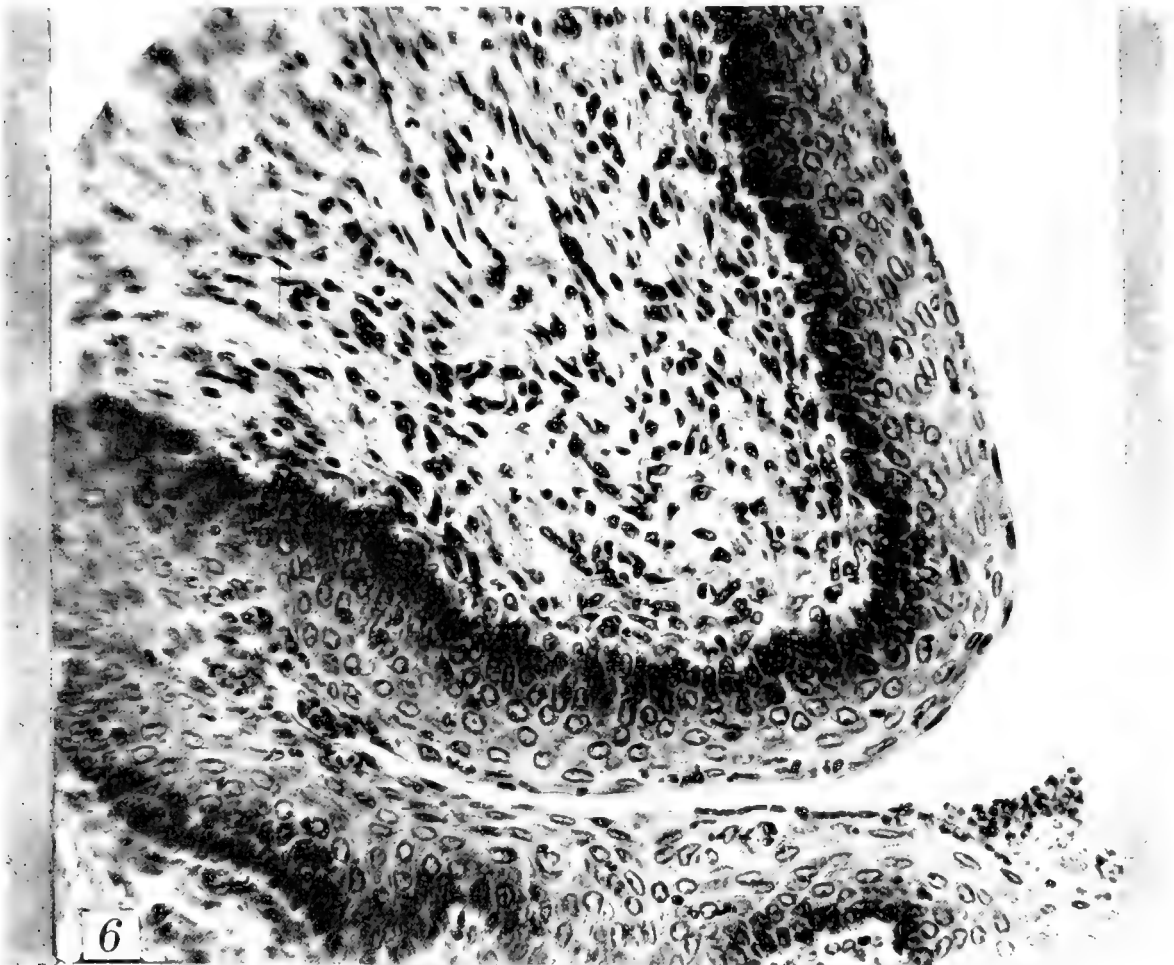
PLATE 3

EXPLANATION OF FIGURES

6 Vaginal epithelium of the D interval. × 275. Leucocytes are still present, but chiefly in the superficial layers. There is no clear-cut basement membrane.

7 Section of half of the vagina of the early P stage. × 60. Two zones are clearly defined by staining reaction before either granular or horny layers appear (animal no. 8).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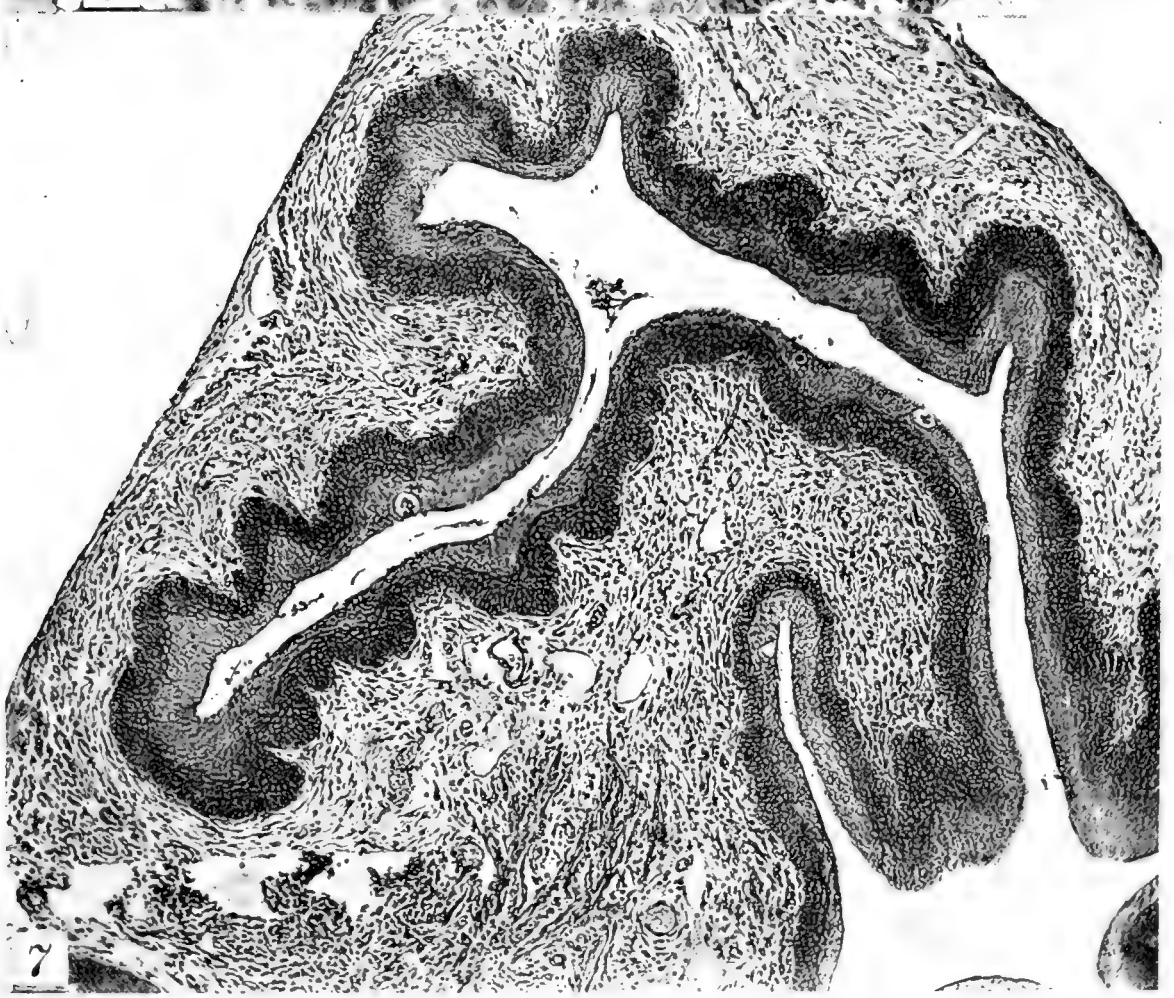
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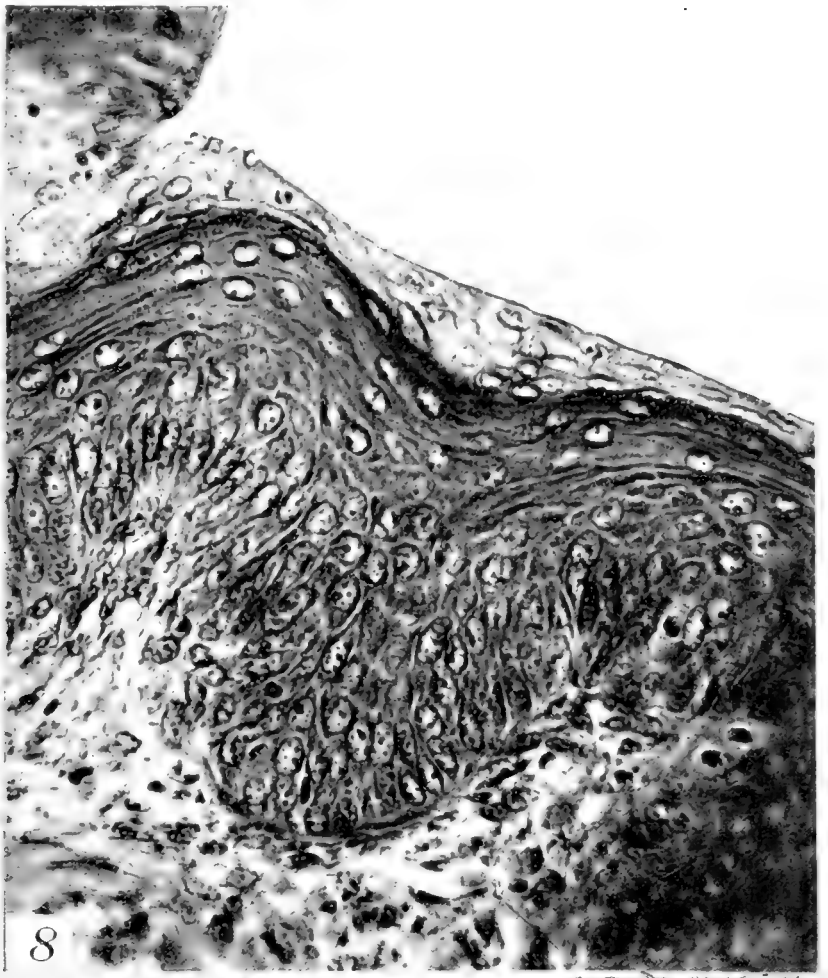
PLATE 4

EXPLANATION OF FIGURES

8 Vaginal epithelium of a later phase of stage P. × 275. The granular layer now clearly separates the two zones figured in 7 (animal no. 9).

9 The stratum lucidum of the corneum is forming. × 265. (Animal no. 10.) Sloughed-off nucleated cells make up the stage P smear.

10 Section of the vagina in stage O. × 45. Corneum is well formed, superficial, and still intact. Free cells form the O smear.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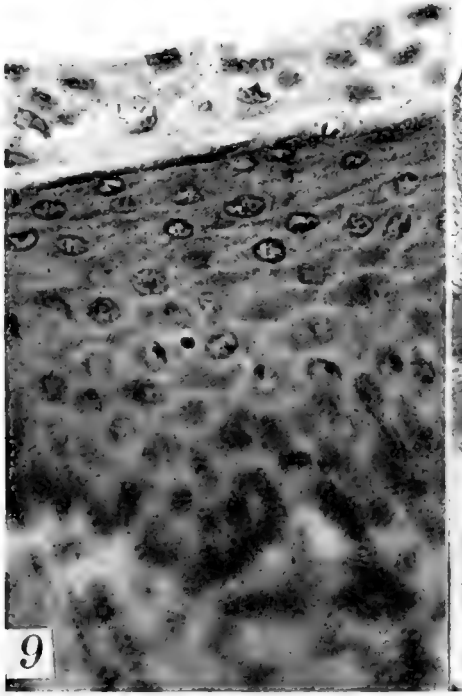
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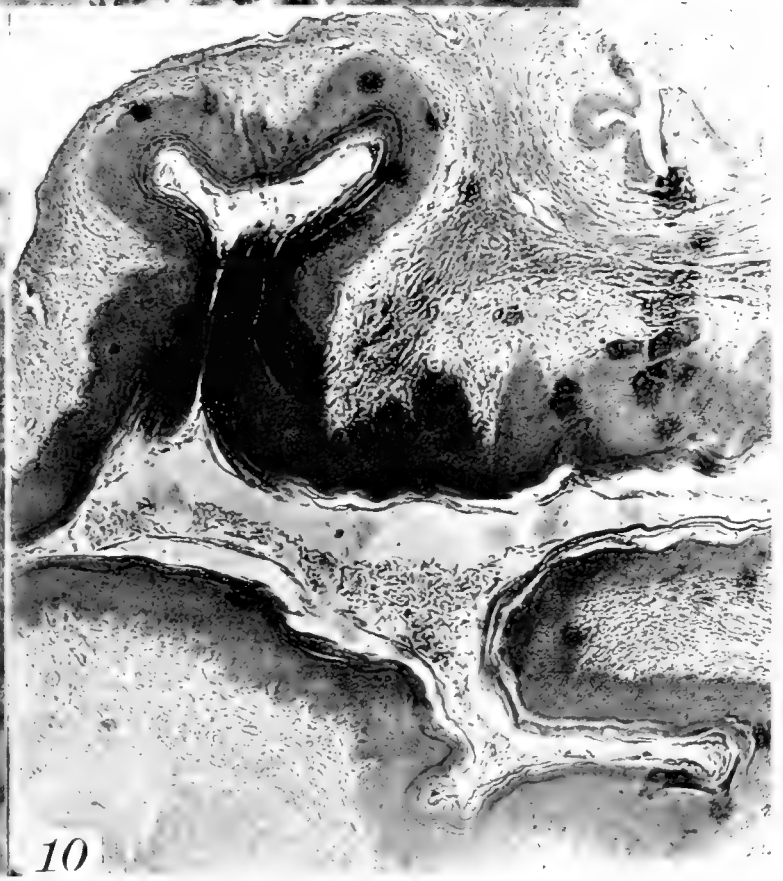
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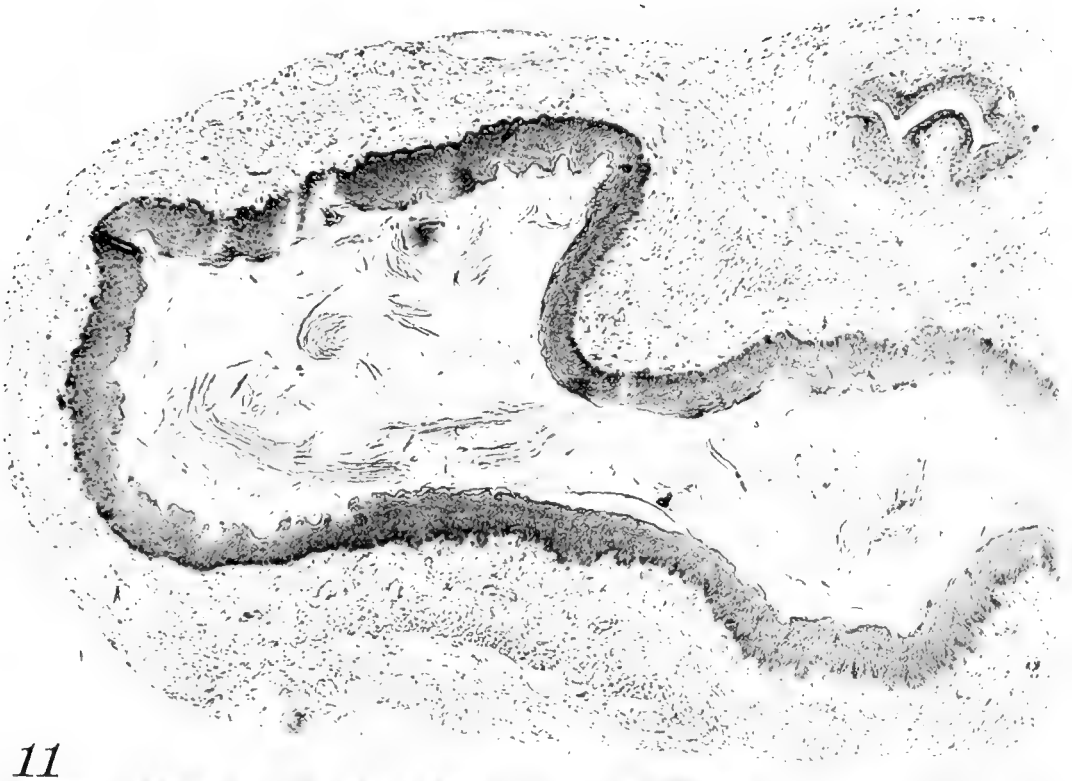
PLATE 5

EXPLANATION OF FIGURES

11 Vagina in stage M_1. × 65. Corneum is completely delaminated into the lumen. Leucocytosis has not yet begun.

12 An early M_2 stage of the vaginal epithelium heavily infiltrated with leucocytes. × 180. Few have as yet entered the masses of cornified cells in the lumen.



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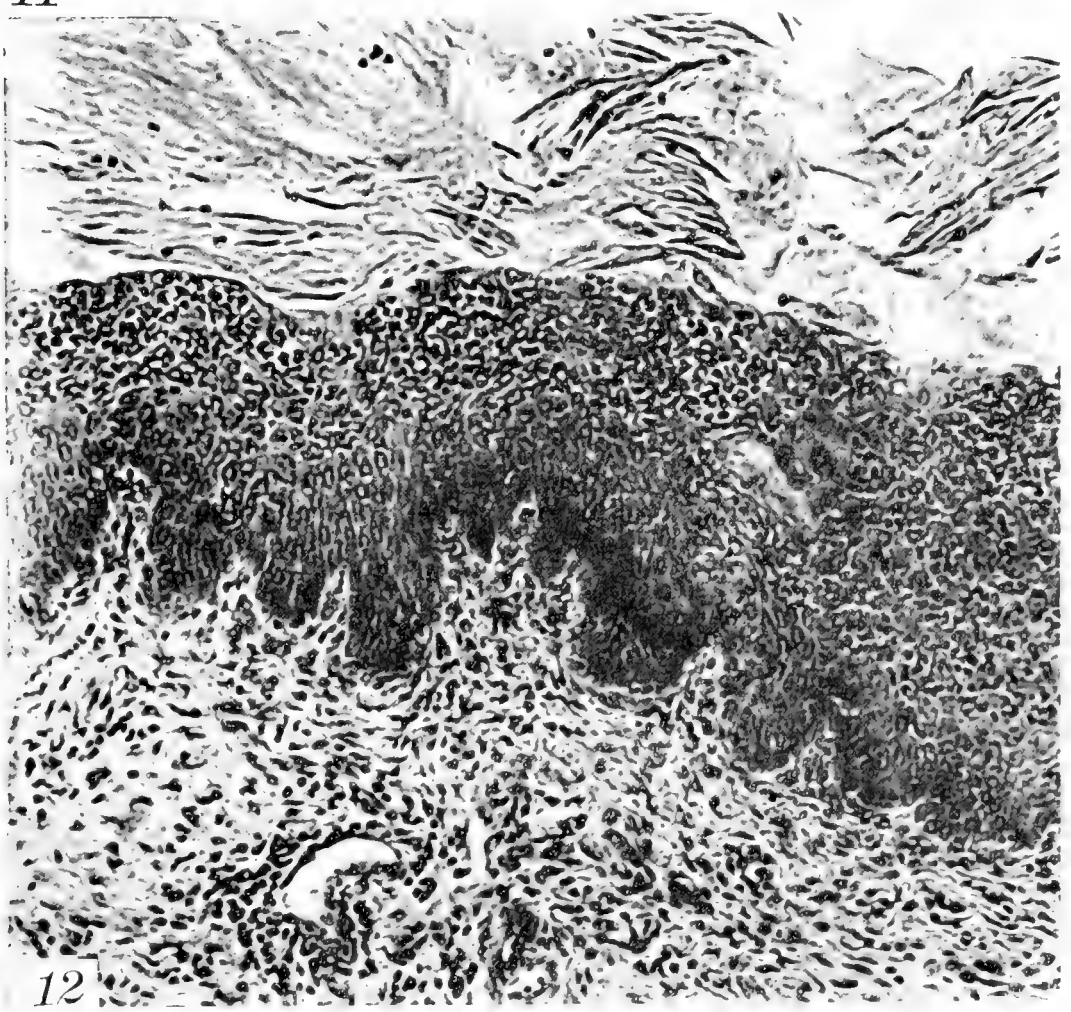

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PLATE 6

EXPLANATION OF FIGURES

13 Vaginal epithelium of a late M_2 stage. × 850. Groups of leucocytes have dissolved out lacunae in the superficial germinativum and enormous numbers have invaded the cornified masses in the lumen.

14 Section of the uterine cornu during stage P. × 55. The section does not show the distention apparent before fixation. Glands are moderately distended.

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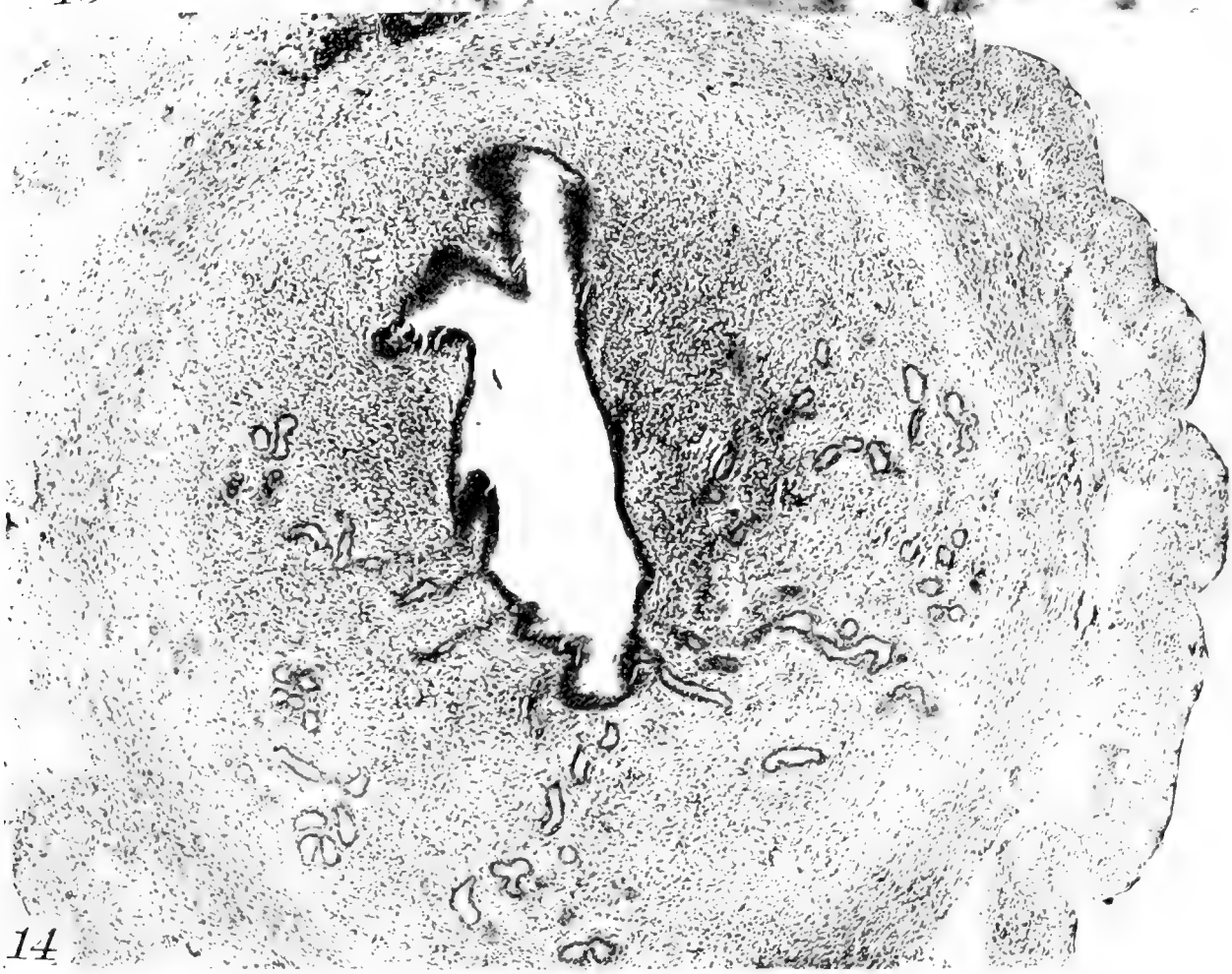

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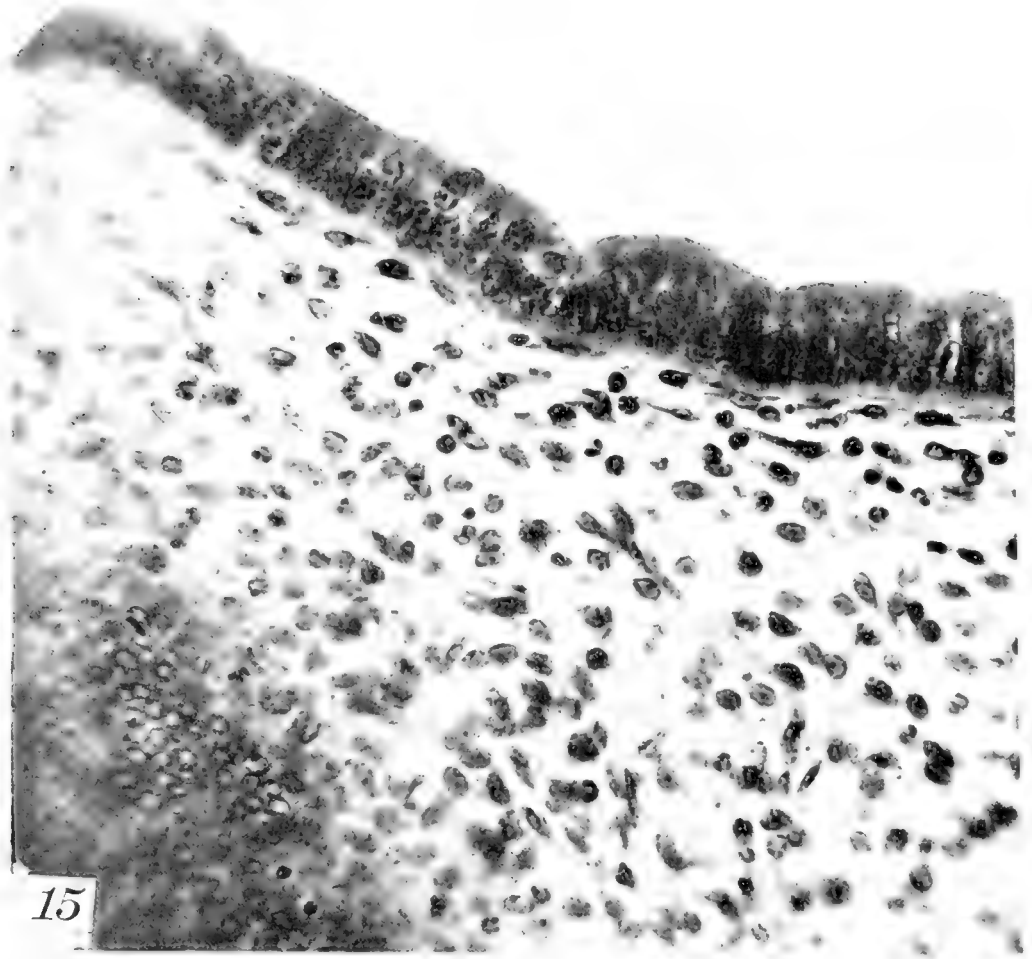
PLATE 7

EXPLANATION OF FIGURES

15 Mucosa of the uterine cornu during stage O. × 365. Note the clear-cut basement membrane and high columnar cells.

16 Uterine mucosa during early stage M_2. × 550. The distinct basement membrane figured in 15 has been replaced by a light pink staining zone containing leucocytes.



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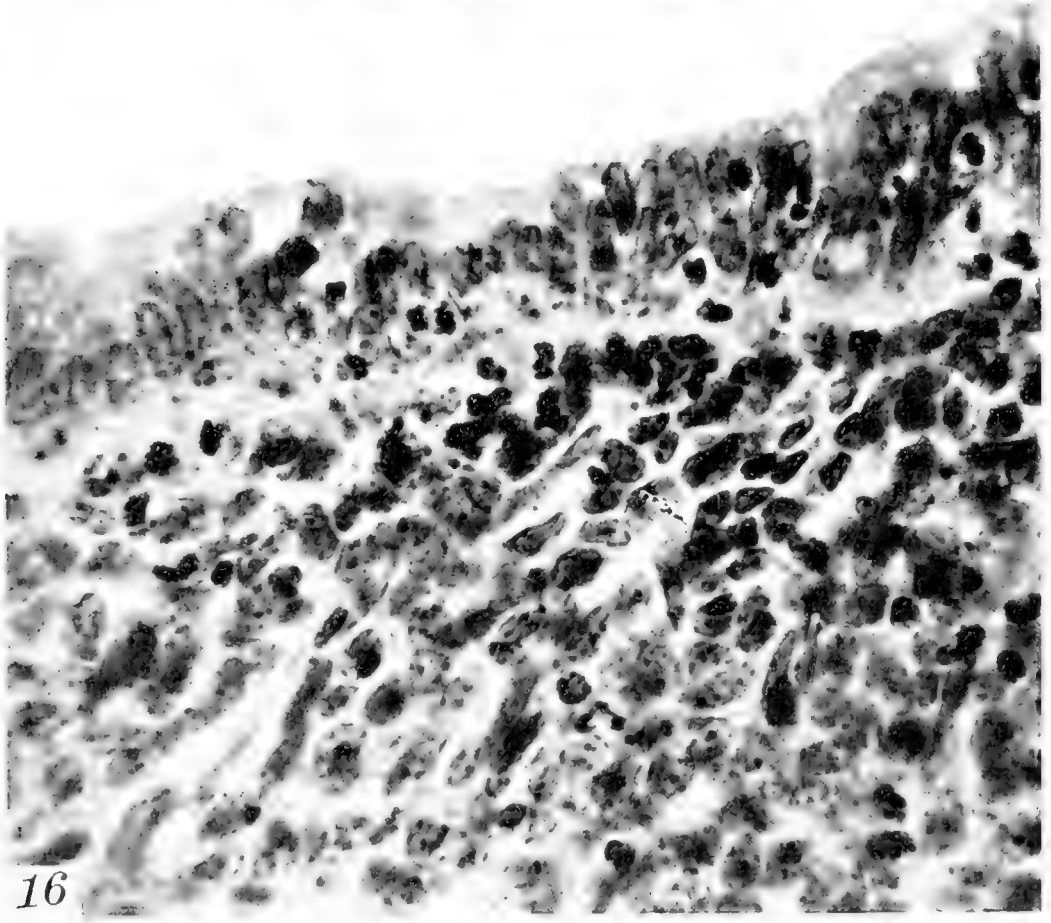

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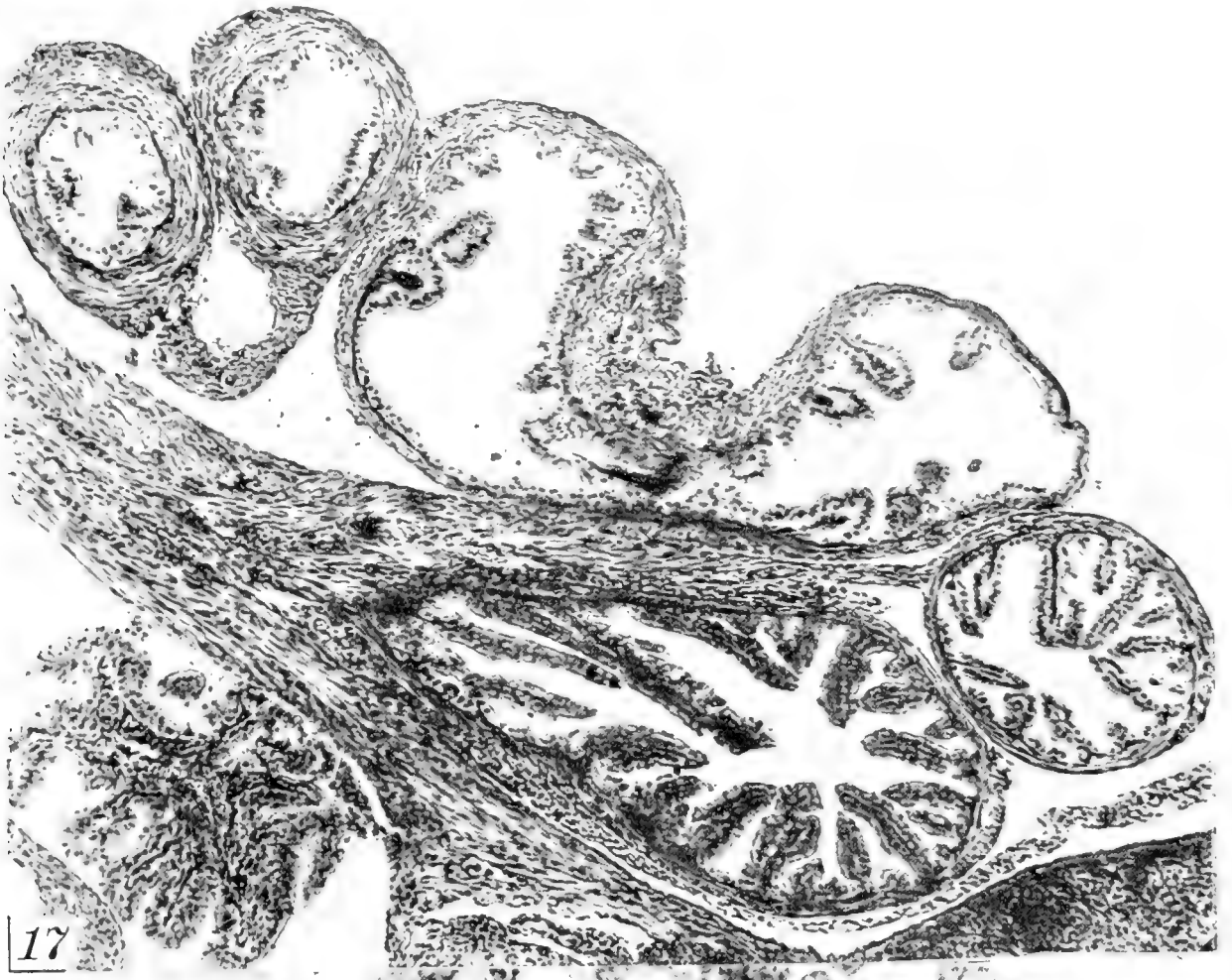
PLATE 8

EXPLANATION OF FIGURES

17 Sections of several loops of the oviducts. × 55. Only that in the lower center is ciliated. Segments are distinguishable by ciliation, degree of folding of the mucosa, and thickness of the muscle walls.

18 Ciliated epithelium of the late stage P oviduct. × 550.



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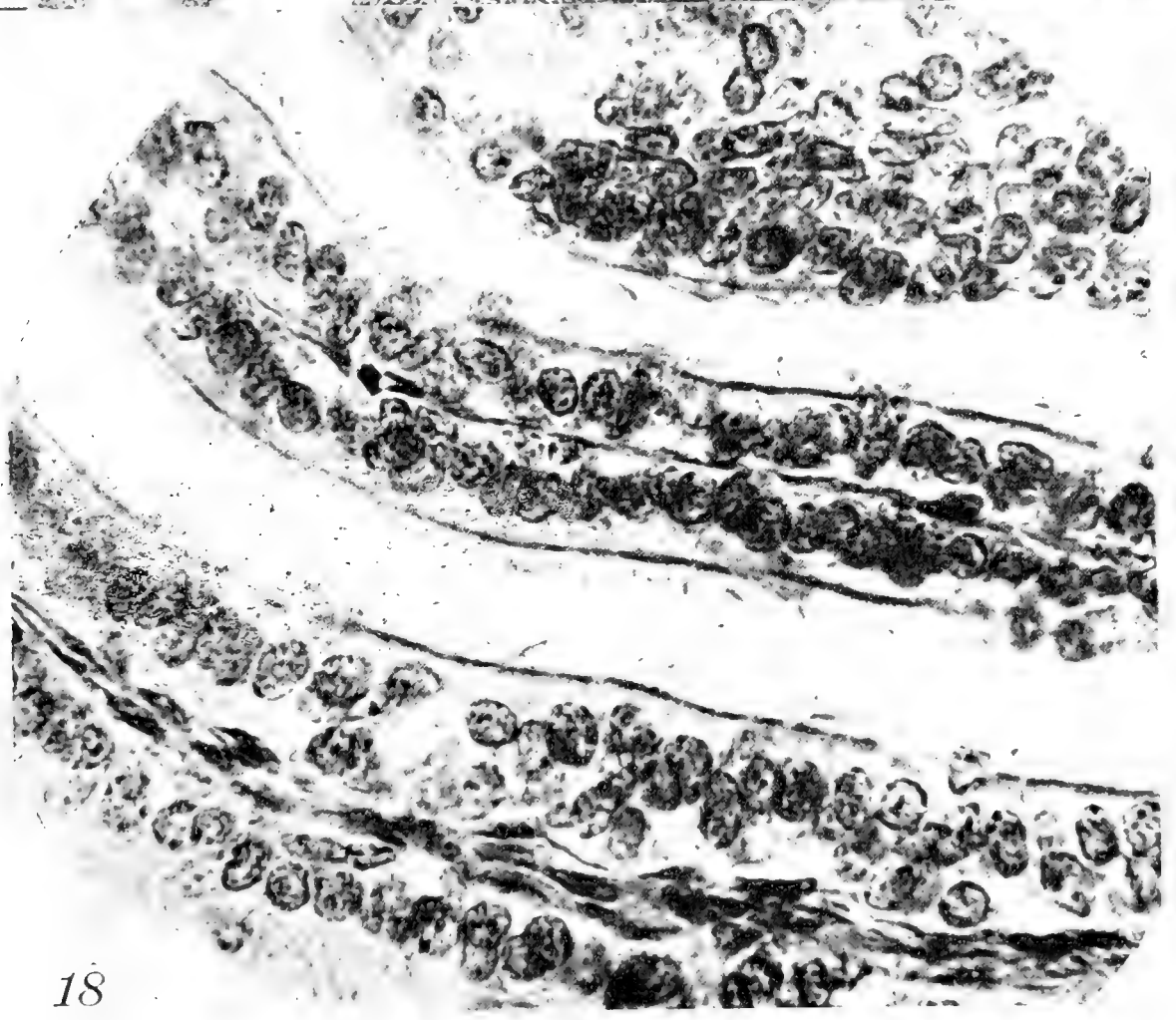

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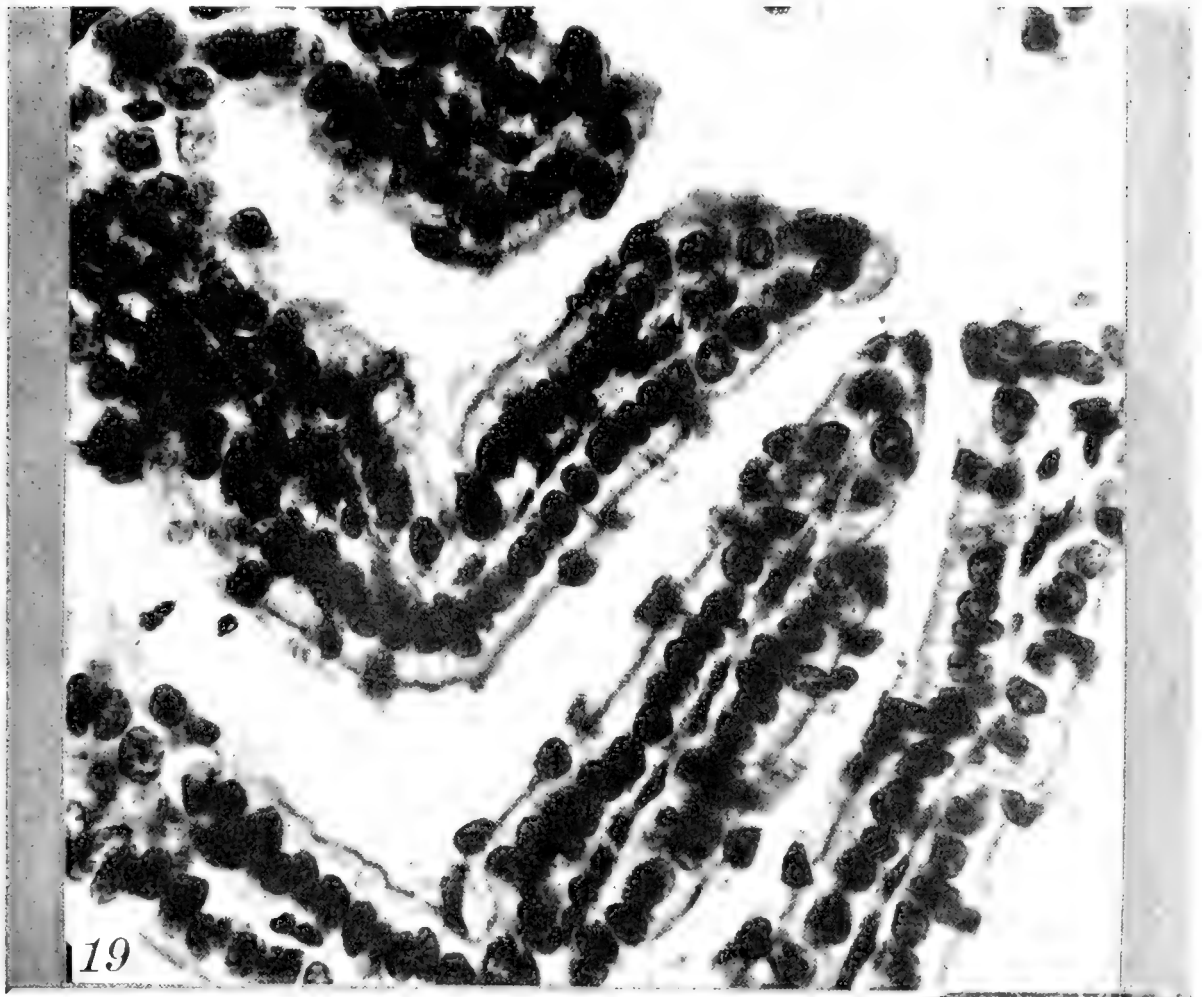
PLATE 9

EXPLANATION OF FIGURES

19 Ciliated epithelium of the oviduct in a late M stage. × 550. The process of extrusion of nuclei is quite general.

20 Largest-sized follicle usually found after ovulation, stage M_1. × 170. Primary liquor folliculi is restricted to two pools. Granulosa contains many mitoses. Note interstitial tissue above to the right.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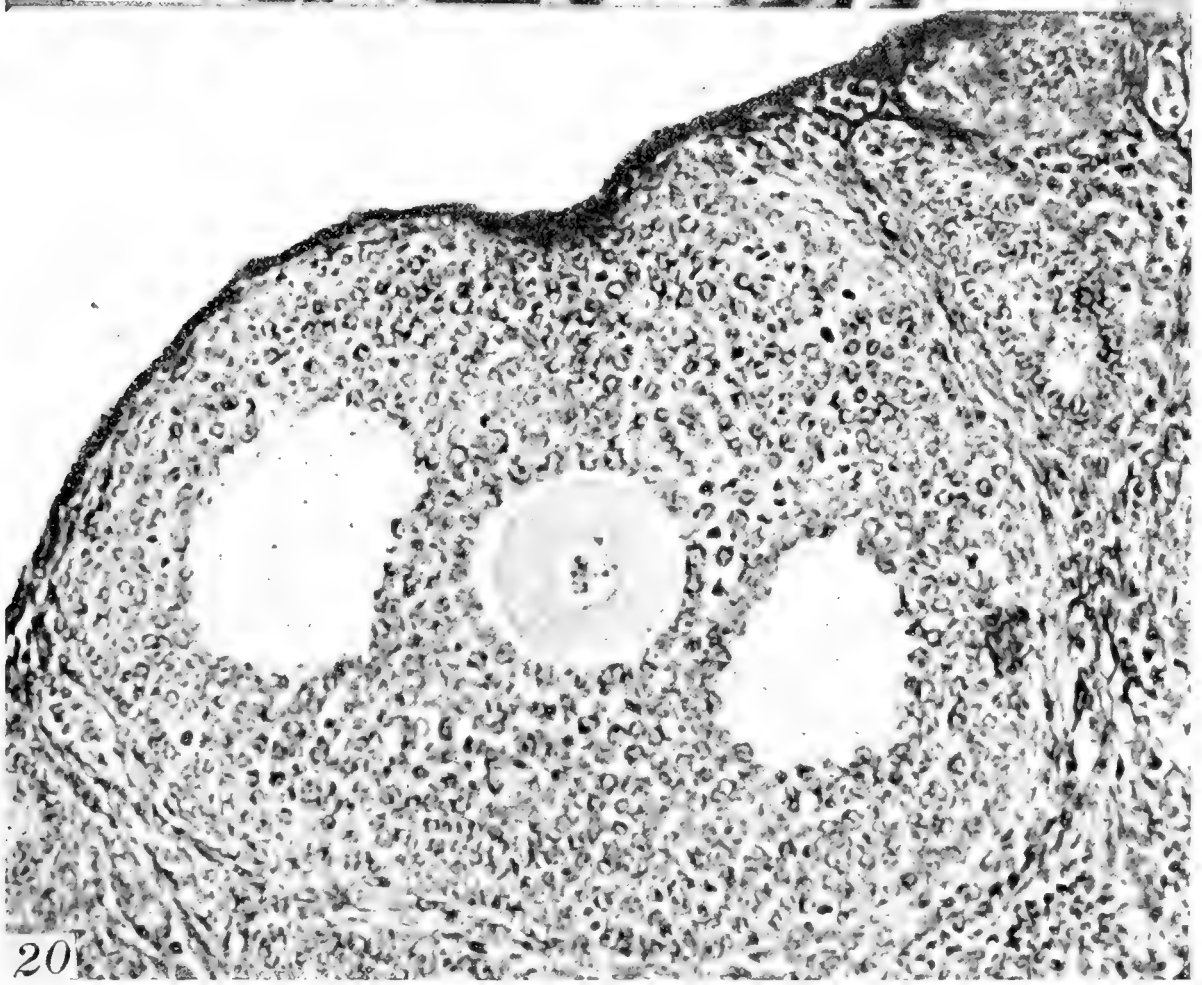
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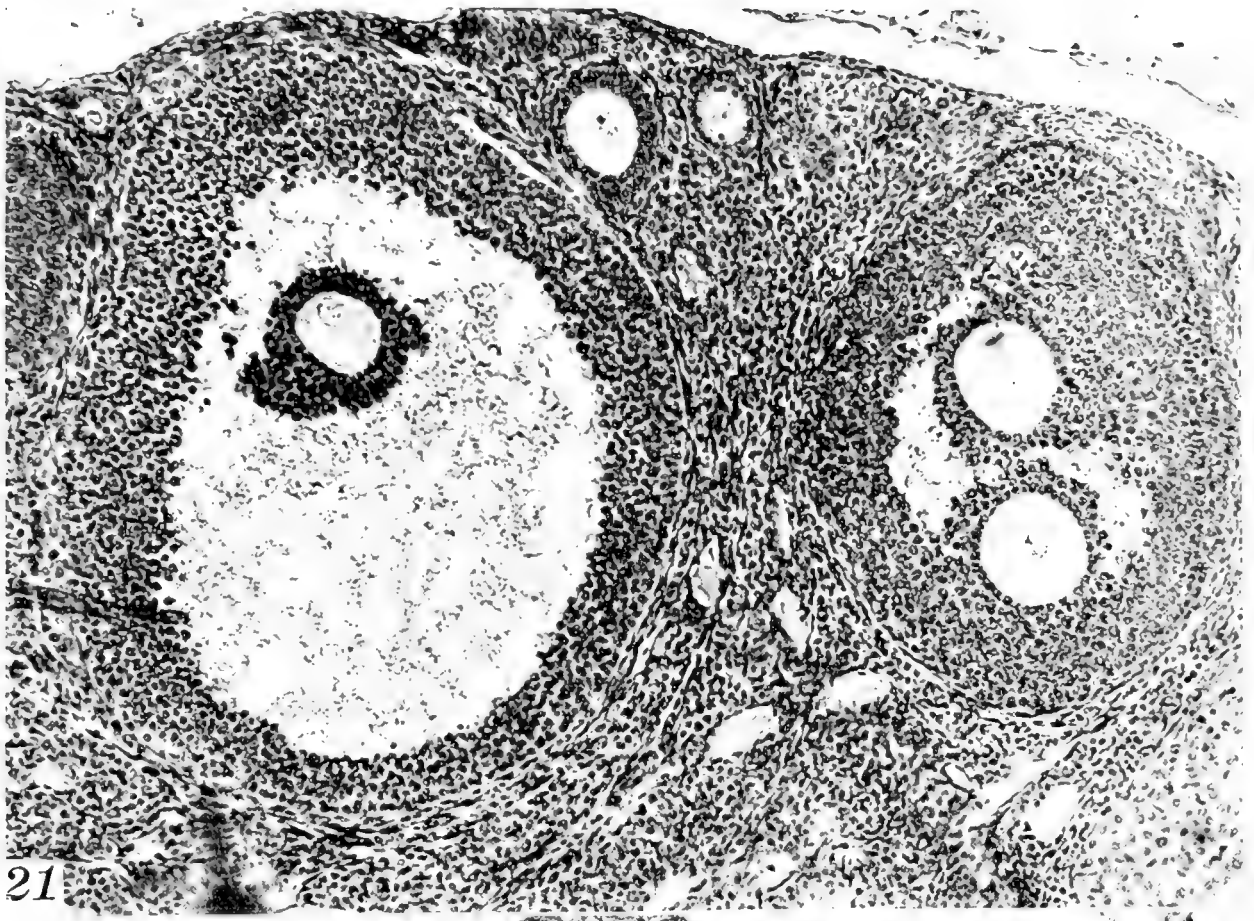
PLATE 10

EXPLANATION OF FIGURES

21 Large follicle (nearly rupture size) in an early stage of atresia. × 125. Several follicles in this set are apparently normal. At right, a medium-sized follicle containing two ova.

22 Ovary of an early M_1 stage. × 58. In upper right field is newly forming corpus luteum, not yet redistended. At lower left is one fully redistended. The age of these corpora is estimated at less than seven hours.



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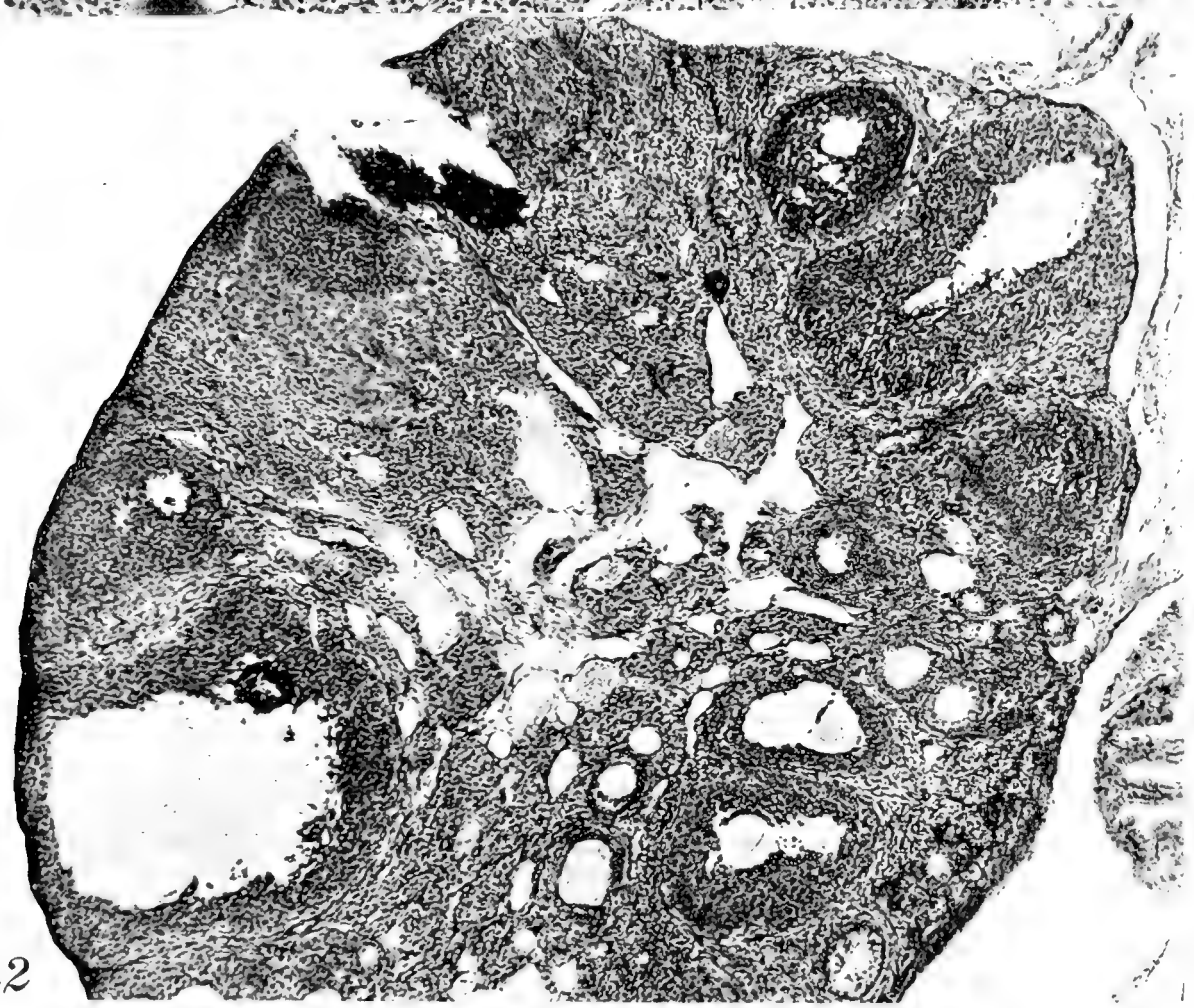

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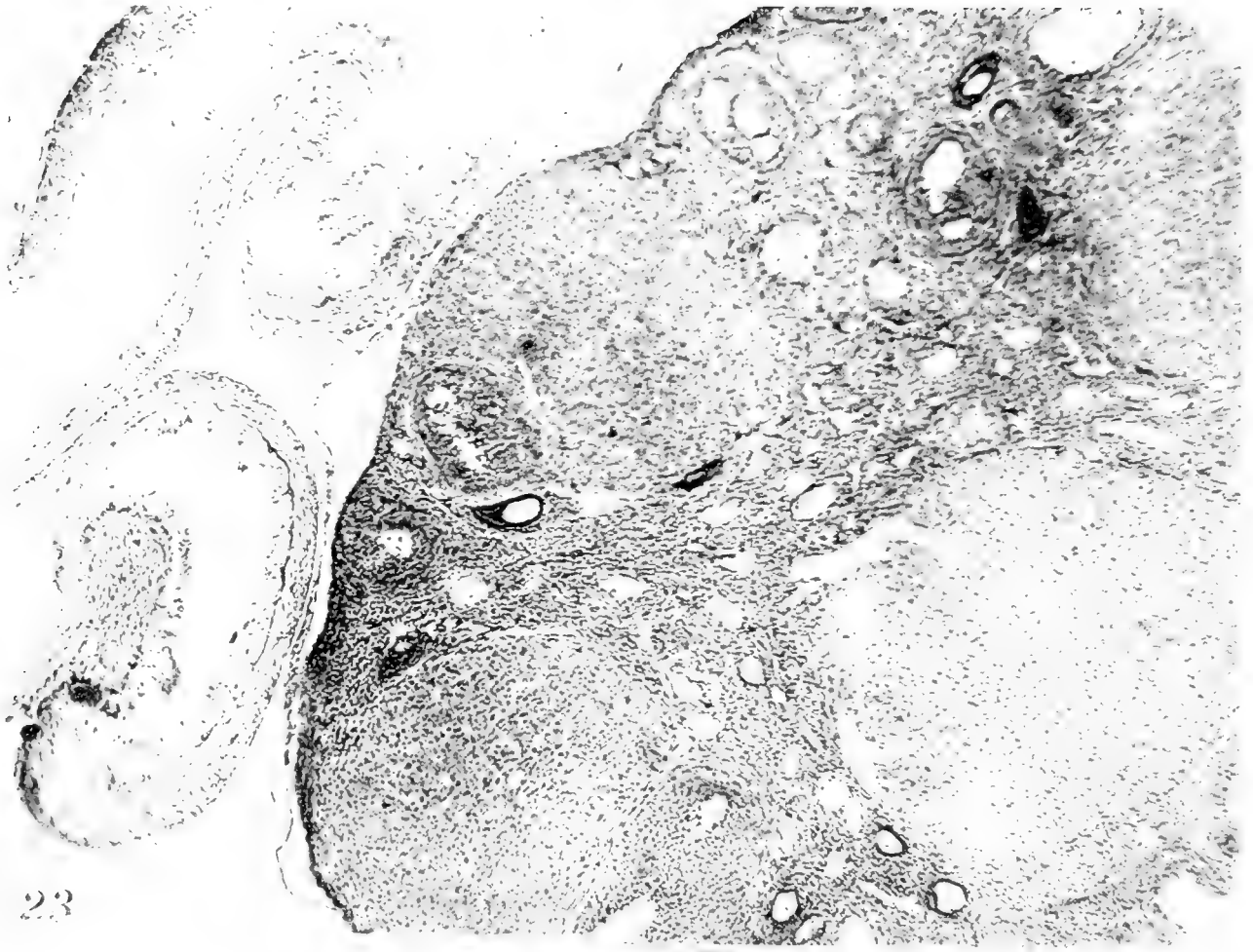
PLATE 11

EXPLANATION OF FIGURES

23 Ovary and third segment of oviduct of late stage M_2 animal. × 501. In upper left field are three ova bunched in the last segment of the oviduct. Of the three corpora lutea included in this field, the two at the left surface of the ovary correspond to the ova in the tubes, the one in the lower right field to the second oestrus recorded before death. They are easily distinguished by staining reaction not brought out in the photograph. The ages of these were estimated at three and nine to ten days, respectively.

24 Two corpora lutea representing follicles which ruptured at the first and third Operiods before death. × 58. That to left stains blue and shows "bleeding into the central cavity." That to the right has a pink tinge, is 'corded,' and deeply placed. Ages are estimated at three and fourteen days, respectively.



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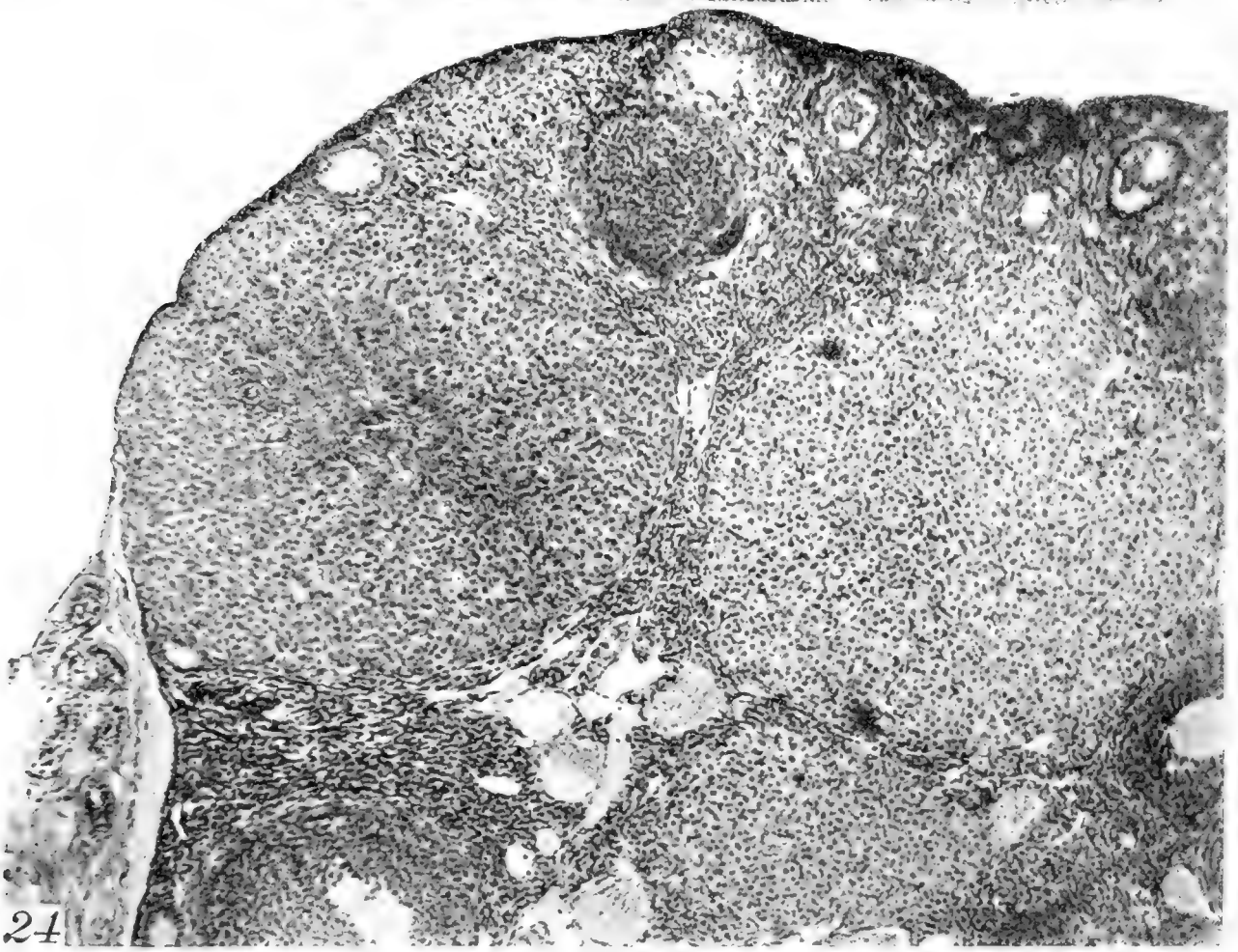

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Resumen por el autor, B. M. Patten.

La formación del asa cardiaca del pollo.

Las fases tempranas del establecimiento del corazón del pollo y los estados ulteriores de división en diferentes cámaras han sido cuidadosamente investigados por diversos autores. El presente trabajo se ocupa de los procesos intermedios algo familiares, pero hasta el presente menos completamente descritos, de la formación del asa y la diferenciación regional temprana, basándose en disecciones, reproducidas en modelos plásticos, y en reconstrucciones en cera. Se ocupa de los siguientes puntos: 1. La formación en el tubo cardíaco de un asa en forma de U dirigida hacia el lado derecho, y de algunos de los factores causativos invocados en este proceso. 2. La formación del asa cardiaca y la relación de la torsión y flexión del cuerpo del embrión con el proceso de la formación de dicha asa. 3. La diferenciación regional del corazón en el bulbo cardíaco, ventrículo, atrio y seno venoso, y los cambios tempranos en cada una de estas regiones.

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Cornell Medical College, New York

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THE FORMATION OF THE CARDIAC LOOP IN THE CHICK

BRADLEY M. PATTEN

Laboratory of Histology and Embryology, School of Medicine, Western Reserve University

TWO TEXT FIGURES AND THREE PLATES

INTRODUCTION

Most of the numerous investigations concerning the development of the heart in birds have dealt either with the very early phases of its establishment or with the relatively late steps of its division into chambers. The intervening process of loop formation, although it is in a general way familiar to embryologists, has received much less attention. When I had occasion to consult the literature for a discussion of the subject, I was unable to find any connected account with adequate figures. It has, therefore, seemed worth while to extend and publish some observations on cardiac-loop formation in the chick which were originally made in the course of other work.

Records of investigations of the development of the chick heart appear in some of the earliest works on embryology. The observations on the heart recorded in such classics as those of Malpighi (1686), Wolff (1759), von Haller (1767), and Pander (1817), though all of them are remarkable for their time, are at present chiefly of historical importance. An interesting summary of the work of these writers appears in the paper of Lindes ('65). The early stages in the establishment of the chick heart have since been dealt with by Afanassiev ('69), Gasser ('77), Duval ('89), His ('00), Rükert and Mollier ('06), Graper ('07), Funccius ('09), Hahn ('09), Miller and McWhorter ('14), and Reagen ('15, '17). Since this work has been summarized from the morphological point of view by Lillie ('08), and from the experimental

point of view by Reagen ('17), it seems unnecessary to go over the ground again here. The abundant information available concerning the establishment of the primordial heart tube places us in a position to take up without preliminaries the processes involved in cardiac-loop formation.

The observations recorded here are confined to the period extending from the establishment of the heart as a nearly straight, double-walled tube to the period at which the process of loop formation has been completed and the main regional divisions of the heart have been definitely established.

The later development of the chick heart, involving the formation of the septa and valves which develop during the division of the heart into chambers, is dealt with in papers by Lindes ('65), Tonge ('69), Masius ('89), Langer ('94), Griel ('06), and Hochstetter ('06).

MATERIAL AND METHODS

At the outset of the work it became obvious that there was considerable individual variability as to heart configuration even among embryos having the same number of somites. The first consideration was, therefore, the selection of a series of embryos which would show as nearly as possible the normal sequence of shape changes undergone by the heart. This phase of the work was greatly facilitated by the availability of some 2000 chick whole-mounts in our laboratory collection. By studying the heart in a large group of embryos having the same number of somites, it was not difficult to determine the characteristic heart configuration for a particular stage of development.

Twelve embryos ranging between 29 and 100 hours of incubation were selected as showing the characteristic steps in the formation of the cardiac loop and the early regional differentiation of the heart. Each of the twelve embryos belonging to the initial series was then carefully matched so that three or four embryos of each stage, exactly like one another as far as could be determined, were available for the work. One embryo in each of these sets was reserved for study as a cleared and stained entire mount, the remaining embryos were used for dissection and serial sectioning.

Camera-lucida diagrams of the cephalic and cardiac regions were made from the whole-mount series. In these diagrams the heart and main afferent and efferent vessels, as far as they could be made out, were drawn in directly. Later in the work, the outlines of the heart and main vessels were completed[1] from dissections and reconstructions of embryos of corresponding stages. These diagrams appear as the text figures and serve to show at the same time the stages worked on and the relations of the heart to the neighboring structures in the body of the embryo.

It was found that the configuration of the heart itself could be worked out very successfully from dissections made in alcohol under a binocular microscope. In such preparations the heart shape is beautifully shown by strong reflected light and can be accurately reproduced with the aid of a camera lucida. Employing this method, drawings of the same heart were made from three aspects to the same scale of magnification. By using dividers to keep the dimensions accurate, it was a relatively simple matter to make a preliminary clay model of the heart from the drawings. This model, with its basic dimensions correct, was then finished directly from dissections of the heart, which could be rotated and thus studied under the binocular microscope from all angles.

Although the contours are less likely to be distorted in dissected hearts than in reconstructions, there are certain details that cannot be made out satisfactorily by the dissection method. The chief point of difficulty is the region of the sinus venosus in the older embryos. The manner in which the veins entering the heart are imbedded in the surrounding structures renders it

[1] Although a consideration of the changes in the aortic arches does not come within the scope of this paper, the condition of the arches at each phase of heart development here dealt with is indicated in the text figures. For discussion of the development of the aortic arches, reference may be made to the works of Boas ('87), Evans ('09), Lillie ('08), and Locy ('06).

The cardinal and umbilical veins are indicated in the figures of the later stages only, because in the earlier stages the position of the embryos is such that these vessels would have to be superimposed on the heart. Moreover, the early stages in the formation of the vessels are shown beautifully in the figures of Evans ('09) and Sabin ('17).

almost impossible to make clean dissections of this region. In the older embryos, therefore, the heart and the main afferent and efferent vessels were reconstructed from serial sections by the wax-plate method of Born. As far as the principal contours of the heart are concerned, these reconstructions were found to conform with the clay models made from dissections. They furnished, moreover, detailed information concerning the sinus region and the entering veins, which it had not been found possible to obtain by means of dissections.

The drawings of the heart shown in the plates were made for the most part directly from dissections. They contain some details, however, that were added from the wax-plate reconstructions. The orientation of the heart in the body of the embryo, and the relations of the vessels are shown in the text figures, which are lettered in correspondence with the plates.

THE FORMATION OF THE CARDIAC LOOP

The youngest stage studied is represented by embryos of 9 somites (approximately twenty-nine hours' incubation), in which the heart is a nearly straight tube (fig. 1, A, and pls. 1, 2, and 3, A). Even when the myo-epicardial folds are first approximated to each other to form the outer wall of the heart tube, there is already a tendency for the right lateral margin of the heart to show a greater convexity than that of the left. This asymmetry is due, at first, more to unequal dilation of the heart wall than to actual bending of the entire tube, as is indicated by the fact that the line of attachment of the dorsal mesocardium lies very nearly in the sagittal plane of the body.

The dorsal mesocardium at this stage forms an unbroken supporting membrane throughout the entire length of the heart. In contrast to the condition in mammals described by Yoshinaga ('21), the ventral mesocardium in the chick is complete, or nearly so, when it is first formed by the approximation of the two folds of splanchnic mesoderm which constitute the medial wall of the cephalic portion of the right and left coelomic chambers. The ventral mesocardium is, however, a more transitory structure

than the dorsal, and even at the 9-somite stage its rupture has begun in the midcardiac region, although its line of attachment to the heart can still be discerned (pl. 1, A).

In the chick heart the endocardial tubes are less irregular in contour and are more completely fused with each other at this stage than in the mammalian heart of corresponding age. Furthermore, the chick endocardium shows no such early foreshadowing of the atrioventricular and the sino-atrial constriction as has been observed in mammals (Murray, '19; Schulte, '16; Yoshinaga, '21). In the later stages studied the endocardium is of secondary interest, its configuration being determined largely by the limitations imposed upon it by the myoepicardium. Although the endocardium has been shown in the figures of our earliest stages by way of bringing this work into continuity with that of other investigators, the later changes in its configuration have not been followed in detail.

Between thirty and forty hours of incubation (10 to 18 somites), there is a marked dilation of the heart, but its most conspicuous change in shape is due to the bending of the entire middle portion of the heart tube to the right (pls. 1 and 3, A to E). In this process of bending, as indeed in the entire series of changes involved in loop formation, there is undoubtedly a considerable factor of mechanical compulsion. The accompanying graph shows how much greater the elongation is in the heart tube itself than is the increase, during the same period of time, in the distance between the attached cephalic and caudal ends of the heart. Under such growth conditions, bending of the heart is inevitable. It is quite logical, furthermore, that this bending should be lateral because of the impediment offered dorsally by the body of the embryo and ventrally by the yolk. Why it should take place to the right rather than to the left is not so clear.

It has been suggested that the bending of the heart to the right might be due to the entry of a stronger current of blood from the left omphalomesenteric vein than from the right, the left vein being conspicuously larger at this stage of development. Sabin ('17) has shown, that while heart contractions begin in the chick as early as the 10-somite stage, the actual circulation

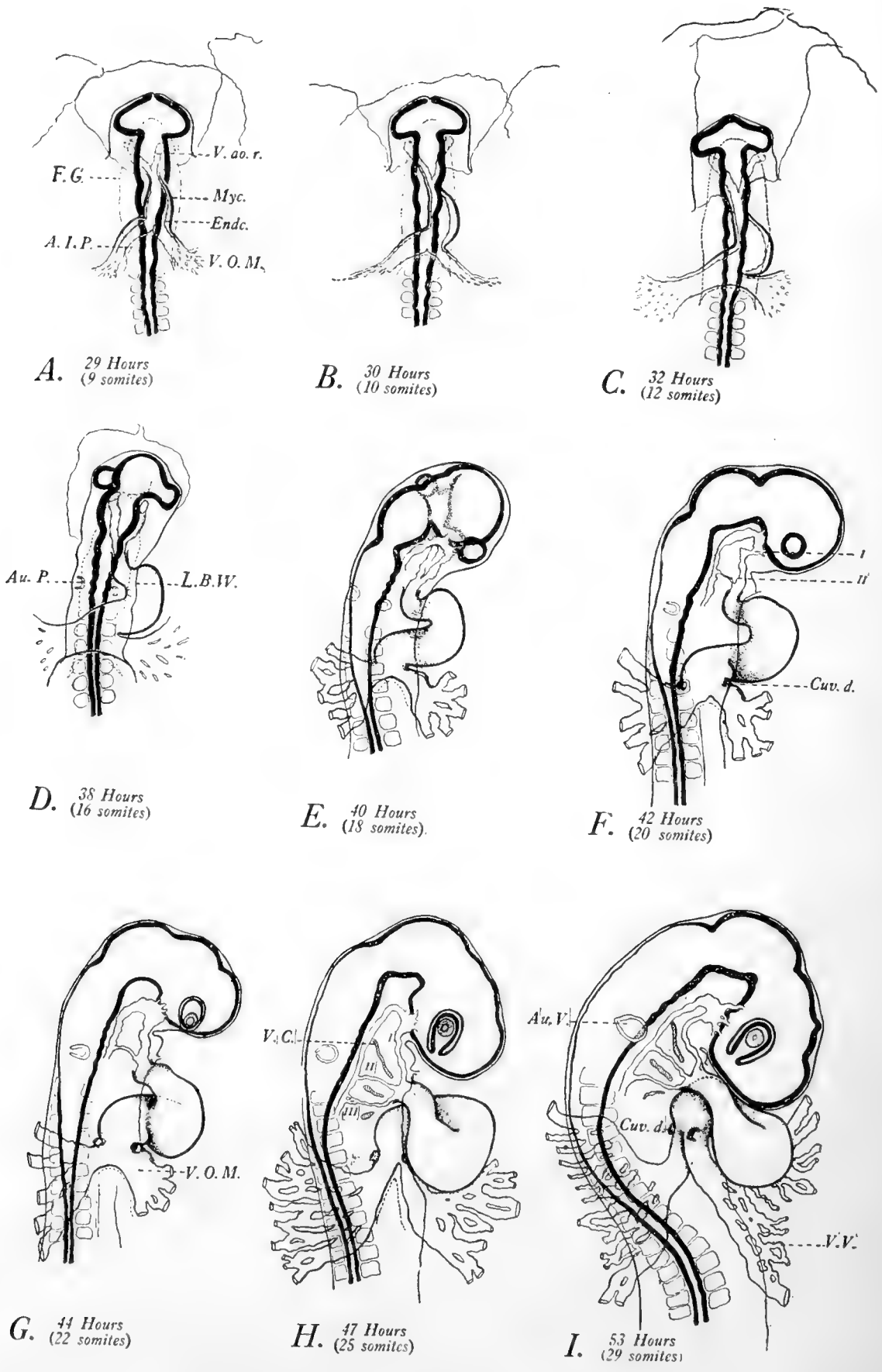
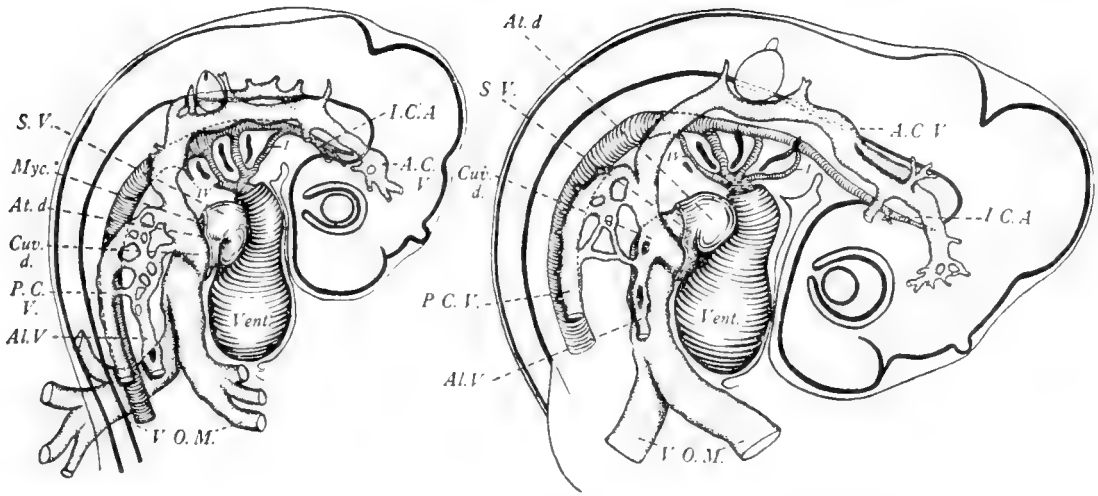
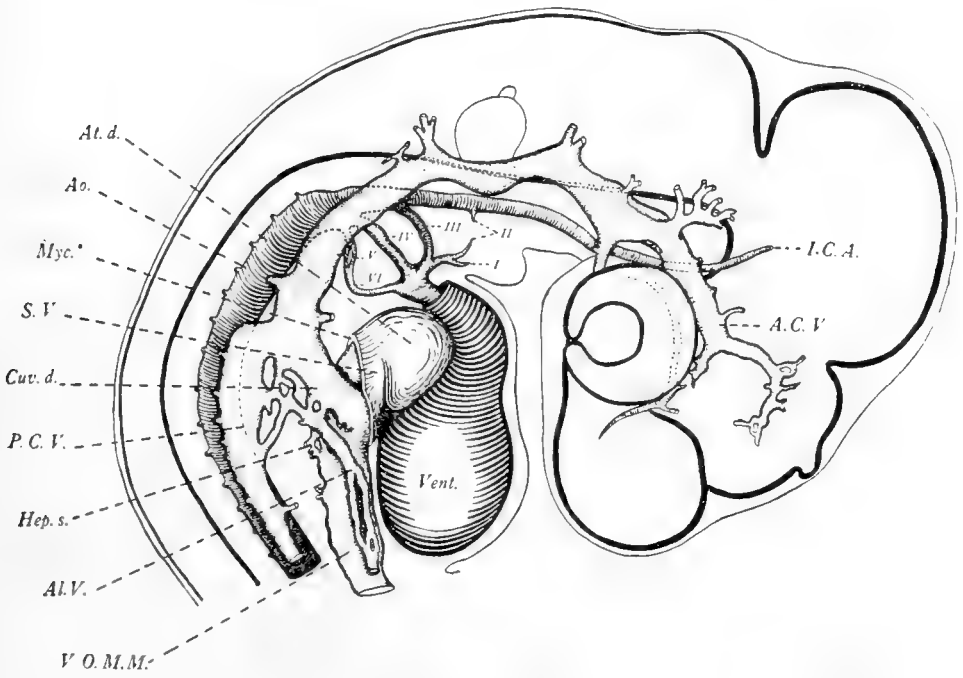


Fig. 1, A to L Camera outlines (× 15) of cephalic and cardiac regions of chick embryos, showing for each stage studied the relations of the heart to neighboring



J. 65 Hours (33 somites) *K.* 76 Hours (38 somites)



L. 100 Hours (45 somites)

structures in the embryo. These figures are arranged and lettered to correspond with the detailed drawings of the hearts shown in the plates. *I* to *VI*, aortic arches I to VI, respectively; *A.C.V.*, anterior cardinal vein; *A.I.P.*, anterior intestinal portal; *Al.V.*, allantoic vein; *Ao.*, aorta; *At.*, atrium (*d.*, right), (*s.*, left); *Au.P.*, auditory pit; *Au.V.*, auditory vesicle; *Bul.*, bulbus cordis; *Cuv. d.*, duct of Cuvier; *Endc.*, endocardium; *F.G.*, foregut; *Hep.s.*, stubs of some of the larger hepatic sinusoids; *I.C.A.*, internal carotid artery; *L.B.W.*, lateral body wall; *Myc.*, myoepicardium; *Myc*.*, cut edge of myoepicardium; *P.C.V.*, posterior cardinal vein; *Sin.-at.*, sino-artrial region of heart before its definite division; *S. V.*, sinus venosus; *Vent.*, ventricle; *V.ao.r.*, ventral aortic roots; *V.C.*, visceral cleft; *V.G.M.*, omphalomesenteric veins; *V.O.M.M.*, fused omphalomesenteric veins; *V.V.*, vitelline veins.

of blood is not established until the 16-somite stage. The fact that circulation does not begin until after the bending of the heart is well advanced excludes inequality of blood-flow from consideration as causative factor ontogenetically. There is still the possibility that the bending of the heart to the right is a recapitulation of development in some ancestral form where lateral inequality of the circulation had become established prior to the bending of the heart, but it would be equally plausible to urge that the bending of the heart to the right was primary, and that the left omphalomesenteric vein became secondarily enlarged owing to the opposition of less resistance to the discharge of its blood.

It has also been suggested that, owing to the direction of the torsion of the embryo's body, the heart tube is free to expand to the right, while it would be obstructed on the left by the swinging of the left side of the body wall toward the yolk. Again we encounter a disregarded time factor. The heart bending is initiated before there is any indication of torsion in the embryo. There is undoubtedly correlation between the two processes in the sense that the development of the heart would be mechanically impeded, if not stopped, by torsion of the embryo in the opposite direction. Here also it might be maintained that the heart bend itself is the primary factor and that the direction of embryonic torsion follows it as a necessary consequence. Certain it is that the bending of the embryonic heart to the right is not peculiar to forms in which torsion is conspicuous. The heart bend is the more deep seated phylogenetically. It occurs in the vertebrate stock as far back as the elasmobranchs (Hochstetter, '06) and Dipnoi (Robertson, '13), and is a characteristic feature of heart development in Amphibia (Rabl, '87). One would scarcely expect to work out the primary causative factors of such a long-established process entirely from the ontogeny of forms as far up the scale as birds.

As has already been stated, the ventral mesocardium has disappeared by the time the bending of the heart becomes apparent. The dorsal mesocardium, which is complete when the bending process begins, soon ruptures in the midheart region

(pl. 3, C, D), and is rapidly obliterated except at the caudal end of the heart (pl. 3, E). Thus the heart tube, being attached only at its two ends, is more free to undergo extensive and rapid changes in shape and position.

Even before the bending of the heart to the right has reached its maximum, torsion of the embryo's body begins to change the mechanical limitations in the cardiac region. As the cephalic part of the embryo comes to lie on the yolk on its left side (fig. 1, D, E, F) the heart, no longer closely confined between the body of the embryo and the yolk, begins to swing somewhat ventrad and lies less closely against the dorsal body wall of the embryo (pl. 2; cf. C and D with E and F).

The initiation of torsion has another very definite influence on the heart. Since torsion involves the cephalic region of the embryo first and progresses caudad, the body of the embryo becomes more inclined toward the yolk at the level of the cephalic attachment of the heart than at the level of its caudal attachment. As a result, the truncus arteriosus is twisted by the carrying of its attached end away from the yolk before a similar twisting effect is exerted upon the sino-atrial region of the heart (fig. 1, E, F, G). This is, I believe, the primary mechanical factor in starting the transformation of the U-shaped bend into the cardiac loop. Once the initial twist is imparted, loop formation progresses extremely rapidly, for the rate at which the heart tube is outgrowing the pericardial chamber in length is exaggerated at this time. The distance between the attached cephalic and caudal ends of the heart is actually being shortened by the progress of flexion in the embryo at just the time when the heart tube is elongating most rapidly (fig. 1, G, H, I, and fig. 2). The attached truncus and sinus ends of the heart are thus brought closer together, tightening the loop as it is formed. In the formation of the loop the truncus and bulbus swing away from the yolk (i.e., toward the embryo's right), and come to lie across the caudal part of the heart, at the atrioventricular constriction (pl. 2, G, H, I). The sino-atrial region being anchored to the body walls by the remaining part of the dorsal mesocardium, the ducts of Cuvier, and the omphalomesenteric veins, undergoes little change in position.

It is during this stage that the individual variability previously alluded to is most conspicuous. The more usual configuration of the heart is indicated in the figures referred to in the preceding paragraph where the loop is shown as rather closely twisted. There were not a few embryos, however, in which the heart stood out from the body, and was more loosely twisted than in the

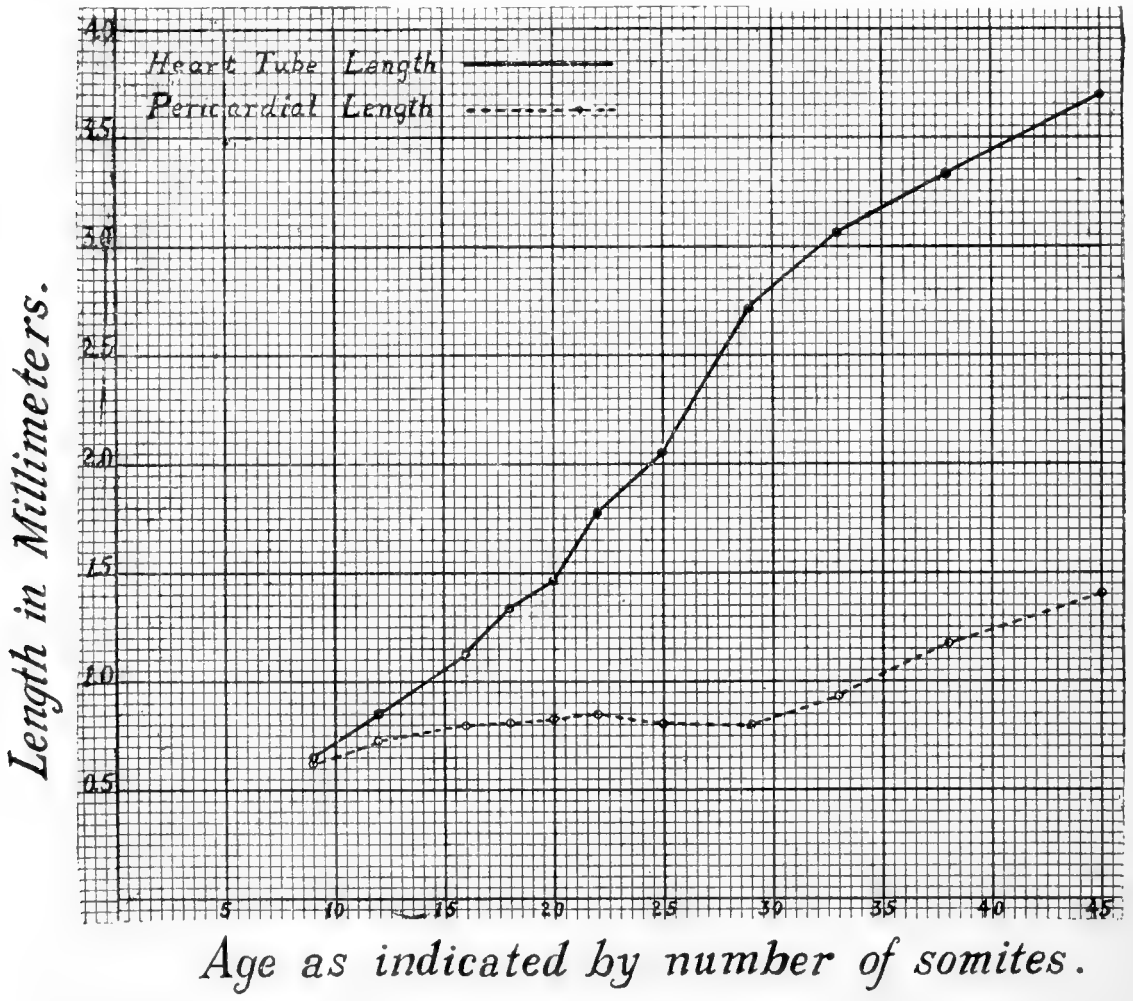


Fig. 2 Graph to show the rate of heart-tube elongation as compared with the rate of elongation of pericardial region. The heart length was measured along the original middorsal line of the heart tube from the point of bifurcation of the aortic roots to the point of convergence of the omphalomesenteric veins. (In the older embryos the omphalomesenteric veins have begun to fuse with each other. The caudal point for the measurements had, therefore, to be approximated by taking it in a given relation to the point of entrance of the ducts of Cuvier.) As an index of the length of the pericardial cavity, the distance between these same two points was measured along the middorsal line of the embryo. All of the measurements were taken on models made to the same scale of magnification, and then converted to actual size.

embryos represented in figure 1, G and H. In the embryos I have studied this condition seems to be correlated with delayed flexion rather than with any abnormality of the heart tube. It is probably to be regarded as within the limits of normal variability.

In the ventral views of the heart it can be seen that as the process of loop formation progresses, the extension of the heart to the right is diminished, and that the loop as it is formed swings not only ventrad, as has been mentioned above, but also distinctly toward the sagittal plane (pl. 1, G, H, and I). This change in position may well be due to the fact that by this stage the body at the cardiac level has completed its torsion and lies on its left side, so that the heart is no longer prevented by the yolk from expanding midventralward.

The heart in the stage when the cardiac loop is first definitely formed (i.e., in embryos of about 25 somites) has been described by many investigators as 'S-shaped.' Neither the dorsal (pl. 3, H) nor the lateral (pl. 2, H) nor the dextrodorsal view of the heart as seen in the ordinary whole-mount (fig. 1, H) can be characterized as 'S-shaped.' Only when a heart model or a heart freed by dissection is rotated, so that a direct ventral view is obtained, does the 'S-shaped' configuration become recognizable (pl. 1, H).

At the close of the second day of incubation, the cranial flexure of the embryo is developing extremely rapidly (fig. 1, G, H, I). As the anterior part of the head is bent caudad, it begins to crowd the heart loop. As a result the ventricular bend of the loop moves at first caudad and then dorsad (fig. 1, H to L). Prior to the formation of the cardiac loop and its dorsocaudal bending, the ventricular portion of the heart is cephalic to the atrium, in the primitive vertebrate position. The bending of the loop brings the ventricle caudal to the atrium in approximately the definitive relationship characteristic for adult sauropsida.

THE REGIONAL DIFFERENTIATION OF THE HEART

Certain text-book diagrams of the chick heart show bulbar, ventricular, and atrial regions, separated by conspicuous constrictions while the heart is still in the straight tubular stage. Although perhaps suggestions of such constrictions are to be detected at this early stage of development, I have not been able to satisfy myself as to their definite appearance until the heart is well bent to the right, and they do not appear at all conspicuously until nearly forty hours of incubation (16 to 18 somites). In the heart of a chick of 20 somites, the bulboventricular constriction, previously but vaguely discernible, has become quite definite (pl. 1, F). The atrioventricular constriction is also well marked by this time (pl. 2, F). The sinus venosus exists rather as the place of confluence of the omphalomesenteric veins with each other and with the atrium than as a definite division of the heart. Nevertheless, the sino-atrial boundary may be said to be foreshadowed by an increased conspicuousness of the grooves formed on either side where the omphalomesenteric veins enter the heart at an obtuse angle to it (pl. 3, F). The apparent deepening of these lateral grooves is, however, due rather to expansion of the atrium than to any actual constriction in this region. There is as yet no demarcation between sinus and atrium dorsally, and no caudal line of demarcation between the sinus and the omphalomesenteric veins.

For convenience in description, the heart at this stage can best be compared to a U with its upright limbs attached to the body of the embryo (fig. 1, F). The ventricle, definitely marked off both cephalically and caudally by constrictions, constitutes the bend of the U. The bulbotruncus portion of the heart tube constitutes the cephalic limb of the U, which is attached to the body by the aortic roots. The sino-atrial region constitutes the caudal limb of the U, which is attached by the omphalomesenteric veins, the ducts of Cuvier, and the remaining part of the dorsal mesocardium.

The early changes in the bulboventricular portion of the heart are already so well known that they require but a brief summary.

In the formation of the cardiac loop, the U-shaped ventricular bend becomes compressed (pl. 1, F, G, H). Coincidently, the apex of the bend is dilated so that the ventricle loses its U shape and becomes more saccular. The same process of dilation shortens, almost to obliteration the ventricular portion of the limbs of the U, so that the atrioventricular canal and bulbus cordis appear to lead off side by side, from a common ventricular sac (pl. 1, I). Meanwhile, as has already been described in connection with the formation of the loop, the entire ventricle has shifted more toward the sagittal plane (pl. 1, F to J). It has at the same time been bent caudad and then dorsad (fig. 1, H to L). In this manner the apex of the ventricle, which was originally the most right-hand portion of the U-shaped heart tube, becomes the most caudal part.

The first external manifestation of the impending division of the ventricle into right and left chambers shows in the oldest stages here studied. During the fourth day, a slight groove appears on the ventral surface of the ventricle, which extends caudad from the angle between the bulboventricular constriction and the atrioventricular constriction (pl. 1, K, L). This groove in later stages extends still farther caudad and marks externally the position at which the septum interventriculorum develops.

In the bulbotruncus region the early changes are shown so definitely by the figures that little can be added by description. The most interesting phases of the development of the bulbus occur in stages of development more advanced than those with which this study is concerned. They have been described in detail by Lindes ('65), Langer ('94), Masius ('89), Hochstetter ('06), and Lillie ('08).

The early differentiation of the sinus venosus has been less fully described and calls for more detailed attention. A definite dorsal line of demarcation between the sinus venosus and the atrium, can first be made out at the close of the second day of incubation (chicks of 25 somites). At this time the middorsal portion of the sino-atrial region of the heart becomes dilated. This dilation is situated just where the persistent caudal portion

of the dorsal mesocardium is attached to the heart. On either side it is marked off by a groove extending from the lateral constriction at the point of entrance of the omphalomesenteric vein, onto the dorsal surface of the heart (pl. 3, H and I, *S-A. c.*). The dorsal dilation thus bounded may now be differentiated definitely from the atrium as the sinus venosus.

The differentiation of the sinus venosus takes place at the same time as the caudal bending of the cardiac loop. It is possible that their appearance may be more than casually coincident. The caudal bending of the loop causes the blood from the omphalomesenteric veins to be directed against the dorsal and cephalic wall of the sino-atrial chamber, rather than toward the atrioventricular ostium, as in earlier stages of development (pl. 2, H, I, and J). It will be noted that the sinus dilation occurs at precisely the point at which the blood current impinges against the heart wall. A deduction that the blood current is a causal factor in the dilation is alluring, but in default of experimental evidence, any suggestion to this effect must be considered as purely tentative.

Whatever molding effect the blood stream may exert in the process, the demarcation of the sinus venosus becomes more and more distinct as the caudal bend in the heart becomes more pronounced (pls. 2 and 3, J to L). The caudal bending of the loop, too, results in the shifting of the sinus from its original position, caudal to the atrium, to the dorsal position it occupies at the end of the fourth day of incubation (pls. 2 and 3). At this stage the sinus venosus is a pouch-like dilation which receives the ducts of Cuvier laterally and the fused omphalomesenteric veins caudally. It is marked off from the atrium by a groove, which is especially strongly developed caudally and dextrally. Already it opens into the atrial chamber somewhat to the right of the midline, foreshadowing its later association with the right atrium.[2]

[2] At this stage the two layers of splanchnic mesoderm which constitute the dorsal mesocardium flare out on either side and are reflected over the ducts of Cuvier at their points of entrance into the sinus venosus (pl. 3, I). These transverse folds of the mesocardium have been designated (Lillie) as the mesocardia lateralia.

The most conspicuous change in the atrial region is its lateral expansion. As early as forty hours the future atrial region is dilated so that its transverse diameter is greater than that of any other part of the heart tube. From the first the dilation to the left is more marked (pl. 3, E, F). When the bulbus is thrown against the right side of the atrium in the formation of the cardiac loop (pls. 2 and 3, H), it seems to crowd the less developed right atrium and retard its development still more. After the configuration of the loop has changed so that the bulbus slips by the atrium, and crosses the heart at the atrioventricular constriction (pls. 2 and 3, I), the right atrium begins to expand more rapidly, but the size of the two atria does not become equalized until after the stages here under consideration.

The first indication of the separation of the atrium into two chambers appears in chicks of 29 to 30 somites (53 to 55 hours). A longitudinal sulcus develops at this time on the ventrocephalic face of the atrium. In a ventral view of the heart this sulcus is at first concealed by the truncus and bulbus; but as it becomes more clearly marked, its caudal portion can be seen extending toward the atrioventricular constriction (pl. 1, J. K. L, *i-a.g.*).

This interatrial groove is an external manifestation of the formation of the septum superius. (The septum superius or atriorum of the chick heart corresponds to the septum primum of the mammalian heart. In the chick no septum secundum is formed.) The formation of the interatrial groove does not appear to be dependent on pressure exerted on the atrium by the truncus arteriosus. When the groove first appears, an appreciable space separates the truncus from the atrium. With further growth, however, the truncus appears to sink into the cephalic portion of the interatrial groove, and the auricles expand rapidly on either side of it. Under these later conditions, the truncus probably does play a secondary part in the division of the atrium in the sense that it acts as a constricting band on either side of which the auricles expand.

SUMMARY

The early phases in the establishment of the chick heart and the later stages of its division into chambers have already been carefully investigated by many workers. This paper covers the somewhat familiar, but heretofore less completely described, intermediate processes of loop formation and early regional differentiation.

The work is based on dissections from which plastic models were made and on wax-plate reconstructions from serial sections. It deals with:

1. The formation in the heart tube of the U-shaped bend to the right and some of the alleged causative factors in this process.

2. The formation of the cardiac loop and the relation of torsion and flexion of the body of the embryo to loop formation in the heart.

3. The regional differentiation of the heart into bulbus cordis, ventricle, atrium, and sinus venosus, and the early changes in each of these regions.

Since these phases of heart development all involve complex changes in configuration and relations, the figures constitute a graphic summary much more satisfactory than a written résumé. The shortness of the intervals between the phases of development figured allows the continuity of the processes to be followed readily.

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EXPLANATION OF PLATES

Series of chick hearts, showing the formation of the cardiac loop and the progress of regional differentiation. The heart contours were drawn directly from dissections with the aid of the camera-lucida outlines. In drawing the older stages, wax-plate reconstructions were used for working out the relations of afferent and efferent vessels and as a check on the configuration of the heart shown by the dissections.

In the stages represented in figures E to I torsion has involved the cardiac region of the embryo. Since torsion affects the more cephalic regions first and progresses caudad, the transverse axis of the body of the embryo is at different inclinations to the yolk at the cephalic, and at the caudal end of the heart. The drawings of the ventral and dorsal views are oriented from the frontal plane, and those of the dextral views from the sagittal plane of the body, at the level of the aortic arches. For this reason the sinus region of the heart appears inclined.

The relation of the heart to neighboring structures in the embryo is shown in the text figures. The lettering of the text figures and plates corresponds throughout.

ABBREVIATIONS

I-VI, aortic arches I to VI
At., atrium (*d.* right), (*s.* left)
A-V.c., atrioventricular constriction
Bul., bulbus cordis
B-V.c., bulboventricular constriction
Cuv.d., duct of Cuvier
Endc., endocardium
Hep.s., stubs of some of the larger hepatic sinusoids
i-a.g., interatrial groove
i.v.g., interventricular groove
Mes.d., dorsal mesocardium
Mes.v., ventral mesocardium
Myc., cut edge of myoepicardium
Myc.F., myoepicardial fusion[3]
S-A.c., sino-atrial constriction
Sin-at., sino-atrial region (before its definite division)
S.V., sinus venosus
V.ao.r., ventral aortic roots
Vent., ventricle
V.O.M., omphalomesenteric veins
V.O.M.M., fused omphalomesenteric veins

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[3] During the fourth day there is formed a curious attachment between the myoepicardium of the ventral wall of the sinus venosus and the myoepicardium of the ventricle (pl. 2, K, L). I have not seen it described elsewhere. From work now in progress on later stages of development, I believe this strand becomes a broad fusion and serves as a pathway over which one of the main coronary veins from the ventricle reaches the coronary sinus.

PLATE 1

Ventral views (× 25) of chick heart in various stages of loop formation. The somite number and the approximate incubation age of each embryo are indicated on the plate.

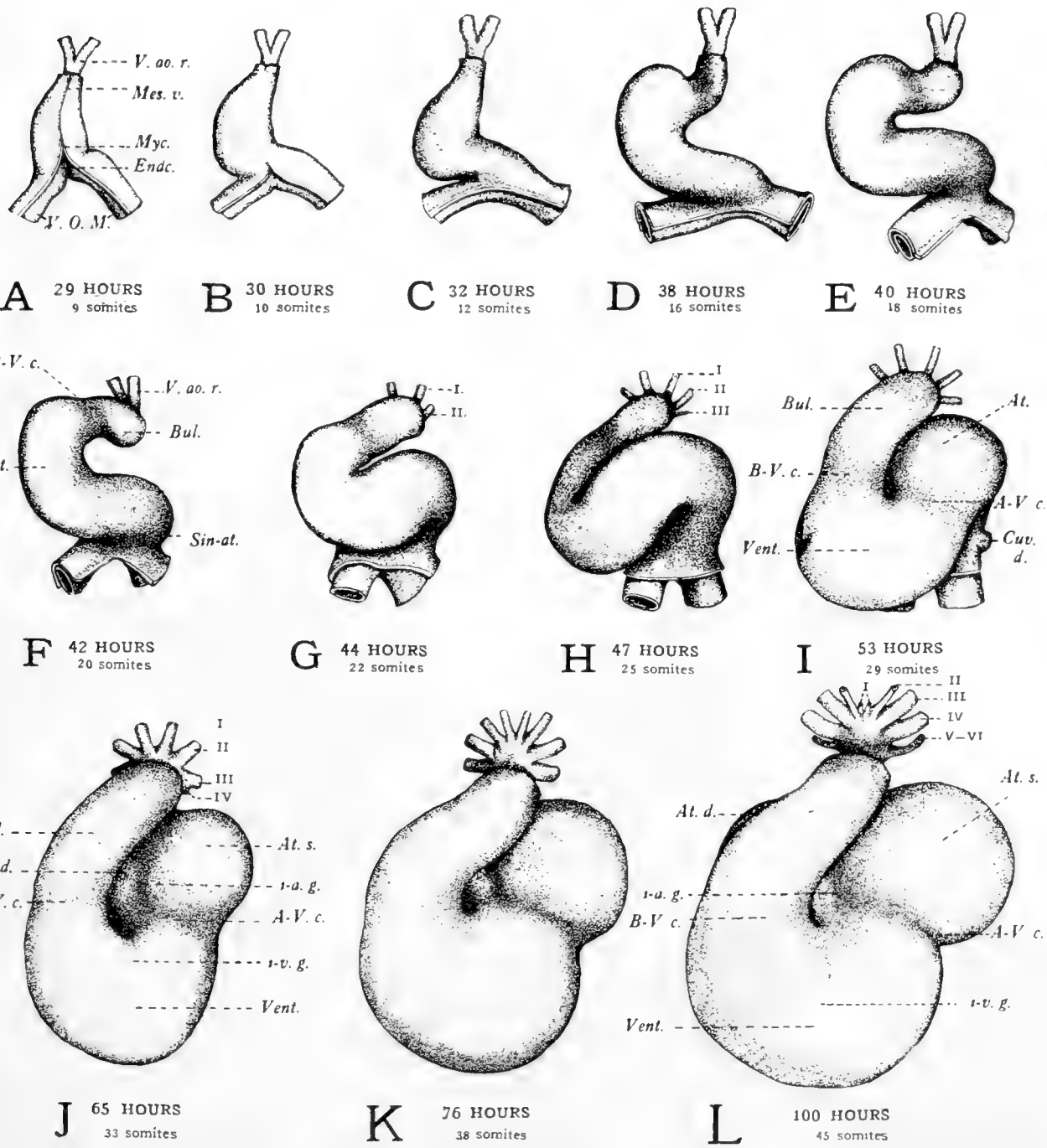
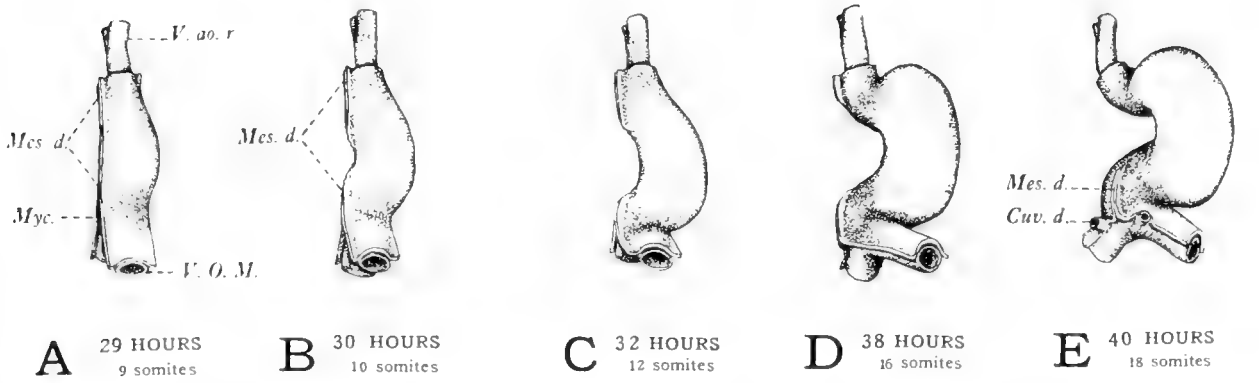
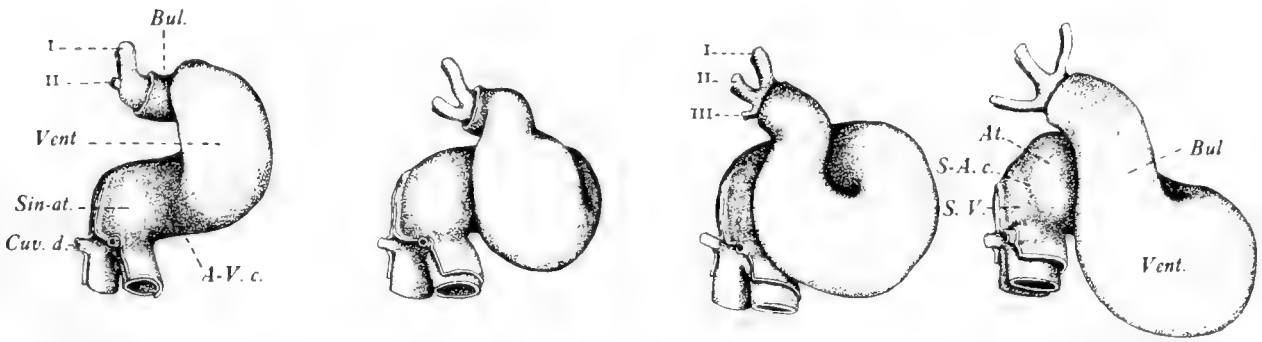

V. ao. r.
Mes. v.
Myc.
Endc.
V. O. M.
A 29 HOURS 9 somites
B 30 HOURS 10 somites
C 32 HOURS 12 somites
D 38 HOURS 16 somites
E 40 HOURS 18 somites
B-V. c.
V. ao. r.
Bul.
Vent.
Sin-at.
I.
II.
I
II
III
Bul.
At.
B-V. c.
A-V c.
Vent.
Cuv. d.
F 42 HOURS 20 somites
G 44 HOURS 22 somites
H 47 HOURS 25 somites
I 53 HOURS 29 somites
I
II
III
IV
Bul.
At. d.
B-V. c.
At. s.
t-a. g.
A-V. c.
t-v. g.
Vent.
I
II
III
IV
V–VI
At. s.
At. d.
t-a. g.
B-V c.
A-V c.
t-v. g.
Vent.
J 65 HOURS 33 somites
K 76 HOURS 38 somites
L 100 HOURS 45 somites

PLATE 2

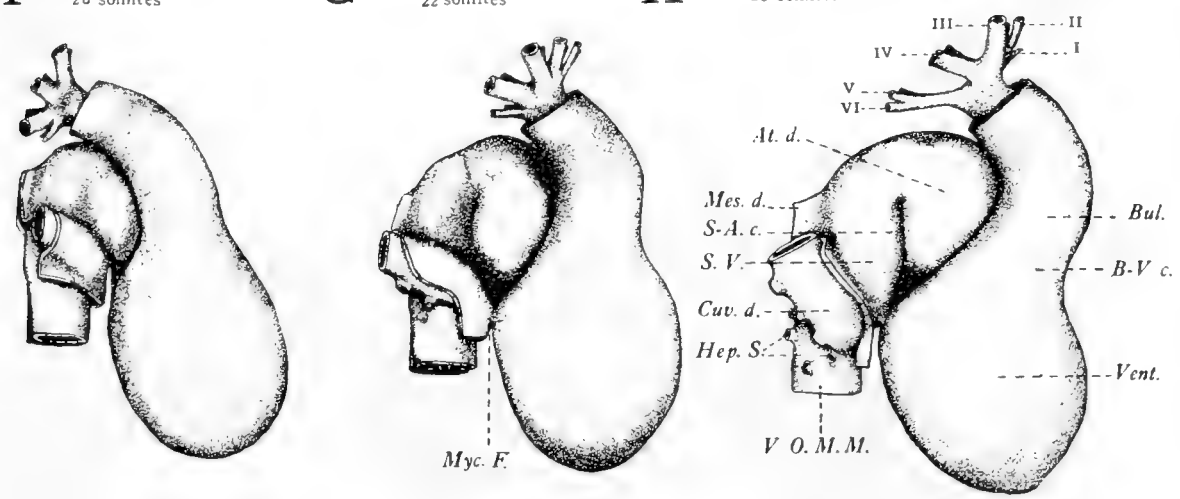
Dextral views (× 25) of same series of hearts shown in plate 1.



A 29 HOURS 9 somites
B 30 HOURS 10 somites
C 32 HOURS 12 somites
D 38 HOURS 16 somites
E 40 HOURS 18 somites



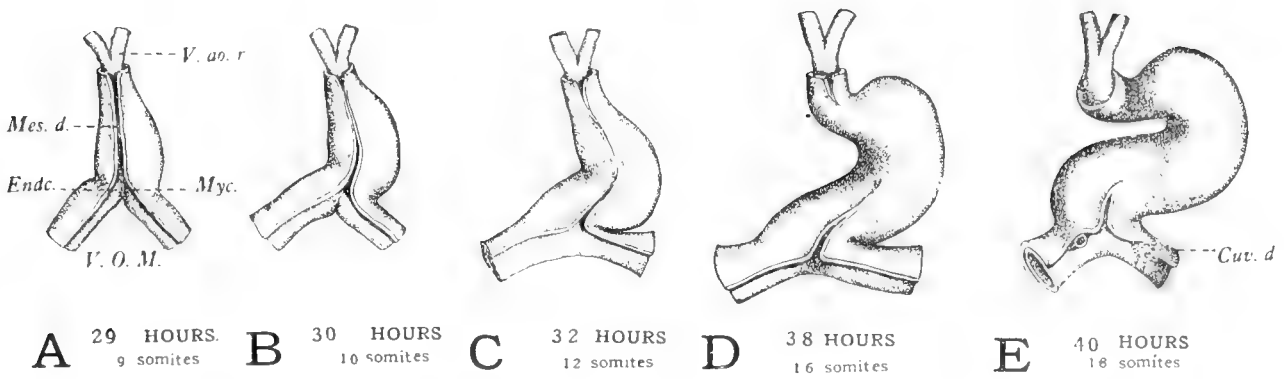
F 42 HOURS 20 somites
G 44 HOURS 22 somites
H 47 HOURS 25 somites
I 53 HOURS 29 somites



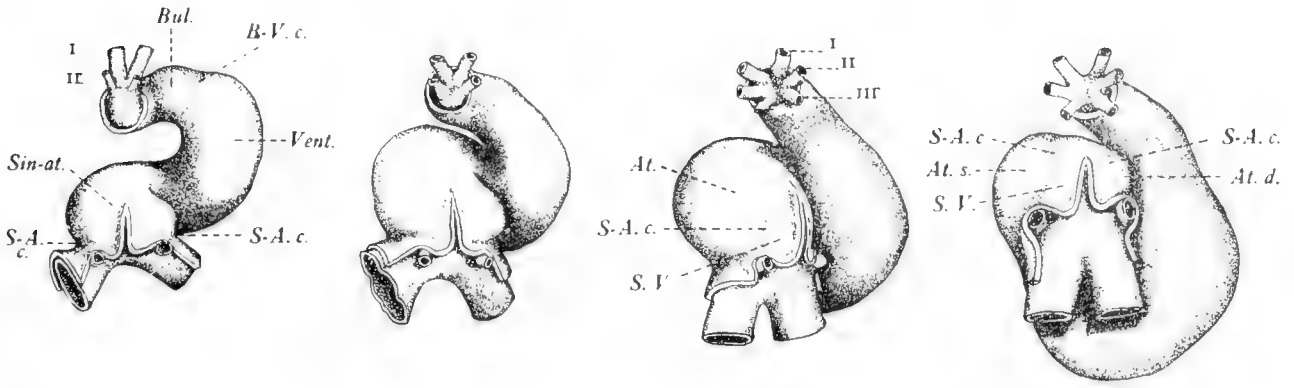
J 65 HOURS 33 somites
K 76 HOURS 38 somites
L 100 HOURS 45 somites

PLATE 3

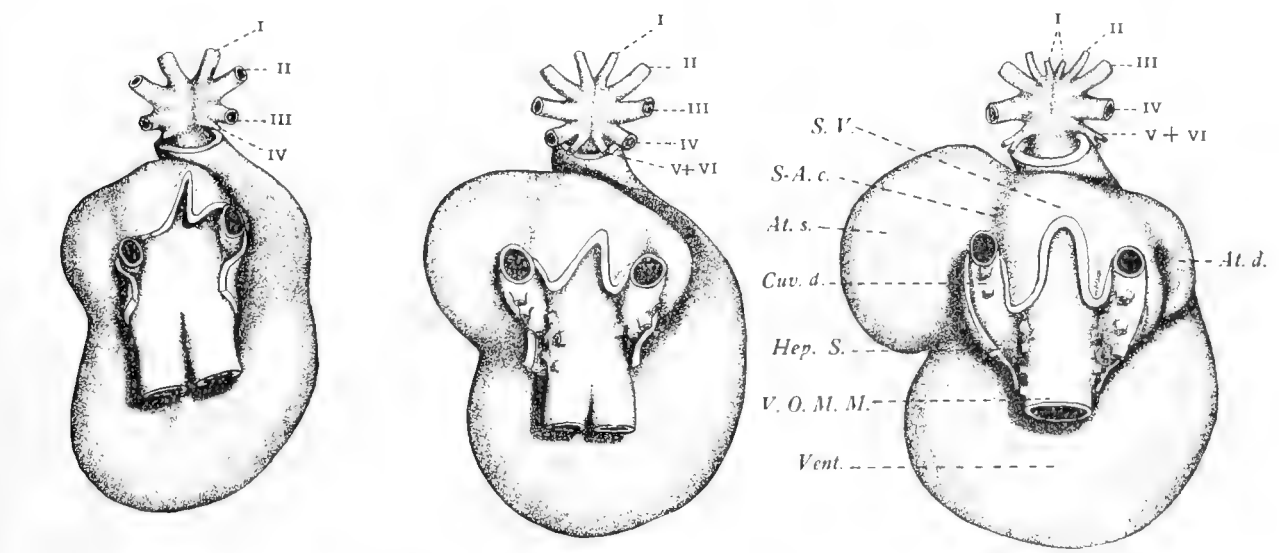
Dorsal views (× 25) of same series of hearts shown in plates 1 and 2.



A 29 HOURS. 9 somites B 30 HOURS 10 somites C 32 HOURS 12 somites D 38 HOURS 16 somites E 40 HOURS 18 somites



F 42 HOURS 20 somites G 44 HOURS 22 somites H 47 HOURS 25 somites I 53 HOURS 29 somites



J 65 HOURS 33 somites K 76 HOURS. 38 somites L 100 HOURS 45 somites

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Resumen por el autor, R. S. Cunningham.

La reacción de las células que tapizan la cavidad peritoneal, incluso el epitelio germinal del ovario, a los colorantes vitales.

El autor ha observado que las células de la serosa que tapiza la cavidad peritoneal almacenan los colorantes vitales de una manera muy característica. Las dos manifestaciones más sorprendentes de esta reacción consisten en la localización de la concentración de los gránulos tintóreos en un área circumscrita del citoplasma de cada célula y en la formación de una roseta perinuclear. Las células mesoteliales de diferentes áreas de la superficie peritoneal presentan ciertas particularidades en sus reacciones con los colorantes vitales, las cuales son suficientes para clasificarlas en grupos mientras que todavía se conforman con el tipo general característico. Las variaciones notadas en las células de diferentes áreas de la superficie peritoneal consisten en diferencias en la cantidad de colorante almacenado, las características de la roseta perinuclear y la orientación de la acumulación localizada de partículas tintóreas dentro de la célula. Las células que cubren al intestino contienen generalmente la menor cantidad del colorante, mientras que las del mesotelio esplénico y las del epitelio germinal del ovario contienen la mayor cantidad. El epitelio germinal almacena los colorantes vitales de una manera especial y característica. Cada célula contiene una masa de gránulos, redonda, oval o en forma de copa, en la zona infranuclear de la célula; esta masa, en los ejemplares bien teñidos, llenaba toda la porción de la célula situada entre el núcleo y la membrana basal. Por otra parte, la roseta perinuclear ha sido hallada solo raras veces en las células del epitelio germinal.

Translation by José F. Nonidez
Cornell Medical College, New York

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THE REACTION OF THE CELLS LINING THE PERITONEAL CAVITY, INCLUDING THE GERMINAL EPITHELIUM OF THE OVARY, TO VITAL DYES

R. S. CUNNINGHAM

Department of Anatomy, Johns Hopkins University

ONE PLATE (EIGHT FIGURES)

The general trend of the large amount of work carried out on the nature and significance of vital staining has been to indicate a definite relationship between all the cells which manifest a reaction to these dyes in the same way and to the same degree. That many observers have been extravagant in regard to the various applications of this theory may be admitted, while the strict value of the principle is still adhered to. Again it has been customary to consider as important only those reactions to this group of dyes in which large, or at least moderate amounts were stored. All cells having a smaller content were more or less huddled together and left unstudied. Yet the careful study of the characteristics in those groups of cells where only minor amounts of vital dyes are stored may prove of as much interest and importance as the elaborate study of those cells in which the power to store these substances is very great. This is, I think, further suggested in the recent brilliant studies of Evans and Scott ('20) on the reactions of clasmatocytes and fibroblasts. This work, though directed towards the differentiation of these two cell-types in as exact a way as possible, revealed much of the reaction of the fibroblasts with different exposures to many vital dyes, and it is practically certain that these observations will later be of very great value in increasing our knowledge of the phases of fibroblastic activity.

Since the cells lining the peritoneal cavity do not store vital dyes in large amounts, they have been among those to which but little attention has been paid. But the finding that the

reaction of these cells is most specific in character, even though the amount of dye stored is much less than in the case of the clasmatocyte, has suggested that this may be a method which will assist in solving some of the long-discussed questions regarding the relationships which the cells lining different areas of the peritoneal cavity bear to each other and to other cells.

Goldmann, in his studies on vital staining, gives very brief consideration to the peritoneal lining cells. In one place ('12, p. 40) he states specifically that the 'endothelial serosal lining cells' do not store any vital dyes, and in another ('09, p. 45) he records the observation that the germinal epithelium of the ovary likewise does not stain in the living animal.

Pappenheim ('13) and Pappenheim and Fukushi ('14) agree with Goldmann that the peritoneal mesothelial cells do not take vital stains. They use this lack of the ability to store vital dyes as an argument against the participation of the lining cells in the formation of the free phagocytic cells of inflammatory exudates. Tschaschin ('13) carried out experiments using collargol, isamine blue, and trypan blue, and states that he found that the serosal lining cells remained entirely unstained.

That peritoneal mesothelium stains to some extent, however, when exposed to vital dyes has been noted by Schlecht, Evans, Kiyono, and Foot. Kiyono ('14) has given the most elaborate description of the vital staining of mesothelium, and his are the only illustrations of vitally stained serosal lining cells which I have been able to find. His figure (a), plate (1), shows two cells containing dye from animals which had been vitally stained by intravenous injections of carmine; these cells he designates as peritoneal lining cells, but he does not state from which surface they were obtained. Kiyono also studied the reaction of these cells to trypan blue in the omentum and found that the distribution of the blue was precisely the same as that found for carmine. He suggests that the reason the trypan blue granules have not always been found in the mesothelial cells is due to too long fixation, during which the blue diffuses out of the surface cells. He fixed his omental spread preparations for one hour and then studied them for the distribution of the dye. He

describes a concentration of the dye-granules near the nucleus, but suggests that this is due to the greater amount of cytoplasm in that region, and he intimates that there are as many granules elsewhere in relation to the amount of cytoplasm. The granules, which he found, were always small and evenly distributed without accumulations in any particular areas, or irregular clumping as has been found in the clasmatocyte. His plate (1) gives a most interesting comparison between the immense amount of carmine which has been stored by the clasmatocytes and monocytes, and the sparse granulation of the serosal cells. On the other hand, the fibroblasts show amounts of carmine not very different either in amount or distribution from that of the mesothelium. Kiyono thinks that his work with vital dyes indicates a very definite and close relationship between the serosal cells and the fibroblasts, though he finds no actual transition between the two cell-types.

Evans ('15) has also described the sparse granulation of the serosal cells, noting that the fine granules of dye were concentrated around the nucleus, but his descriptions are very brief and do not indicate whether or not he agrees with Kiyono in regard to the more general distribution of dye-granules throughout the cytoplasm.

Schlecht ('07) also describes the peritoneal mesothelium as taking vital dyes, but does not differentiate very sharply between the surface-cells and connective-tissue cells, which probably also include some true clasmatocytes. His observations are not very exact with regard to the serosal lining cells, but are sufficiently so to indicate that there are some vital dye-granules to be found in the serosal mesothelium.

Tschaschin, on the basis of his studies with vital dyes ('13 a) and on experimental inflammations ('13 b), has concluded that the serosal lining cells constitute a specific group, having no relationship with the connective-tissue elements.

Evans and Scott ('20, p. 47) have referred to the subject of the specificity of mesothelial cells as distinct from fibroblasts and clasmatocytes in their monograph on the connective-tissue cells. They agree entirely with Tschaschin in considering the

lining cells as a distinct strain and state that the use of the azo-dyestuffs has furnished, in part at least, a means of differentiating these cells. They do not give any very specific discussion of the characteristics which the surface-cells display other than that the granules are always minute and that the spleen manifests them in the most marked degree. That they have observed many of the characteristic differences which the cells lining the peritoneum manifest towards vital dyes is undoubted, and their opinion is clearly in favor of their specificity, but the analysis is insufficient for establishing any basis regarding the especial peculiarities of these cells. Undoubtedly an examination of these cells with a large number of dyes would yield further interesting facts.

Ribbert ('04), Schlecht ('07), Goldmann ('09), Kiyono ('14), and Evans ('16 a, '16 b) have described the vital staining of the atretic follicle of the ovary. But none of these authors describes any staining of the germinal epithelium, Goldmann alone referring specifically to these cells, stating that they do not store any vital dye at all.

Foot ('19, p. 366) reports the observation that the serosal cells from the omentum stain deeply with vital dyes, and uses this reaction to suggest their having a genetic relationship to free cells formed during inflammatory reactions. In a later communication ('21, p. 635) he describes the serosal lining cells of the omentum as containing a few fine granules, and concludes that most of the dye appears to pass through the cells.

Most of the work that has been done on the cells lining the peritoneal cavity has been directed to the solution of the problem of relationship between these cells and the free cells of inflammatory exudates. But the serosal lining cells are of interest in other connections, among these the most important are their relationship to fibroblasts and to the fundamental physiological activities in which the surface-cells are adapted to mediate. The phenomena of vital staining is of very great importance in differentiating cell-groups from cell-groups and has already assisted very much in outlining the absolute separation of serosal cells from clasmatocytes. Can it also be used to assist in establishing

the relationship which exists between the fibroblast and the serosal cell? Finally, a careful study of the way in which serosal lining cells react to vital dyes may throw some additional light on the interrelationships of different areas of mesothelium and on the physiology of these cells, both individually and as a living membrane.

MATERIAL AND METHODS

It is obvious at once to anyone who has studied the cells lining the peritoneal membrane that sections cut in the ordinary manner perpendicular to the flat surface of the cells could yield but little information as to the extent or arrangement of the dye concretions in the entire cell. It is, I think, this fact that has caused so many observers to conclude that the cells are entirely free from the dye; while most of those who have described the granulation have studied the cells in the omentum where they can be observed in their entirety by means of spread preparations.

The observations reported here have been made from a series of experiments on rabbits. The animals were stained with Grübler's trypan blue or with carmine prepared according to Kiyono's method. Both dyes were usually administered intravenously at intervals of twenty-four or forty-eight hours, the animals received from six to thirty doses of carmine, from three to twenty doses of trypan blue, and were usually sacrificed one to two days after the last injection.

In the study of the cells several methods were used. The animal was anesthetized and the abdomen opened, cells were scraped from the surfaces of all the viscera, diaphragm, and body-wall, and were studied immediately. Smears were also prepared in a similar way, fixed by heat or alcohol and then stained in an appropriate manner to obtain good contrasts. Spread preparations of omentum and mesentery were studied fresh. Omental spreads were fixed by immersion for one hour in osmic acid or neutral formalin, washed quickly and stained; others were treated with weak ($\frac{1}{4}$ to 1 per cent) silver nitrate, exposed to sunlight, and then fixed in alcohol. Very thin blocks of all the organs which border the peritoneal cavity were fixed in neutral formalin for one hour, and then transferred to 80 per

cent alcohol, dehydrated, and embedded as rapidly as possible. Blocks were also fixed in 95 per cent alcohol, it having been found that the dye did not diffuse out to any appreciable extent from tissue fixed in strong alcohol. Sections were cut parallel to the surface and all the surface sections carefully preserved in series. In this way some slight shrinkage of the tissues occurred as determined by control preparations, but any loss of dye from the cells was reduced to a minimum. Finally, very thin membranes were obtained by stripping small bits of the surfaces of organs while the blocks of tissue were in 80 per cent alcohol; these were stained and cleared, and while many were entirely too thick for careful study, others were obtained which were as thin and easily analyzed as omental spreads. In general there seems to have been some loss even in these preparations, as could be determined by the control studies in fresh material, but the loss was in no way sufficient to prevent cellular orientation, which is obviously the one difficulty in the interpretation of the cells removed by scraping.

It is a pleasure to thank Mr. Didusch for the care and accuracy with which he made the excellent drawings.

EXPERIMENTAL RESULTS

The cells which line the peritoneal cavity may be divided into four classes by means of the reactions to vital dyes. These are all closely related to each other, agreeing in certain particulars which seem to be very fundamental, while they differ in minor points which are probably in some way related to the function of the organs which they cover. The classification into four groups is not to be considered as absolute, because variations in one may approach the normal in another, but the general types are sufficiently distinct to require separation.

1. General serosal mesothelium, including the cells covering:
   a. intestine.
   b. body-wall.
   c. liver, pancreas, etc.
   d. mesentery.
   e. diaphragm.

2. Cells covering the omentum.
3. Cells covering the spleen.
4. Cells covering the ovary, the so-called germinal epithelium.

The first division is a composite one, including all the cells which have no very sharp differences from each other, and besides all of these cells agree in general in storing smaller amounts of the dyes than do those of the spleen and ovary and differ from the cells of the omentum in certain peculiarities other than the amount of dye stored. The lining cells of the mesentery are very much like those of the omentum, but have certain local differences which are more like the general lining cells elsewhere, thus placing the cells covering the mesentery midway between the ordinary serosal lining cells and the cells covering the omentum. In this way there are all gradations between the cells covering the intestine which take less vital dye than any other serosal cells, and the cells covering the spleen and the ovary, both of which show a characteristic vital staining with considerable ease. The other variations and what evidence there is of a parallel physiological relationship will be taken up later.

The most obvious characteristic which has been observed in the manner in which the lining cells store vital dyes is the collection of the dye concretions into an area more or less regular in shape in a definite portion of the cytoplasm. The distribution of dye-granules in a perinuclear rosette is also characteristic of the serosal cells, but requires longer exposure to the dye for its development and is never so universally present as the specific separate mass in the cytoplasm. These two specific types of staining are often found combined, but each and particularly the clump of granules may appear alone or with many minor variations, which are in part characteristic of the cellular surface, in part the result of the kind of dye employed, but to a greater extent due to the length of time during which the cells have been exposed to the dyes. Again it has been noted in general that carmine tends to remain more definitely collected in a small area, while trypan blue tends much more to form perinuclear rosettes, usually with, but sometimes without, local concentrations. It

is quite likely that still other variations than those described would become apparent if a larger series of dyes could be employed, and they might prove of value in further subdividing the serosal cells into smaller groups.

General serosal mesothelium

In animals acutely stained with trypan blue (i.e., having received six to eight doses), the cells covering the intestine, the body-wall, the liver, the mesentery, and the diaphragm had in common the distribution of dye-granules in a fine irregular, perinuclear rosette with a more or less evident concentration at some area in the cytoplasm. There were a few scattered granules besides, usually just adjacent to the small clump. In animals having had only three to five doses, the perinuclear rosette was usually represented by an occasional granule and the small cytoplasmic clump at this earlier stage was present as a small irregular ring of granules. This ring was usually the first evidence of dye in the cell and often formed an almost perfect ring or oval, usually about one-half or one-third the size of the nucleus. The most conspicuous individual differences between the individual surfaces included in this group, as shown in acute staining with trypan blue, were the greater number of diffused granules in the diaphragmatic cells, the more sharply formed ring in the mesentery, the greater tendency to perinuclear rosettes in the liver, and the generally sharper localization and less amount of dye in the body-wall and intestine. These differences are given more because they indicate the relative lack of extremely sharp differentiation than because they seem of great importance within themselves.

In animals in which staining was carried beyond eight to ten doses of trypan blue, the most important changes were a general increase in the amount of dye around the nucleus in all the cells of these surfaces except the diaphragm where there was a more definite increase in the granules diffusely scattered around the circumscribed area of earliest staining (fig. 2).

Turning now to the reactions of this group of cells to intravenously administered carmine: in the early stages when the blue was found in a ring with a few scattered perinuclear granules the carmine was almost always in a small round or oval patch, always sharply localized in the cytoplasm. These carmine granules were almost always very small, uniform in size and evenly distributed. The regularity with which the early carmine staining showed this precise arrangement was quite remarkable. When the experimental animal had received more dye, ten to fourteen injections, the principal difference noted was the increase in the size of the patches of carmine granules and the beginning of the perinuclear rosettes. In animals in which the staining was carried much further—fifteen to thirty doses—the size of the isolated groups of dye-particles increased still more, and in many cases there were some granules which had apparently strayed away from the central fold so that these areas were no longer so sharply outlined. This distribution of the dye was particularly characteristic of the cells covering the diaphragm, the irregularity of the granules almost furnishing sufficient degree of difference to permit the classification of these cells in a separate group. These diaphragmatic mesothelial cells were the first to show perinuclear rosettes, though these were often most irregular and diffuse. Figure 8 shows two cells which were obtained from the surface of the diaphragm of a rabbit which had received twenty-five doses of carmine on alternate days; both the diffuse perinuclear arrangement and the irregularly scattered cytoplasmic granules are shown, but nevertheless there is quite definite evidence of a greater concentration of the dye in a definite part of the cell. Only in very rare instances have cells been found from any of the surfaces of the general serosa that did not present this wholly characteristic type of staining.

In animals stained over a considerable length of time, surface-cells other than those of the diaphragm also contained more carmine granules, but distributed usually with greater regularity. In those over the stomach and intestine the additional granules were usually added to the preexisting small ring or mass, while

a few scattered granules sometimes developed about the nucleus. In the cells covering the liver particularly and the other surfaces to some extent, the carmine granules, with increased staining, often developed into a long, oval belt which sometimes crossed the nucleus only on one side, and sometimes extended partially if not wholly around it. So that instead of having the circumscribed perinuclear rosette which has been described as characteristic of trypan blue, these cells had a rosette in the form of a belt running all, or part way, around the nucleus anteroposteriorly.

Finally, it seems important to emphasize the question of the orientation of the dye distribution in relation to the nucleus and the position of the cells in situ. These observations were made both upon living cells and upon sections, the latter being cut perpendicular to the surface. In sections the perinuclear rosette so typical of trypan blue was clearly seen as a patch of granules at either end of the nucleus, one or the other containing more blue corresponding to the area of localization. This was often extended somewhat over the surface of the end of the nucleus. But the characteristic arrangement was the principal amount of the blue close to one end of the nucleus. In the early staining with carmine the small sharply localized area of granules was usually between the nucleus and the surface of the cell, but placed laterally to the central axis of the nucleus, so that as the dye increased in amount with longer exposures the cross-sections showed red granules in both outer and inner zones of the cell. This again was the formation of the perinuclear belt, already described, from the lateral or anterolateral group of granules. Considering the relationship of the early areas of carmine staining to the center of the nucleus as distributed along its long axis, they were found to be usually somewhat nearer one end, but were often close to the center. As the carmine increased in amount to the maximum, the granules often covered the entire length of the nucleus, but more often formed the perinuclear belt.

The omentum

When the omentum from a rabbit that had been vitally stained with trypan blue was spread on a cover-slip, treated lightly with silver nitrate and stained with carmine, the large pavement cells of the serosa were found to be very characteristically stained. They almost always contained a small ring of blue granules in one part of the cytoplasm, sometimes this ring was entirely regular and smooth in contour and again it was broken or oval shaped. These granules appeared in animals which had received only a few doses of blue; when the staining was carried further, the cytoplasm in the neighborhood of this first ring-like group of granules began to show a few additional granules, and finally the perinuclear rosette developed. In an animal in which the staining was carried out with the usual amount of dye, six to eight doses, the sharply defined area was very characteristic and the ring was still present though obscured by the development of some diffuse granules adjacent to it. The perinuclear rosette was present in about one-half to one-third of the cells, but was usually quite irregular. Perhaps the most interesting and characteristic finding was that, in the majority of the cells, the ring and its subsequently attached granules were opposite the long axis of the oval nucleus (fig. 5). Most of the mesothelial cells covering the omentum were rather elongated, and with the patches of dye-granules located in one end they presented a very characteristic appearance. With longer exposures to blue, the granules gradually extended throughout the end of the cell and a few appeared irregularly elsewhere in the cytoplasm, increasing the sharpness of the perinuclear rosette, and sometimes appearing over or under the nucleus. Occasionally there were two sharp areas of localization in a single cell, but this was not common in the normal cells except in those where there were two nuclei; in such instances the arrangement was usually of one or two types. In many cells the dye-granules were arranged about each nucleus as though the cell might be in process of division, suggesting that each group of granules eventually would be entirely characteristic of an individual cell. In others there

were granules massed between the nuclei, and this mass extended arms of dye which surrounded the two nuclei in more or less irregular rosettes. In the one case the arrangement suggests that the two nuclei are functionally the centers of separate cells, while in the other the arrangement implies that the cell is truly binucleate and functionally a single unit. The collection of granules opposite the long axis of the nucleus was not always present, a few cells in every preparation having the principal mass at the side of the oval nucleus, but the proportion was greatly in favor of the other type. A few granules were often seen scattered between the nucleus and the surface or in the infranuclear zone, but the principal mass was very seldom in either of these two regions.

In animals stained with carmine the same distribution was observed with certain modifications. The mass of carmine in the end of the cell often extended until it almost filled the entire cytoplasmic area, but always the borders were more regular than with the trypan blue, the irregular scattered granules being much less numerous. The perinuclear rosettes were more infrequent, or rather were relatively later in appearing in the case of carmine. However, in very heavily stained animals they were to be seen in many cells, long fine arms of granules extending irregularly across or around the nucleus, or perhaps out into the free areas of cytoplasm. A similar condition is shown in figure 7, which is from the spleen. It is interesting to recall that the cells of the omentum show much more diffusely scattered granules after they have been irritated than do the cells of the other areas, especially of the spleen (Cunningham, '22), but the significance of this still remains to be studied.

The spleen

In the cells covering the spleen the perinuclear rosette developed very early in the course of staining with trypan blue, but it was usually accompanied by some condensation into the type of arrangement which has already been described for the general mesothelium. As has been noted, this blue rosette

surrounded the nucleus parallel to its flattened surface, so that the condensation did not, as a rule, develop between the flattened surface of the nucleus and the surface of the cytoplasm, but rather it was adjacent to some point of the periphery of the nucleus. The numerous apparently different arrangements are all easily comprehensible if this fundamental type of the distribution of the dye is clearly understood. Despite the fact that in surface cells of the spleen the rosettes developed early and quickly, they contained only a small amount of dye before the condensation of granules became the most obvious part of the staining. These collections of dye very rapidly increased in amount until in smear preparations the cells often seemed to contain only this single large mass of dye. The location of the mass of dye in relation to the nucleus was very interesting, and while every conceivable appearance was found both in smears and sections, careful study revealed that the dye-masses were, in the vast majority of cells, located lateral to the flat surface of the nucleus and near the termination of its shortest diameter in the periphery. The rapid increase in the mass produced the type of cap seen in figure 4, this cell having been selected because the perinuclear rosette was represented by only a few granules, while the entire lateral surface of the nucleus was covered by the dye. In animals having received about eight to ten injections of trypan blue, this type of arrangement was very common, though more granules were usually present in the perinuclear rosette; the cells shown in figures 3 and 4 were both from an animal in this stage of staining. In sections from the spleen at this stage the nuclei of the mesothelial cells were bordered at either end, the 'ends' being obviously the optical appearance—rather than the anteroposterior poles—of the long axis of the nucleus, by groups of blue granules varying in amount from a small number to a large patch, practically filling the cytoplasm adjacent to the nucleus. The amount of the dye in such sections was obviously dependent upon where the section divided the cell in relation to the principal mass of dye.

On further increasing the amount of dye administered, the edges of the principal dye-mass extended irregularly further and

further around the nucleus, until, in many cases, in sections, granules of dye were found in either or both of the supranuclear and infranuclear zones of the cells. With such increased amounts of dye administered, the cell-body increased considerably in thickness usually throughout the entire length, though a supranuclear bulge was occasionally present. This arrangement was constantly found in most of the cells, but there was a small proportion in which certain characteristic variations appeared. In some cells the location of the primary concentration was between the center of the nucleus and the pole of the long axis; in such cells the extension of the mass led to considerable irregularities in the relation between the nucleus and its cap of blue. Some of these caps extended around or partly around the pole of the nucleus in an irregular fashion. Again, a few cells were noted in which two localized aggregations were situated on opposite sides connected by arms of blue extending around the nucleus from one to the other. These double caps were found on the two poles as well as on the two flattened sides of the nucleus, although they were more numerous in the latter location. Again, it was found that the lateral mass had extended almost entirely into the infranuclear zone and had produced a reaction somewhat similar to that seen in the germinal epithelium of the ovary. Finally, it must be noted that the lateral location of the dye-mass, extending a fine outline of granules around the nucleus to form the typical perinuclear rosette, may, I think, be considered as the one most striking characteristic of the splenic mesothelial cells of the rabbit when vitally stained with trypan blue.

Turning now to the results obtained with the intravenous administration of carmine, the cells of the splenic mesothelium were found to be equally as characteristic as when stained with trypan blue. In the early stages the carmine was found distributed in the usual sharply localized area just lateral to the center of the nucleus and always superficial to it. As the amount of dye was increased, the area extended more and more on all sides, sometimes in a regular fashion, sometimes with fine lines and a few detached granules; figures 6 and 7 illustrate two stages in this progressive increase. The perinuclear rosette was some-

times found at about this time, but was always most irregular and never so sharp and characteristic as seen with trypan blue. The area of the cell just over the nucleus, which contained the localized mass of dye, was humped up often to a considerable extent. When the staining was carried to the maximum used in these experiments, the edges of the supranuclear mass extended irregularly around the nucleus and reached the infranuclear zone, in this way forming a perinuclear belt, similar to, but much more extensive than, those found in the cells of the general serosal mesothelium. The cells had by this stage increased considerably in size and over a larger surface than at the stage when there was only a supranuclear swelling. In some few cells the mass of dye seemed to be around the pole of the nucleus, probably the first-formed area was somewhat displaced from the region of the center of the cell. This cap formation around the longitudinal pole of the nucleus was not as common as with trypan blue. The principal difference observed in the reactions of the cells to the two types of dyes was the greater tendency of the carmine to enter the supranuclear zone and to remain circumscribed, while the trypan blue extended towards the periphery and usually towards the infranuclear zone. In general the cells covering the spleen contained considerably more dye than any other serosal cells at the same general stage of staining.

It has been reported elsewhere (Cunningham, '21, '22) that, in cats in which the splenic mesothelial cells had been irritated, the trypan blue granules tended to collect in the infranuclear zone of the cell together with many highly refractive droplets which were developed during the progress of the irritation. Further, it was found that in experimental hydremia in cats the fluid always collected in the infranuclear zone. The normal vital staining in the cat likewise tends towards the perinuclear rosette, but with a considerably greater tendency to the infranuclear extension. Whether these differences are very important cannot be stated as yet, sufficient data on long-continued irritations of the serosa of heavily stained rabbits not being completed. The area which shows the most active vital staining

probably is important in relation to other activities, or potential activities, of these cells. No further conclusions can legitimately be drawn from these observations at the present time.

The ovary

The cells covering the ovarian ligaments in the rabbit are the ordinary, normal, flat mesothelial elements, but at the point of attachment these cells pass by rather an abrupt transition into the cuboidal cells which constitute the investiture of the ovary proper. These cells, commonly known as the germinal epithelium of the ovary, are cuboidal in shape and have clearly defined, sharply staining nuclei. They seem to rest upon a very definite basement membrane and the nuclei are placed in general about midway between the surface and the base.

When vitally stained these cells stored the dye in the infranuclear zone, but only in relatively small amounts in comparison with the amounts which were taken up at the same time by the clasmatocytes and other cells commonly known as the more active in their reaction to the vital dyes; so that in lightly stained rabbits there were very few granules to be seen in these cells, not more than were found in the cells of the peritoneum generally. An increase in the amount of stain given caused a greater increase in the amount of dye which was stored by the cells covering the ovary, and a very prolonged course of staining produced an astonishing picture; the amount of dye in the cells increased very greatly until the bases of the whole layer seemed entirely filled with the dye (fig. 1). A more accurate description of the vitally stained germinal epithelial cells is given from one experiment that demonstrates especially well the very large amount of dye that can be stored: the cells of the germinal epithelium varied considerably in size and shape, usually they were cuboidal with a tendency to columnar form, the outer border occasionally tended to be rounded and to show slight indentations between the cells. The nuclei were relatively clear and stained well. They were located near the center of the cell, but there was a slightly wider zone between the nucleus and the basement

membrane than between the nucleus and the surface. Here and there a cell contained a vacuole in the base of the cell, though it was very difficult to determine whether these were inter- or intracellular. Most of the cells had no vacuoles. But every cell, without exception, contained a group of trypan blue granules near the basal pole of the nucleus and often forming a slight cup around it. Careful examination showed that there were a considerable number of types of arrangement; some cells contained an irregular circle of dye-granules; others a solid, round clump; others, a cap on the lower pole of the nucleus, and some, an irregular patch with knobs, projections, etc. Here and there an occasional granule appeared in the upper part of the cell, or a line of them extended up around the nucleus in an arm-like manner. Some of the cells were very heavily laden with granules which extended often up around half of the nucleus. Practically all the blue granules were the same size, about $\frac{1}{4}$ to $\frac{1}{2}$ μ in diameter. None of them was very large, as in clasmatocytes, and yet few were very fine, as in ordinary normal mesothelial cells. Here and there one found cells which were considerably flatter, being about half cuboidal, and here the dye-granules were again located in the region of the cell just medial to the nucleus. These flattened cells seemed to be from the edge of the ovary near its attachment and suggested a transition to normal peritoneal mesothelium. When the transition from the flat mesothelium to the cuboidal germinal epithelium was studied it was found to be most interesting. Here transition cells showed less and less staining in the characteristic area down to the ordinary mesothelial cell with its patch of granules and perinuclear rosette.

Animals stained with carmine presented precisely similar pictures, the variations from the reaction to trypan blue were very considerably less than in areas such as the spleen or diaphragm. But there was slight tendency to surround the nucleus, and the irregularities of staining were always more sharply shown in the animals stained in trypan blue than in those stained in carmine. In many areas in the sections of the ovaries from animals stained with carmine, cells were found just beneath the layer of the germinal epithelium proper and forming a nest-like

mass bordered beneath by the basement membrane. These cells were stained exactly like the cells of the surface, having a small patch of the red granules around one pole of the nucleus, though they were not oriented in any especial manner. Whether these cells are derivatives of the layer of germinal epithelium or whether they are other cells reacting in a similar manner to the dyes is not yet clear.

DISCUSSION

The characteristic reaction to vital dyes which has been described for the general peritoneal mesothelium certainly suggests that there is a similarity in function which extends throughout the membrane, and perhaps may even include the germinal epithelium of the ovary. The differences noted between diverse areas such as spleen and diaphragm may eventually prove to be specifically bound up in the peculiar functions of the organs which they cover, or may be due to differences in environment, such as blood supply, movements of viscera, or other physical factors. The characteristic location of the vital dye-granules in the various types of mesothelial cells may have some bearing on the differences in the physiological activities of the organs which the cells cover, but our information is far too limited at present to even hazard a guess concerning these interpretations.

Obviously, the observations reported here may assist in the settlement of two general questions. In the first place, there has been much discussion regarding the relationship between the fibroblasts and serosal lining cells, some authors having maintained them to be the same fundamental type of cell manifesting different physical characteristics under different environments, and others having considered them as entirely different final types. The second question, in regard to which these findings may be of importance, is the relationship between the germinal epithelium and the general serosal mesothelium, on the one hand, and between the germinal epithelium and the structures of the ovary proper, on the other.

Evans and Scott ('20) have succeeded in separating the fibroblasts from the clasmatocytes by their reactions to vital dyes,

but they have done even more in giving us a very large number of figures of fibroblasts stained with various dyes. In studying their plates it becomes evident that there is no pattern which one can consider as in common between even a small number of the fibroblasts.

The typical fibroblast therefore shows no characteristic staining in a definite area of the cytoplasm such as is so evident in the serosal cells from the entire peritoneal membrane. Indeed, the reactions of the fibroblast to vital dyes would seem from these plates alone to ally them more closely to the clasmatocytes than to the serosal cells. Many observers have described reactions of connective-tissue cells and serosal cells as being quite similar during the different stages of inflammations, and on such grounds as these have considered that they are interchangeable (Cornil, '97; Ranvier, '93; Schott, '09; Weidenreich, '07; Roloff, '94, '96, and Dominici, '01, '02).

Clarke ('16), who introduced celloidin and paraffin into the general connective tissues, considered that he had produced a true experimental mesothelium which, according to him, was entirely comparable to the lining cells of the peritoneum. Among the older workers many thought that the mesothelial cells were capable of transformation into a 'variety of forms,' one of which was the fibroblast. Schott ('09) and Weidenreich ('07) suggest that the surface-cells of the omentum are merely flattened fibroblasts. Mallory ('20), in studying tumors of the arachnoid, has concluded that they are of connective-tissue character, and because of these tumors arising from the arachnoidal cells, he concluded that these cells must be considered as flattened fibroblasts.

The work done on the differentiation of fibroblasts into serosal cells seems to me inconclusive. That the normal serosal lining cell has a different reaction to vital dyes from that manifested by the fibroblast is undoubted, but no effort has yet been made to determine whether the cells which have formed around foreign bodies or those which have recovered denuded areas of peritoneum give reactions to vital dyes similar to those reported here for serosal cells. It seems, therefore, that the evidence so

far obtained tends to indicate that the serosal cell is a specific cell-type and is not a transformed fibroblast, in the sense of being interchangeable morphologically and physiologically.

The relation which the germinal epithelium bears to the ovary and to the cells lining the general peritoneal cavity has been extensively studied. In the course of these investigations there have developed two principal questions, both of which must be considered in the light of the reactions which these groups of cells manifest towards vital dyes. In the first place, are the germinal epithelial cells closely related to the cells lining the general peritoneum, or are these two groups entirely different genetically and functionally? In the second place, to what extent do the follicular cells, interstitial cells, and definitive ova have their origin in these cells which constitute the ovarian envelope?

Waldeyer ('70), after long and careful study of many species, decided that the cells of the general peritoneal mesothelium were in early stages entirely similar to, and continuous with, the germinal epithelial cells. But later in the developmental history, all of these cells except those over certain specific areas, differing in different species, were destroyed or desquamated and their place taken by subjacent connective-tissue cells. He found that in the amphibia the cells of the general serosa retained their primitive characters in wider distribution than in the mammals, and so he considered that the peritoneal sac might be thought of as fundamentally a reproductive pouch which gradually was narrowed until, in mammals, this property was retained only by the cells of the ovarian envelope.

Gatenby's work ('16) on the frog, in which he found that the general peritoneal lining cells could bud off and produce ova, is in support of Waldeyer's idea. But, on the other hand, many observers have considered the cells covering the genital ridge as being differentiated out of the general coelomic layers.

Neumann ('75) found that there were interesting interrelationships between the cells covering the ovary and those lining the general peritoneal cavity in the frog. He considered them genetically identical, because they both arose from the low

cylindrical layer of cells which lines the peritoneal cavity during its formation from the coelomic layers. Later, the special characteristics of the germinal epithelium are developed in connection with their further differentiation into sexual elements. Furthermore, he found that the flat serosal cells of other areas in the peritoneal cavity of frogs may be transformed into typical germinal epithelium when sexual maturity is reached.

MacLeod ('80) suggests that the peritoneal epithelium also undergoes modifications in other regions, e.g., spleen and pancreas, and Kolossow ('93), in his careful studies of the normal peritoneal epithelium in both mammals and amphibia, comes to the conclusion that the germinal epithelium of the ovary is a part of the true layer of peritoneal lining cells, which, owing to specific stimuli, has undergone a certain amount of differentiation. He also found certain differences in the cells covering the spleen in mammals and in those covering the stomach of amphibia.

Coert ('98) believes that the germinal epithelium is formed from the general coelomic epithelium and denies its specificity in Waldeyer's sense, but agrees with him that the germinal epithelium gives rise to primordial germ cells.

Van Beneden ('80), Allen ('04), Sainmont ('06), working with the cat, Firket ('14), ('20 a), using the chick, and others have come to the conclusion that the germinal epithelium of the ovary is a part of the peritoneal mesothelium which has become cylindrical. Even Waldeyer in his more recent work has modified his earlier opinions indorsing the conclusions of Coert. In general all modern work has tended to establish the genetic continuity of the general peritoneal serosa and the germinal epithelium; whatever the factors may be that determine the specific changes which have been found in the cellular layers, particularly of those over the ovaries, but also to some extent of those covering the spleen, stomach, etc., they must be associated with some stimulus coming from the structures over which the changes take place.

The results of the vital staining of the germinal epithelium and the cells of the general peritoneal lining indicate a certain similarity in the reactions of these two groups of cells. There

are gradations between the intestine, body-wall, diaphragm, omentum, and the spleen; the latter approaching the type of staining manifested by the ovary more closely than the others. These cells have in common the localization of the dye in a particular part of the cytoplasm of the cell, and while there is no direct evidence that similarity in the storage of vital dyes represents similarity in physiological function, yet it is permissible to consider such findings suggestive. The extension of such studies to species such as the frog would be helpful because there is evidence suggestive of a greater similarity between the general serosal lining cells and the germinal epithelium in these species.

With regard to the second question concerning the relationship between the germinal epithelium and the internal structures of the ovary, a very large amount of work has been done. Elaborate reviews of the literature have been given by Firket, Coert, von Winiwarter, and others, and it is entirely unnecessary to repeat these here. In brief, it has been accepted by most workers that the medullary cords are downgrowths of the germinal epithelium, and many also believe the so-called cords of the second proliferation are likewise derived from the germinal epithelium. The question about which most of the discussion has been centered is the relation which the germinal epithelium bears to the definitive ova.

Waldeyer ('70) found large numbers of cells in the germinal epithelium which he interpreted as young germ-cells, and concluded that these cells were developed entirely from the cells of the ovarian envelope. This view has been supported in general by many workers, among whom are von Winiwarter ('01), von Winiwarter and Sainmont ('09), Sainmont ('06), Lane-Claypon ('06), Gatenby ('16), and von Berenberg Gossler ('12). In this way a general school has been developed whose principal belief is the origin of the definitive ova either directly from the germinal epithelium or from the cellular cords which have been developed as downgrowths from this superficial layer of cells.

On the other hand, Rubaschkin ('07, '12) and Swift ('14) have strongly supported the theory, advanced by Nussbaum ('80, '01), that the primordial germ-cells do not originate in the germinal epithelium, but come from cells which have not given

up their embryonic character and have not been differentiated into any especial somatic structures. Eigenmann ('91), Beard ('04), and Hoffmann ('92) have also supported this view. Swift ('15) derives the primordial germ-cells from a particular part of the germ-wall entoderm at the edge of the area pellucida during the primitive-streak stage. When this area becomes vascularized by the extension of the mesoderm, these cells gain entrance to the circulation and settle out in the region of the developing gonad. He agrees that the medullary cords are formed from downgrowths of the germinal epithelium, but considers the cords of the second proliferation as formed by rapidly proliferating, primordial germ-cells or oogonia. Finally, the oogonia form the definitive ova, while the cells derived from the germinal epithelium present in the cortical cords become follicular epithelium.

A third group of workers have assumed that there are two sources for the definitive ova, one the primordial germ-cells which furnish a few ova, while a second generation of germ-cells develop from the germinal epithelium. This view has been supported by Felix ('06), Allen ('04), Dustin ('07), and particularly by Firket. Firket ('14, '20 b) has described the early stages in the chick and rat, and in both species he finds the total number of the primordial germ-cells far too few to permit of their being considered the sole source of definitive ova, so that he is forced to assume an additional development of ova from the germinal epithelium. Using his two species as types of the bird and mammal, he suggests that this process may be a phylogenetic recession.

The discussion of the origin of the definitive ova from the germinal epithelium deals almost entirely with changes which take place during embryonic life, and hence, in mammals at least, would be most difficult to examine by the vital staining technique. However, it is well known that in some species there is formation of ova after birth, and in these it seems that the vital staining of the germinal epithelium might prove a useful adjunct to the study of the question regarding the origin of the definitive ova. My experiments, being confined so far to the

rabbit, do not offer any assistance in settling this point. On the other hand, the similarity between the reactions displayed by the cells of the germinal epithelium and the general serosal lining cells towards vital dyes suggest a closer relationship between these two groups of cells than has usually been assumed.

SUMMARY

1. The serosal lining cells have been found to store vital dyes in a very characteristic manner. The two most striking manifestations of this reaction consisted in the localization of a concentration of dye-granules in a circumscribed area of the cytoplasm of each cell and in the formation of a perinuclear rosette.

2. Mesothelial cells from different areas of the peritoneal surface presented certain peculiarities in their reactions to vital dyes which sufficed to classify them into groups, while they still conformed to the general characteristic type.

3. The variations noted in the cells from different areas of the peritoneal surface consisted in differences in the amount of dye stored, the characteristics of the perinuclear rosette, and the orientation within the cell of the localized collection of dye-particles. The cells covering the intestine usually contained the least amount of dye, while those of the splenic mesothelium and the germinal epithelium of the ovary contained the largest amount.

4. The germinal epithelium was found to store vital dyes in an especially characteristic manner. Each cell contained a round, oval, or cup-shaped mass of granules in the infranuclear zone of the cell; this mass, in well-stained animals, filled the entire portion of the cell between the nucleus and the basement membrane. On the other hand, the perinuclear rosette was found only rarely in the cells of the germinal epithelium.

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PLATE 1

EXPLANATION OF FIGURES

1 Section of the germinal epithelium of a rabbit which had received eight intravenous injections of trypan blue.

2 A binucleate mesothelial cell from the diaphragm of a rabbit which had received twelve intravenous injections of trypan blue.

3 and 4 Mesothelial cells from the spleen of a rabbit which had received ten intravenous injections of trypan blue.

5 A mesothelial cell from the omentum of a rabbit which had received six intravenous injections of trypan blue.

6 A mesothelial cell from the spleen of a rabbit which had received twelve intravenous injections of carmine.

7 A mesothelial cell from the spleen of a rabbit which had received eighteen intravenous injections of carmine.

8 Mesothelial cells from the diaphragm of a rabbit which had received twenty-five intravenous injections of carmine.

Fig 1 (x 2180)

Fig. 2 (x 925)

Fig 3 (x 925)

Fig. 4 (x 925)

Fig. 5 (x 925)

Fig 6 (x 925)

Fig. 7 (x 1300)

Fig. 8 (x 1300)

Resumen por el autor, Raymond M. Selle.

Cambios en el epitelio vaginal del conejillo de Indias durante el ciclo éstrico.

El ciclo éstrico del conejillo de Indias presenta cuatro periodos o estados bien definidos, además del intervalo, que corresponde a estados semejantes de la rata. En el primer estado, que en apariencia no ha sido notado por Stockard y Papanicolaou, el frote vaginal contiene solamente grandes células epiteliales granulosas y vacuolares. Estas células epiteliales derivan de las capas superficiales de la mucosa, debajo de las cuales ha tenido lugar la cornificación. La mucosa alcanza su mayor altura en este periodo. En el estado 2, las células epiteliales superficiales han desaparecido, dejando como capa más externa el estrato córneo. Este último mediante descamación susministra las células típicas, anucleadas, en forma de copos, del frote. Hasta este momento la semejanza con la rata es muy estrecha. Sin embargo, en el conejillo de Indias toda la capa córnea, no todo el epitelio, con algunas de las células no cornificadas pueden ser expulsadas en masa. Mientras que en el estado 3 de la rata el material granular y caseoso consta de elementos del estrato córneo, descamado rápidamente, aisladamente o en pequeños grupos, hasta desaparecer por completo, en el conejillo de Indias la masa caseosa descrita por Stockard y Papanicolaou está constituída por las células epiteliales no cornificadas situadas más profundamente, las cuales se desprenden en grandes números. En el último estado (4) los leucocitos descritos por Stockard y Papanicolaou en su estado 3 aparecen. El autor no ha hallado estado alguno que corresponda a su estado 4. No existe prueba alguna sobre la importante contribución del útero a los elementos celulares de la vagina, pues la última es prácticamente idéntica en los conejillos normales y en los histerectomizados.

Translation by José F. Nonidez
Cornell Medical College, New York

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CHANGES IN THE VAGINAL EPITHELIUM OF THE GUINEA-PIG DURING THE OESTROUS CYCLE

RAYMOND M. SELLE

THREE PLATES (ELEVEN FIGURES)

INTRODUCTION AND LITERATURE

The last few years have witnessed a considerable advance in our knowledge of the cyclical changes in the reproductive organs of the mammalian female, chiefly as the result of the introduction of a new method by Stockard and Papanicolaou; a method by which the course of the cycle may be followed in the living animal. The results obtained during the preceding years involved the sacrifice of animals at various times with relation to some definite event such as parturition or heat (when the latter is easily determined) and the microscopic study of sections. Obviously, it was not possible to study successive cycles in the same animal.

Nevertheless, data of some importance have been accumulated from the time of Lataste, Morau, and Retterer to the present. Since the literature of the period is dealt with more completely by Long and Evans ('22), it is unnecessary here to do more than call attention to those papers dealing more directly with the guinea-pig and the development of the method.

As early as 1892, Retterer observed that regular changes occurred in the vaginal mucosa of non-pregnant guinea-pigs. Following parturition he killed animals at regular intervals and was the first to describe a stratum cornium in the guinea-pig, although such a layer had been described in mice by Morau in 1889. He found that on the fifteenth day postpartum a layer of cornified cells was beginning to form under the superficial layers of the stratified vaginal mucosa.

In a number of papers Leo Loeb reported his results on the study of ovulation and the formation of corpora lutea in guinea-pigs. He came to the conclusion that it recurred at intervals of

about twenty-one days. Similar investigations on the incidence of ovulation, etc., in rats and mice were being carried on in this laboratory by Doctor Long and several students.

No further work of importance on the vaginal cycle of the guinea-pig was recorded until 1917, when Stockard and Papanicolaou published their paper on "The existence of a typical oestrous cycle in the guinea-pig with a study of its histological and physiological changes." They studied the vaginal cycle by taking samples of the contents of the vagina at regular intervals. They obtained these samples by introducing a small nasal speculum into the vagina, the arms of which were held apart by means of a thumb-screw, allowing them to observe the entire lumen of the vagina. They were thus able to recognize an oestrous rhythm consisting of four stages, each having a characteristic vaginal fluid which contained cells from the walls of the vagina.

The vaginal fluid during the first period contained a great amount of mucous secretion, many desquamated epithelial cells, and non-nucleated, or cornified cells, which appeared toward the end of the stage. The second stage could be recognized by the thick, cheese-like contents of the vagina due to the accumulation of the desquamated epithelial cells. During the third stage the vaginal fluid became thinner because of the solvent action of leucocytes which invaded the epithelium and entered the lumen. The fourth stage, during which a small amount of blood was found in the vagina, was of short duration and did not always occur. These stages were followed by the dioestrum, or intermenstrual period, during which the vaginal fluid contained mucus, atypical squamous cells, and many leucocytes. They then correlated these stages with the conditions of the uterus and ovary at corresponding periods.

A second paper by Stockard and Papanicolaou appeared in 1919 in which they described the vaginal closure membrane and the time of copulation in relation to the oestrous cycle. They found that there was formed regularly a very delicate epithelial membrane which closed the orifice of the vagina soon after the 'heat period' and remained closed until the next cycle or until parturition in case of pregnancy.

As the result of following the suggestions contained in Stockard and Papanicolaou's earlier paper, the cycle in the rat was worked out (Long, '19) and later elaborated and verified (Long and Evans, '22). Smears taken from the vagina of the rat by means of a small spatula were studied and correlated with sections of all parts of the reproductive system. By means of the smears, it was possible to recognize four definite stages besides the interval. Smears taken during the first stage contained nucleated epithelial cells of uniform size; during the second stage, few, large, cornified cells; in the third stage they were made up of a great abundance of cornified cells; in the fourth stage, by many leucocytes admixed with cornified cells. Throughout the interval between stage 4 and stage 1, or the dioestrous pause, the vaginal fluid contained leucocytes with only a few epithelial cells.

The sections revealed a cyclical growth and desquamation of the vaginal epithelium. The epithelium is lowest during the interval, increasing in thickness just before the advent of stage 1. One of the striking features is the occurrence, during this stage, of the processes of cornification not in the superficial layers but at a depth of almost two cells, the upper two layers of cells retaining their epithelial character, later to be shed as the cells of the smear of stage 1. The cornified stratum so denuded becomes superficial, increases in thickness, and is later detached, thus furnishing the cells of stages 2 and 3. Leucocytes, of which the epithelium is devoid during stages 1, 2, and 3, now invade the mucosa and escape into the lumen in stage 4.

Long and Evans attempted to correlate the cycles in the rat with the cycles in the guinea-pig as found by Stockard and Papanicolaou and as observed by themselves in twenty-two guinea-pigs for a couple of months. In a section of the vagina of the one guinea-pig which they killed, Long and Evans observed what they believed to be the cornified layer under a layer of epithelial cells. Since they found that such a condition occurred regularly in the rat, they conjectured that an epithelial layer superficial to the cornifying layer must be normal in the guinea-pig, and if such a condition were true, then it was evident that Stockard and Papanicolaou had overlooked it.

With these results in mind, it seemed worth while to study the changes in the vaginal epithelium of the guinea-pig in order to ascertain whether or not a layer of epithelial cells occurred superficial to the stratum cornium as observed by Retterer; to supplement the work of Stockard and Papanicolaou, and to correlate the rat and guinea-pig more exactly. The problem was undertaken at the suggestion of Prof. J. A. Long and was carried out under his general direction in the Zoölogical Laboratories.

METHODS

The guinea-pigs chosen for these experiments were common white, brown, and mixed colored virgin females about a year old. They were kept at a relatively constant temperature averaging 68° to 72°F. and were fed a constant, daily ration of green grass or alfalfa hay, carrots, and rolled barley, because there is reason to believe that a lower or a higher temperature as well as variation in the ration tends to affect the span of the oestrous cycle.

Since the determination of the state of progress of the cycle, as shown by the previous work cited on the guinea-pig and rat, is dependent on our knowledge of the character of the contents of the vagina, the method of sampling the contents becomes a matter of some importance. Several methods of taking samples were employed. For reasons explained later, the method employed by Stockard and Papanicolaou was discarded, and because of the extreme length of the vagina in the guinea-pig the spatula method as used by Long and Evans on the rat was not of much value.

The pipette method, suggested by Doctor Long and developed by Miss E. Fisher in this laboratory for making vaginal smears from mice, was tried, and after several improvements a satisfactory method for taking samples was perfected. The instrument used was made by inserting into a 25-cc. oval, rubber bulb a piece of thin-walled, glass tubing 14 cm. long and 1 cm. in diameter, about 5 cm. of which was drawn down to a diameter of 5 mm.

When a sample was to be taken, the animal was held ventral side up in the left hand allowing it to lie on its back along the left forearm with its head toward the elbow. The left thumb

was placed in front of the animal's left hind leg and exerted pressure just in front of the vulva; at the same time with the left forefinger between the vulva and the right leg, it was easy slightly to open the orifice of the vagina. The syringe, containing about 1 cc. of warm, normal, salt solution, was then introduced into the vagina to a depth of 25 to 40 mm. By squeezing the bulb and forcing the contents of the syringe into the vagina once or twice, it was possible to get a characteristic sample of the cells in the lumen. If, while withdrawing the syringe, pressure was applied anterior to the vulva with the thumb of the left hand, the sample could be drawn up readily into the syringe. Enough of a sample was obtained at one operation by this method to make several smears. This proved a most satisfactory method, because each time a sample was taken the vagina was washed out and the cells which were free in the lumen were removed; consequently, it was reasonable to suppose that all of the cells which were found in any one sample had been shed during the interval immediately following the preceding sample.

In order to study the cycle, samples were taken at 8 A.M., 12 M., 4 P.M., and 8 P.M., and the results recorded. Animals were then killed during each stage as revealed by the character of the smears. A small amount of 0.75 per cent solution of table salt immediately followed by Bouin's fixing fluid was injected under pressure posteriorly into the aorta just anterior to the diaphragm. By this method the vagina in situ was fixed in its normal position at the earliest possible moment and its excision greatly facilitated. After removal from the body the vagina was immersed twenty-four hours longer in the fixing fluid. Most of the picric acid was extracted in 50 per cent alcohol containing lithium carbonate. Portions of the vagina including the cervix were then dehydrated, cleared, and imbedded in paraffin and sectioned at $7\frac{1}{2}\mu$. The condition in the vaginae as revealed by these sections was then correlated with the smears taken just before killing the animal.

The uterine glands in the guinea-pig secrete an abundance of mucus which flows down into the vagina (Stockard and Papanicolaou, '17), often obscuring the nature of the smear. Indeed, Stockard and Papanicolaou found uterine epithelial cells thus

carried into the vagina. In order to avoid any confusion that might be caused by the presence of uterine mucus and cells, in seven of the twenty females the uterine horns were tied off posterior to the oviducts and anterior to the cervix and the intermediate pieces excised. The value of this operation was not sufficient to warrant its recommendation. Although the contents of the vaginae in the animals which had been hysterectomized were freer from mucus and consequently more easily diagnosed, there was not a great difference between smears made from these animals and smears made from normal guinea-pigs.

A careful scrutiny of successive vaginal smears of twenty guinea-pigs over a period of seven months disclosed a succession of cell changes in the contents of the vagina substantially similar to that described by Stockard and Papanicolaou for the guinea-pig and by Long and Evans for the rat. The enumeration of the stages in this paper follows that employed by Long and Evans.

CHANGES IN VAGINAL SMEARS AND HISTOLOGY OF VAGINAL EPITHELIUM

Interval of dioestrum

If the vaginal smears of a number of guinea-pigs were to be examined, it would be found that most of them would contain many leucocytes and few, small, round epithelial cells; in fact, this condition would be found to be characteristic during three-fourths of the time in any one pig. While the nature of the smear does not seem to vary during this period there are, however, histological changes in the mucosa.

The lining of the vagina of a guinea-pig consists of a stratified epithelium, the upper surface of which is nearly even, while the side in contact with the submucosa, from which it is clearly demarked, appears in section to be deeply lobed as though furrowed, the effect being produced by tongue-like projections of the submucosa extending up into the epithelium. The epithelium thus seems to vary in thickness (figs. 1 and 8). The whole mucosa is further thrown into larger longitudinal folds.

At about the middle of the interval the epithelium was found to be thinner than at any other time, being only one or two layers of cells high at the thinnest places. At the deep ends of the downward projections of the epithelium were numerous mitoses—a condition which apparently substantiated Stockard's and Papanicolaou's statement that the vaginal epithelium was regenerated from the base of the infoldings.

Towards the end of the interval the epithelium rapidly increased in height until it reached a maximum of ten to twelve cells. The uppermost of these cells which bordered the lumen had become enlarged and vacuolated, with their nuclei usually at their bases. The cells lining the inner surface of the vagina were the most vacuolated, while the layers beneath were smaller and gradually approached the normal size of the epithelial cells at about three or four layers below the surface (figs. 2 and 9).

Stage 1. Large irregular epithelial cells only

(No corresponding stage described by Stockard and Papanicolaou)

Stage 1 marks the beginning of the oestrous cycle. A smear made early in this stage exhibited, in marked contrast to that of the interval, first, numerous, large, round or odd-shaped, epithelial cells containing many vacuoles, and, secondly, very few leucocytes. The latter undoubtedly remained over from the interval which, as just described, was characterized by the presence of many leucocytes and a very few epithelial cells. These epithelial cells appeared in the smear singly or in groups of three or more.

A section of the mucosa early in stage 1 showed the epithelium to be practically as high as at the end of the interval, i.e., about ten to twelve cells; also that a characteristic transformation had already made its appearance. This transformation was exhibited in cornification of certain of the epithelial cells as shown in figures 3 and 10. It will be observed that the most superficial cells were enlarged, were odd-shaped, and contained vacuoles; that the next deeper cells were somewhat smaller and more spherical, and that the next deeper levels showed the beginning

of the process of cornification. The former of these were the cells which were cast off and furnished the cells found in a sample taken during this stage. They are called superficial epithelial cells for the reason that they were above a region in which cornification later took place. The cornifying process began rather suddenly and continued at a rapid rate simultaneously throughout the entire vagina. It began two to four cells below the surface of the superficial epithelium, and involved four to six cell layers. In some cases it extended up into the superficial epithelial cells before the latter had been entirely cast off. This is shown in figure 4. Beneath the stratum cornium may be distinguished the typical layers of stratified epithelium.

A little later in this stage the leucocytes had entirely disappeared from the vaginal fluid and the only cells found in the lumen were the superficial epithelial cells. As a result of the sloughing off of these cells, the vaginal mucosa became reduced in height and the cornified layer exposed.

It usually happened that the shedding of these superficial epithelial cells occurred while the entrance to the vagina was closed by the vaginal closure membrane described by Stockard and Papanicolaou, often making it necessary to break the membrane in order to get a sample.

Stage 2. Cornified cells only

(Stockard and Papanicolaou's stage 1)

A vaginal sample taken during this stage contained many flattened, horny, non-nucleated or cornified cells. No leucocytes were found in the lumen. Stockard and Papanicolaou in their first paper mentioned two kinds of cornified cells. These are apparently two different stages of the same process of cornification. The various intermediate degrees of cornification can be demonstrated both in smears and sections counterstained with eosin, the amount of stain taken by the cells varying with the extent of cornification.

Because of the fact that there were almost always a number of cornified cells at the external orifice of the vagina, smears of the

succeeding stages were constantly contaminated by them, especially in those obtained by means of a nasal speculum or a spatula; hence the value of the syringe method.

As the process of cornification continued, the cornified cells became loosened from the underlying layers and were cast off into lumen singly or in flakes of many cells.

An interesting occurrence not described by any writer was the shedding of the entire cornified layer as a cast which showed the natural shape of the lumen of the vagina. It was possible in several animals by the use of the syringe to get several succeeding cornified casts at regular intervals of sixteen days. When pieces of the casts were examined under a microscope, the structure was readily recognized as continuous layers of superimposed cornified cells. Some very perfect casts were obtained, even showing the folds about the cervix. Fourteen complete cornified linings or casts were obtained and preserved, besides a great many more casts broken in the process of taking the sample. This shedding of the entire lining of the vagina occurred at the end of stage 2.

The shedding of the entire cornified layer would seem to have been mistaken by Stockard and Papanicolaou to be the whole vaginal epithelium separated from the underlying connective tissue, for, according to them ('19, p. 234), "the epithelium is now expelled as one continuous tube forming the cover around the vaginal plug instead of sluffing off in smaller pieces as occurs during the fourth stage when copulation has not occurred. However, the vaginal epithelium may occasionally be shed en masse without copulation. . . . . It is clear, therefore, that what was termed by Lataste the 'enveloppe vaginale' is the layer of epithelium separated from the underlying connective tissue. . . . . ."

That the entire vaginal epithelium did not separate from the connective tissue, but that only the whole cornified layer became loosened from the underlying epithelium and lay free in the lumen, is shown in figure 5. The condition of the vaginal epithelium immediately after the shedding and expulsion of the cornified cast is shown in figures 6 and 11.

Stage 3. Cornified cells and small epithelial cells

The characteristic smear for this stage consisted of cornified cells, and of nucleated, small, epithelial cells. There are as yet no leucocytes found, but as the stage progressed fewer cornified cells and increasing numbers of epithelial cells appeared. Some of the latter contained granules in the cytoplasm. In case the cornified layer had been shed in a cast, this stage lasted only a short time during which the epithelial cells were cast off.

The active shedding of these two kinds of cells reduced the height of the stratified epithelium to from four to seven cells.

This stage was called the cheesy stage by Stockard and Papanicolaou, because of the appearance of the mass of epithelial cells. Since in this investigation the vagina was regularly washed out with warm saline solution in the process of taking samples and was thereby kept clean, the cells could not accumulate to form such cheesy masses.

During this stage leucocytosis began in earnest. Leucocytes invaded the submucosa and the epithelium, in the latter of which it was common to see aggregates of three to ten leucocytes. In many cases leucocytes had become imbedded in the epithelial cells, as many as five having been counted in a single cell. Cornified cells had completely disappeared from the smears and did not appear again until the next cycle.

Stage 4. Small epithelial cells and leucocytes

(Stockard and Papanicolaou's stage 3)

This marks the advent of leucocytes in the lumen of the vagina. In the previous stage the leucocytes were migrating into the vaginal epithelium, but had not yet reached the lumen. Smears made during stage 4 contained the nucleated epithelial cells of the preceding stage and also increasing numbers of polymorphonuclear leucocytes. At first the epithelial cells predominated, but gradually ceased to become detached; while at the same time the number of leucocytes rapidly increased until they were in a majority.

At the end of this stage the epithelium was only two to four cells high over the finger-like processes of the submucosa (fig. 7).

Following this stage, Stockard and Papanicolaou described another, which they called the fourth, during which blood was found in vaginal samples. In these investigations only two cases were observed in which blood was found in the smears, and in both cases it occurred at the end of stage 4. It is hardly probable that this is a common occurrence. Because of the thin epithelial covering over the submucosa at this stage, it seems highly probable that an instrument inserted into the vagina might injure some of the many blood vessels in the submucosa and thus permit blood to escape into the vagina.

Because of the differences in the stages of the cycles of the guinea-pig as found by Stockard and Papanicolau and in these investigations, the following table (1) has been constructed in

TABLE 1

A comparison of the stages in the oestrous cycles in the guinea-pig and rat

GUINEA-PIG (STOCKARD AND PAPANICOLAOU)	GUINEA-PIG (NEW)	RAT (LONG AND EVANS)
stage	*stage*	*stage*
1. First period: Squamous epithelial cells Second period: Cornified cells	1. Large irregular epithelial cells only. (Superficial epithelial cells)	1. Superficial epithelial cells only
2. Cheesy mass, epithelial cells	2. Cornified cells only	2. Cornified cells only
3. Leucocytes and epithelial cells	3. Cornified cells and small epithelial cells, or small epithelial cells only	3. Many cornified cells (cheesy)
4. Appearance of blood in lumen	4. Leucocytes and epithelial cells	4. Leucocytes and cornified cells (sometimes epithelial cells)
5. Interval: Leucocytes and epithelial cells	5. Interval: Leucocytes (few epithelial cells)	5. Interval: Leucocytes and epithelial cells

order to point out the similarities as well as the differences. It will be seen that these newly determined stages in the guinea-pig correspond more closely to stages in the rat as described by Long and Evans.

Interval

At the end of stage 4 or at the beginning of the interval the vaginal closure membrane began to form as described by Stockard and Papanicolaou, growing very rapidly and, if not disturbed, closing the vagina in one to three days. This membrane remained intact until mechanically destroyed or until the next cycle, when it was normally broken during the cornified stage by the tension produced by the swollen vulva and accumulated fluid in the lumen. When samples were taken daily, the membrane was prevented from forming for an indefinite period.

It will be recalled that toward the end of stage 4 and the beginning of the interval the rapid rate of desquamation of the epithelial cells gradually diminished, while the leucocytes increased until they were in the majority. The transitional period between stage 4 and the interval is indicated by the decreasing numbers of epithelial cells and increasing numbers of leucocytes in the smears. Samples taken up to the middle of the interval contained a few epithelial cells, but for the next week or more only leucocytes. From this time on until the beginning of stage 1 vaginal smears were found to exhibit leucocytes and some atypical cells.

A section through the vagina during the middle of the interval showed the epithelium to be only one or two cells high. This reduction in height was no doubt caused by the continued dropping off of the epithelial cells which were found in the vaginal fluid following stage 4.

Toward the end of the interval and immediately before the beginning of stage 1 the epithelium was rebuilt to a height of ten to twelve cells (fig. 2). The uppermost of these cells became vacuolated as described under the interval at the beginning of the discussion on stages.

It is interesting to note that, although there was the above rapid transformation in the epithelium, such a condition could

not be suspected by a mere study of the smears. Consequently, the method of obtaining material for histological study at this critical time involved a departure from the ordinary procedure. In the ordinary procedure it was only necessary to keep records of smears made from the animals under operation and to kill the pig at the desired period in order to correlate the smear with the condition in the vagina as shown in sections. In the above exception, the one killed for the high epithelium shown in figures 2 and 9, the method used was to study the length of consecutive cycles in all the animals and then to select the animal with the most regular recurring cycles and to kill it twenty-four hours before the calculated cornified stage.

In this connection the question arises, may not frequent douching of the vagina with warm saline solution affect the length of the cycle, and also may not the operation for hysterectomy affect the following cycles in the animal. By referring to table 2, the following facts will be made clear. The average length of the one hundred eleven cycles observed in twenty-four guinea-pigs was found to be 15.87 days. This corresponds to the length of the cycle as found by Stockard and Papanicolaou who found the average length of the cycle to be 15.73 days for sixty-seven cycles. There was no perceptible difference in the length of cycle in the normal and hysterectomized animals (table 2).

SUMMARY

1. The syringe method for taking vaginal samples is more satisfactory than any other method yet described for the guinea-pig.

2. The length of the oestrous cycle of the guinea-pig was found to be 15.87 days, the same as determined by Stockard and Papanicolaou (15.73).

3. The oestrous cycle has four well-defined periods or stages besides the interval.

4. Stage 1. The epithelium is at its greatest height at the beginning of this stage, ten to twelve cells. Cornification has been going on beneath the superficial layers. Vaginal smears contain large, vaculated, granular, odd-shaped, epithelial cells.

5. Stage 2. This is the stage of desquamation of the flattened, scale-like, non-nucleated cells—cornified cells. The inner cornified portion of the vaginal mucosa loosens and may be shed as a cast.

TABLE 2

Showing the length of consecutive cycles in twenty guinea-pigs from August, 1920, to January, 1921

ANIMAL	LENGTH OF CONSECUTIVE CYCLES IN DAYS	AVERAGE LENGTH OF CYCLE
199	16, 17, 16, 17, 16, 17	16.50
200[1]	15, 14, 15	14.66
206[1]	15, 16, 16, 16, 16, 17, 15, 16	15.75
208	17, 16, 15, 16, 16, 17, 17, 16, 16	16.22
211	16, 16, 16, 16, 17, 15, 16	16.00
213[1]	17, 16	16.50
220	15, 16, 16	15.66
232	15, 15, 15, 15, 15, 16, 17	15.42
233	16, 17, 16	16.33
246	14, 14, 16, 16, 16, 16, 16, 15	15.37
262[1]	15, 16, 15, 16, 16, 15, 15, 16, 17	15.66
272[1]	17, 16	16.50
277	16, 17	16.50
279	16, 16	16.00
280	15, 16, 15, 16, 16, 16, 15, 15, 15, 15	15.40
281[1]	16	16.00
282	17, 16, 17	16.66
285	15, 16, 16, 17, 16, 17, 17, 17, 17	16.44
296[1]	17, 15, 16, 16, 16, 16, 16, 16, 16, 16	16.00
298	16, 15, 15, 14, 15, 16, 16	15.28

Number of 14-day cycles	4
Number of 15-day cycles	28
Number of 16-day cycles	57
Number of 17-day cycles	22
Total number of cycles	111
Mode	16.0 days
General average	15.87 days

[1] Animals having both horns of the uterus excised.

6. Stage 3. Vaginal smears made during this stage contain round, nucleated, epithelial cells and some cornified cells which have remained from the preceding stage. Leucocytosis begins during this stage but leucocytes do not enter the lumen.

7. Stage 4. During this stage leucocytes appear in the lumen of the vagina for the first time since the beginning of the cycle. Smears contain epithelial cells and leucocytes.

8. Stage 5 or interval. Vaginal smears contain leucocytes and mucus, but very few or no epithelial cells. The epithelium has become reduced to its lowest condition, one to two cells. The epithelium is rapidly regenerated at the end of the interval immediately preceding the next cycle.

9. Stage 4 of Stockard and Papanicolaou, marked by the appearance of blood, is very doubtful.

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PLATE 1

EXPLANATION OF FIGURES

1 (Microphotograph.) Longitudinal section of the vagina at the middle of the interval, showing the finger-like processes of the submucosa projecting up into the epithelium. The submucosa contains many blood vessels. The epithelium is thinnest at this time, being only one or two cell layers. × 115.

2 (Microphotograph.) Longitudinal section of the vagina near the cervix at the end of the interval, about twenty-four hours before stage 1, showing the epithelium at its greatest height, about ten cells. The uppermost cells are greatly enlarged and vacuolated and are the superficial epithelial cells found in vaginal smears during stage 1. Leucocytes are seen in the lumen. × 115.

3 (Microphotograph.) Longitudinal section of the vagina during stage 1. Some of the large irregular epithelial cells characteristic of stage 1 are seen in the lumen along with a few leucocytes. The uppermost layers of cells have begun to slough off. About three or four cells below the surface of these superficial epithelial cells the cornifying layers are seen as flattened and more darkly stained cells. × 115.

4 (Microphotograph.) Longitudinal section of the vagina during stage 1, showing the well-formed cornified layers splitting from the stratum granulosum below and the process of cornification extending up into the superficial epithelial layers above. × 115.

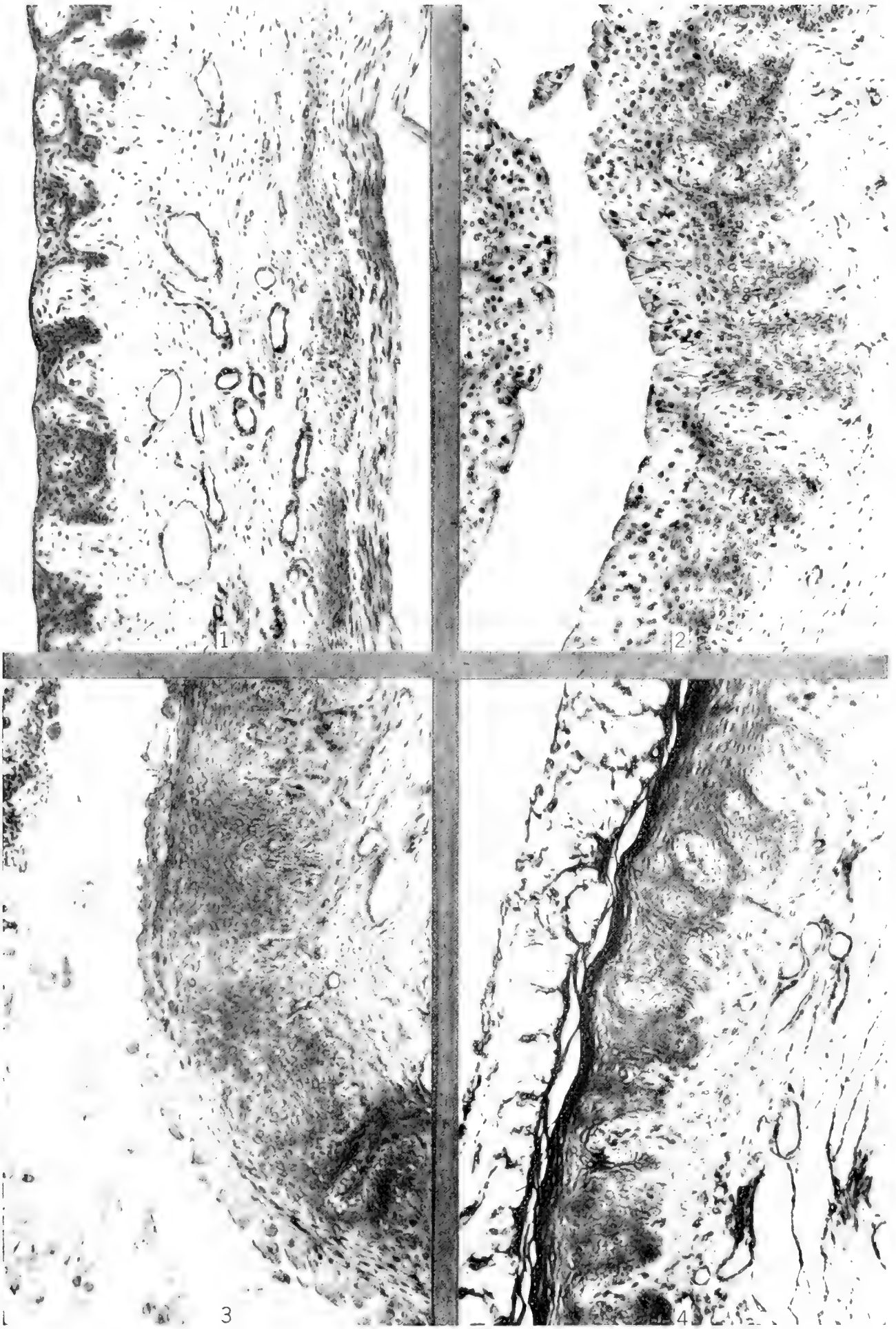
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PLATE 2

EXPLANATION OF FIGURES

5 (Microphotograph.) Transverse section of the vagina near the cervix, showing the cornified layers free in the lumen. The animal was killed at the beginning of stage 2. Notice the few superficial epithelial cells between the two cornified layers. × 115.

6 (Microphotograph.) Longitudinal section of the vagina near the cervix, showing the condition of the epithelium immediately after the entire cornified layer was shed as a cast. The epithelium is only four to seven cells high. Stage 3. × 115.

7 (Microphotograph.) Longitudinal section of the vagina near the cervix, showing the condition of the epithelium during stage 4 when epithelial cells and leucocytes are found in the lumen. The epithelium is about three to four cells high. × 115.

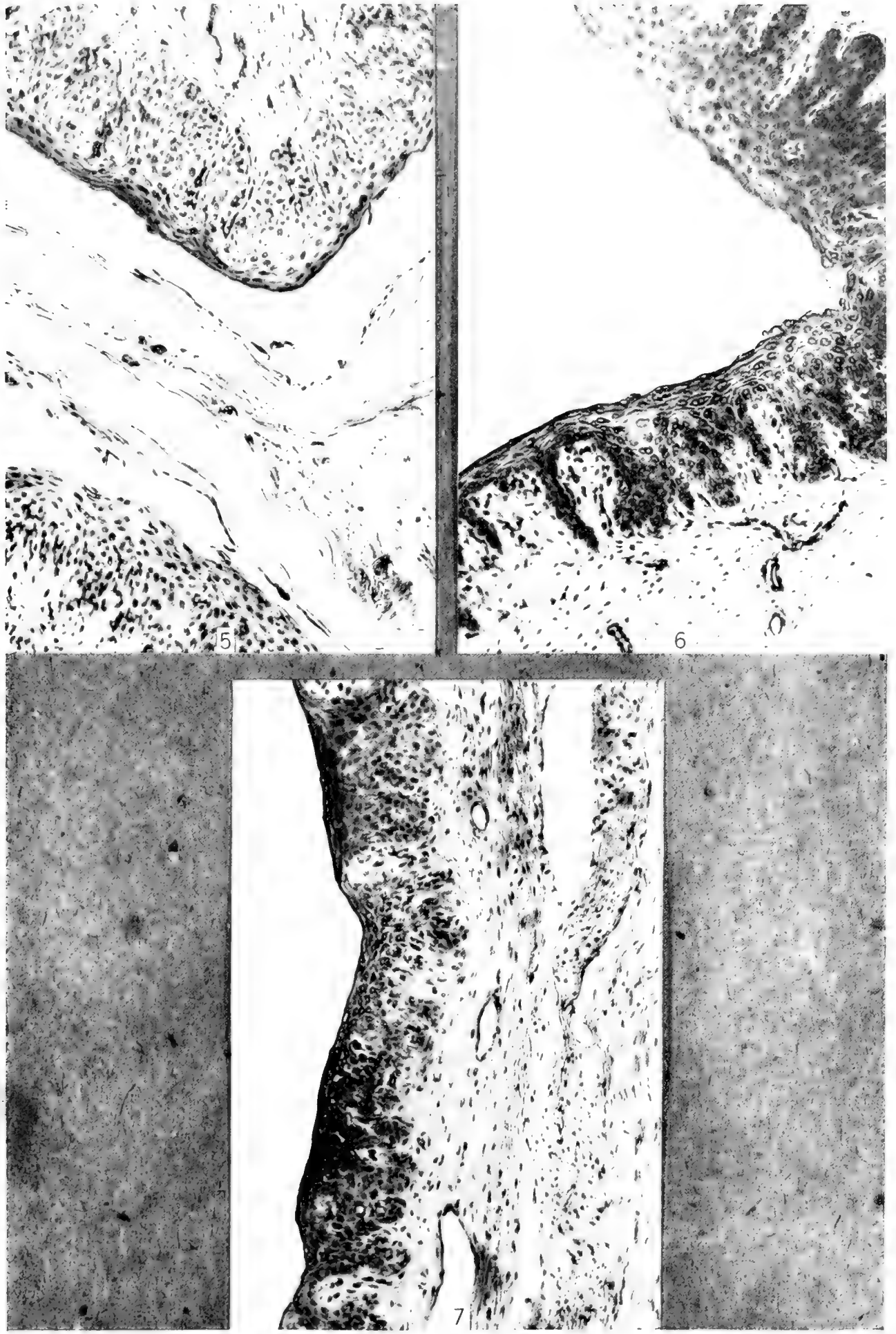
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PLATE 3

EXPLANATION OF FIGURES

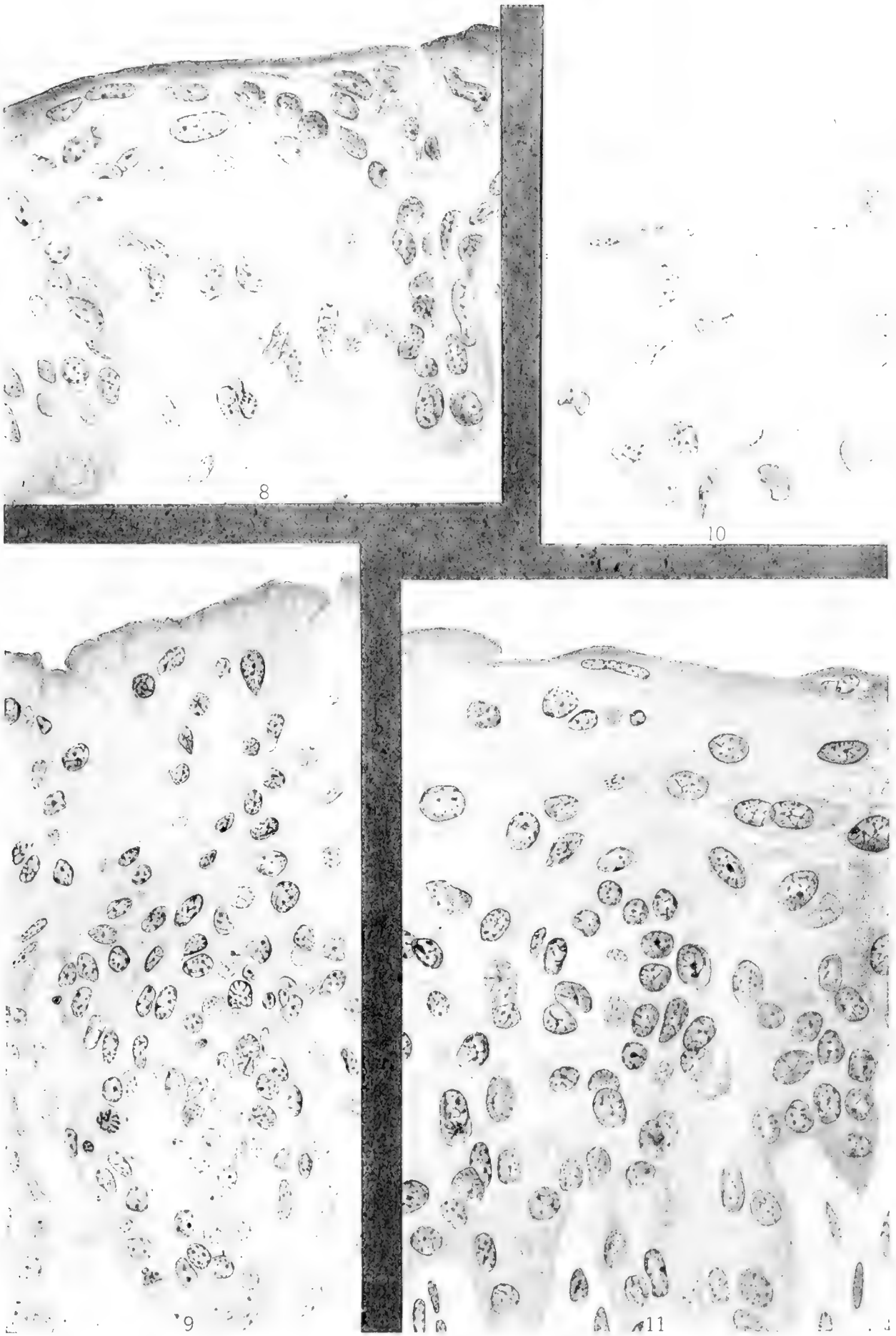
8 Part of mucosa in figure 1 enlarged. Middle of interval. × 624.

9 An enlarged portion of the epithelium in figure 2. End of interval. Note the enlarged superficial epithelial cells. × 572.

10 The upper part of the mucosa in figure 3 more highly magnified. Stage 1. The superficial epithelial cells are vacuolated. Under them is the beginning of the cornified layer. × 624.

11 More highly magnified portion of epithelium in figure 6. After the shedding of the cornified layer. Stage 3. × 624.

(Drawn by Miss Edna Fisher)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Resumen por el autor, Ivan E. Wallin.

Sobre la naturaleza de las mitocondrias.

III. La demonstración de las mitocondrias mediante los métodos bacteriológicos.

El autor ha preparado frotes de tejidos animales en porta-objetos. Las mitocondrias se tiñen con los colorantes bacteriológicos en estas preparaciones.

IV. Un estudio comparativo de la morfogénesis de las bacterias de los nódulos de las raíces y de los cloroplastos.

El Bacillus radicicola experimenta un desarrollo morfológico en los nódulos de las raíces, semejante a la morfogénesis de los cloroplastos. Una nueva forma de bacteria ha sido descubierta por el autor, quien discute la simbiosis y hace notar la analogía de la simbiosis en los líquenes con su concepto de las mitocondrias.

Translation by José F. Nonidez
Cornell Medical College, New York

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ON THE NATURE OF MITOCHONDRIA

III. THE DEMONSTRATION OF MITOCHONDRIA BY BACTERIOLOGICAL METHODS

IV. A COMPARATIVE STUDY OF THE MORPHOGENESIS OF ROOT-NODULE BACTERIA AND CHLOROPLASTS

IVAN E. WALLIN

Department of Anatomy and the Henry S. Denison Research Laboratories, University of Colorado, Boulder

TWO PLATES (NINE FIGURES)

III. THE DEMONSTRATION OF MITOCHONDRIA BY BACTERIOLOGICAL METHODS

In a former paper ('22) the author submitted evidence to show that the special mitochondrial technique in general use, including the vital janus-green method, is not specific for mitochondria, but will also stain bacteria. While, from a purely theoretical consideration, the properties of mitochondria are of such a nature that one could hardly expect them to respond to bacteriological methods, an analysis of results in this direction is not only interesting, but also instructive.

The author has not been able to find any references in the literature to mitochondrial staining with bacteriological methods. The results recorded below will serve to indicate not only the reactions of mitochondria to such methods, but will also indicate possibilities in mitochondrial manipulation.

MATERIALS AND METHODS

The tissues used in this study have consisted of various samples from young rabbits, kittens, and mature dogs. These tissues have included lymph nodes, liver, pancreas, kidney, salivary glands, suparenal, thymus, and other tissues. Immediately after

removal from the previously killed animal, smears of the organs and tissues were made on microscopical slides. The smears were then permitted to dry in the air without any other fixation.

A large number of bacterial staining methods were later applied to the smear preparations. Most of these staining methods have no selective action on bacteria, and consequently the entire smear was stained and mitochondria could not be distinguished with any important degree of clarity. In a few instances Gram's stain appeared to give a little clearer differentiation. One bacterial staining method was found, however, that gave a sharp differentiation—Pappenheim's pyronin-methyl green. This stain has had very extensive use in bacteriological technique. Todd ('18) recommends it especially for the demonstration of bacteria in cells on account of its selective action. In this study saturated aqueous solutions of pyronin and methyl green have been used in various proportions of mixture. In some instances it was found that a special proportion of the two stains was necessary to produce sharp differentiation. As Todd has recommended for the demonstration of bacteria, it is necessary to experiment with the proportions of the two stains to attain the best results.

RESULTS OF BACTERIOLOGICAL STAINING METHODS ON TISSUE SMEARS

Figure 6 is a camera-lucida drawing of a part of a young rabbit pancreas smear after pyronin-methyl-green staining. There was only a small area in the entire smear that appeared like the illustration. It is only in a very few cases out of a great number of attempts that anything resembling mitochondria were present in pancreas preparations. The bodies in the smear, represented in figure 6, appear like mitochondria, but not like the typical mitochondria of pancreas cells. Obviously, I am not in a position to definitely state that these bodies are mitochondria. It appears probable to the author that these bodies are the fragments of the original mitochondria of the pancreas cells. They may be artifacts. If they are, then, what evidence do we have of the reality of mitochondria in stained preparations?

Figure 7 is a camera-lucida drawing of a part of a rabbit-liver smear after pyronin-methyl-green staining. The small stained bodies in this preparation appear like the typical mitochondria of liver cells. I can find no alibi for their mitochondrial nature.

Figure 8 is a camera-lucida drawing of a portion of a rabbit-kidney smear after pyronin-methyl-green staining. It is difficult to find a place in the kidney smears where mitochondria-like bodies are distinct or present. The group of kidney cells illustrated was more or less intact. The minute bodies in the illustration were sharply differentiated and are not unlike the typical kidney mitochondria.

Figure 9 is a camera-lucida drawing of a part of a rabbit lymph-node smear after pyronin-methyl-green staining. The minute bodies represented in the illustration were sharply differentiated not only in the cells intact, but also in the cytoplasm of ruptured cells. They appear like the typical lymph-node mitochondria.

In a number of lymph-node-smear preparations it was observed that in some cells the entire cytoplasm, which was apparently intact, was homogenously stained with pyronin (the member of this stain combination which stains the mitochondria). Further, in practically all tissue-smear preparations, the ruptured cytoplasm is distinctly stained by the pyronin. This latter action of the stain is interesting on account of the supposition (original contention of Pappenheim?) that pyronin-methyl green is a selective stain for certain lymphocytes, staining their cytoplasm pink or red.

It appears reasonable to the author that in the cases where the cytoplasm stains with the pyronin the mitochondrial substance has diffused into the cytoplasm. If this interpretation is correct, it assumes that mitochondria are composed of a substance that is miscible with cytoplasm. It, further, assumes that under normal conditions mitochondria have a wall, pellicle, outer membrane, or some limiting structure. The diffuse character of the mitochondrial substance has also been observed in the ordinary fixed and stained mitochondrial preparations of lymph nodes.

These illustrations, it seems to me, are sufficient evidence that mitochondria may be demonstrated by bacteriological methods. In this work various problems in connection with the behavior of mitochondria have arisen. These problems have no fundamental bearing on the major problem of these studies, and consequently have not been pursued.

DISCUSSION

Aside from the demonstration of staining mitochondria by means of bacterial technique, certain facts observed in this study are of importance in connection with the main problem.

In a number of attempts to demonstrate the mitochondria in adult dog tissues with the above-described bacteriological technique, I have practically failed. Only in a very few instances have I been able to distinguish mitochondria-like structure in these preparations. These attempts were made with stains from a different source than the ones originally used on kitten and rabbit tissues. The original were Grübler's stains. However, with the kitten and rabbit tissues the results were not the same for all tissues. It was exceedingly difficult to demonstrate any mitochondria-like bodies in the pancreas. I have not been able to demonstrate any mitochondria in the salivary glands, thyreoid, and suprarenal. On the other hand, it appears quite easy to stain the mitochondria in the cells of lymph nodes. It must be borne in mind that in all these preparations the tissues were crushed when the smear was made.

Two prominent facts stand out in these results: first, mitochondria are not as delicate as it has been supposed; second, mitochondria vary in fragility.

Regarding the delicacy of mitochondria, Cowdry ('18) maintains that the slightest desiccation of a tissue is sufficient to alter them. The above-recorded results with bacteriological methods demonstrate the fact that mitochondria may retain their form and be stained after the degree of desiccation present from complete drying in the air.

These staining reactions, further, indicate the danger in formulating hypothesis and drawing too extensive conclusions on the basis of staining reaction alone.

IV. A COMPARATIVE STUDY OF THE MORPHOGENESIS OF ROOT-NODULE BACTERIA AND CHLOROPLASTS

In a growing conception of a bacterial nature of mitochondria, one naturally would seek an example of an undisputed symbiotic bacterium for study. The root-nodule bacteria, Bacillus radicicola offers an example of a relationship between two organisms that is, at least, of a partial symbiotic nature. The bacterial organism in this case, apparently, exists as a free-living organism in the soil. Under favorable circumstances, it enters the root hairs of Leguminosae and ultimately may be found in the cytoplasm of the cells of the root-nodules. The host plant responds to the infection by developing the root-nodules.

The degree of symbiosis in this example is, perhaps, not absolute.[1] The bacterium can live as an independent organism in the soil. Its symbiotic qualities are of the nature of partial adjustment. In other words, the organism has not changed to such a degree that it cannot exist independently of the host organism. This status of its existence is undoubtedly responsible for the ease with which the organisms from a root-nodule may be grown on artificial culture media.

Compared to absolute parasitism, the root-nodule bacteria are not as dependent on the host as is an absolute parasite. In the case of an absolute parasite, as well as an absolute symbiont,[2] the adjustment is so complete that the organism does not normally live outside the host. However, the Bacillus radicicola offers an illustration of a microscopic organism that may live and flourish within the cytoplasm of the cells of a higher organism.

[1] Various classifications of symbiosis may be found in the literature. The terms employed in these classifications have been based upon individual examples and conceptions and consequently can not be employed with clarity in an enlarged conception of symbiosis. Schneider's (1897) terms "mutualism," "individualism," and "contingent mutualism" are vague and misleading. The terms 'absolute' and 'incomplete' symbiosis have been introduced by the author on account of their simplicity, clearness, and direct significance.

[2] The author chooses to use the term "symbiont" employed by Schneider (1897) in preference to the term "symbiote" introduced by Portier (1918) for the reason that "symbiont" refers to either one of the two organisms entering into symbiosis, while "symbiote" refers particularly to mitochondria in a bacterial conception of their nature.

Further, it offers evidence of the fact that the chemical products of the bacterium in this case are essential to the life of the host plant. True, the host plant may procure these chemicals by absorption from the soil, and it may even do so in spite of the nodule organisms. This fact, per se, has no bearing on the importance of the phenomenon. The point at issue may be clarified by an illustration: The thyreoid gland produces a chemical substance that is essential to normal metabolism in higher animals. The gland may be removed from an animal, but the chemical substance must be supplied artificially if life is to be maintained normally. If it were possible to stimulate the production of this chemical substance from another organ of the animal, then the thyreoid would be unessential and in all probability would degenerate.

Lewitsky ('10), Guilliermond ('12), Regaud ('11), and other investigators have ascribed to mitochondria the property of plastid formation in plants. According to these investigators, the original mitochondria transform into plastids. Accompanying this transformation the mitochondria take on the various functions characteristic of plastids. Various kinds of plastids are to be found in plants. From the standpoint of evolution, the more important of these plastids are the chloroplasts, or the plastids containing chlorophyl.

According to Guilliermond, chloroplasts in higher plants are formed from mitochondria. He has, apparently, observed the various intermediate stages in the metamorphosis from a minute body, the mitochondrium, to a fully formed chloroplast. Such a morphogenesis is so strikingly similar to the morphogenesis of the Bacillus radicicola in the root-nodules of Leguminosae that the writer feels justified in presenting his observations on these forms.

MATERIALS AND METHODS

Root-nodules found on the roots of the common white clover, growing on the University campus, were fixed in the modified Flemming's fixative described in a former paper (Wallin, '22). After they were washed, dehydrated, cleared, and embedded

in paraffin, they were cut into sections 3μ in thickness, mounted, and stained with Bensley's aniline fuchsin methyl green.

The author has made no original observations on chloroplast formation. The work of Lewitsky, Regaud, Guilliermond, and other investigators in this field is accepted as fully demonstrated.

Bacillus radicicola

A longitudinal section of a root-nodule of the white clover, when examined with a low magnification of the microscope, reveals three distinct areas in the nodule. When a higher magnification is employed, the three distinct areas may be seen to be composed of cells containing three distinct types of bacteria-like organisms. Figures 1, 2, and 3 are camera-lucida drawings of portions of these three areas from a single nodule.

In figure 1 the typical Bacillus radicicola may be recognized. Most of the organisms are rod-shaped. A few of the characteristic Y-shaped individuals may be seen. It is this type, represented in figure 1, that, I believe, is generally considered the active individuals in nitrogen fixation. They may be designated the 'mature forms.'

In figure 3 the cells contain small bodies that are not unlike mitochondria in appearance. These forms, undoubtedly, are the young bacilli that have recently entered the nodule, or they represent a young form that will later metamorphose into the mature type. They may be designated the 'juvenile forms.'

Prazmowski (quoted by Marshall, '12) and other investigators have demonstrated that the source of Bacillus radicicola is from the soil. The free-living forms are quite small. They enter the roots through the root-hairs. The host plant responds with the production of the nodules into which the juvenile forms migrate and later metamorphose into the mature type. I have not been able to find a comprehensive description of the metamorphosis.

In figure 2 the organisms found in the cytoplasm of the nodule cells are spherical in shape. I have been unable to find any mention of these forms in bacteriological literature. The American text-books on bacteriology have no reference to them. They may be designated the 'senile forms.'

These senile forms occupy the older part of the nodule. That is, they are present almost exclusively in the cells of the nodule nearest to the attachment to the root. Apparently, there is nothing indicated concerning their activities. Their position in the nodule suggests the possibility that they are senile. It is possible that they no longer concern themselves with nitrogen fixation. However, one is justified in concluding that they are a stage in the morphogenesis of Bacillus radicicola.

The failure of investigators of this interesting and well-known organism to notice the existence of the senile forms is, assuredly, an excusable error. The object of my study, as well as my histological training, suggested the study of the root-nodule cells intact. The bacteriologist would crush the nodule and make a smear to study the contained bacteria. Again, the bacteriologist is interested in the cultural behavior of bacteria. The nodule must be crushed to transplant the contained organisms.

I have never observed the spherical or senile forms of the bacillus in smear preparations of the root-nodules.

Figure 4 is a camera-lucida drawing of a portion of the stem of a clover leaf after mitochondrial fixation and staining. Figure 5 is a camera-lucida drawing of a portion of an unfolded clover leaf after mitochondrial fixation and staining. These preparations are introduced to furnish a basis for comparison of apparent mitochondria in this plant with, particularly, the juvenile forms of bacillus radicicola as represented in figure 3. It is apparent from an examination of these illustrations that the mitochondria are similar in appearance to the juvenile bacteria. While it is obvious that form in itself does not necessarily indicate relationship, the question may properly be asked: What evidence is there to indicate that these structures are cytoplasmic organs and not representatives of the organisms that are known to be present in the root-nodules of this plant? Again, it may be asked with equal pertinence: What evidence may be submitted to indicate that these bodies, generally considered cytoplasmic organs, are not bacteria that have gained entrance to the plant through the root-hairs? The author has not been able to find any evidence that would satisfactorily answer these questions.

To recapitulate, the Bacillus radicicola is a minute organism that may be found as a free-living bacterium in the soil. Under favorable conditions, it may enter the root-hairs of Leguminosae and enter into a partial symbiotic existence. The host plant responds to the infection with a production of nodule cells. The invading organism comes to lodge in the cytoplasm of the root-nodule cells. In a mature root-nodule these forms, apparently, represent three stages in the morphogenesis of the bacillus after it acquires the symbiotic relationship.

DISCUSSION

The morphogenesis of Bacillus radicicola from the juvenile to the senile forms is strikingly suggestive of the morphogenesis of chloroplasts as described by Guilliermond and other investigators. Both forms develop from minute structures that have similar form and staining reactions. Both forms have a similar shape when they have reached their fullest development and are apparently alike in fragility. Both forms have a similar relationship to the cytoplasm of host cells. The morphogenesis of chloroplasts, as described by Guilliermond, appears to imitate more closely the morphogenesis of an organism than the development of a cytoplasmic organ.

The senile forms of Bacillus radicicola have a particular interest and will serve a useful existence in our conception of the nature of mitochondria. It is quite obvious that absence of these forms in bacteriological literature is due to the fragility of the organism. In ordinary bacteriological technique these forms are destroyed; both in smear preparations and planting on culture media they would be destroyed in the procedure. I have not used any other fixation for root-nodules than the one indicated above (a mitochondrial fixative). It is probable that the senile forms are destroyed by many fixatives just as mitochondria are, and this condition may account for some of the failures of previous investigators to observe them.

If my interpretation of the senile forms is correct, then, they furnish excellent proof of the contention I stated in the 'ad-

dendum' to a former article (Wallin, '22), namely, that fragility is a resultant of symbiosis. Further, their fragility refutes Regaud's contention that no bacteria are known which exhibit the fragility of mitochondria.

The apparent fragility of the senile forms of Bacillus radicicola has a bearing on the problem of growing mitochondria on artificial culture media. It is apparent from the absence of these forms in bacteriological literature that they have not been cultivated. This failure to grow them on culture media may be caused by various factors. It may be possible that they require a special culture medium. It is more likely, however, that their fragility does not permit a transfer. A third possibility presents itself, namely, that they may have lost the power of reproduction. This latter possibility appears the least likely explanation. It it quite certain that if success is to attend attempts to grow mitochondria in artificial culture media, the fragility of mitochondria, as well as the proper culture medium, must be taken into account.

There is no reason to suppose that all mitochondria and symbiotic bacteria develop and possess the same degree of fragility. To the contrary, I have presented evidence in the preceding section of this paper in support of the contention that mitochondria vary in fragility.

It is perhaps excusable at this time to digress into the realm of theory. The question naturally arises: Would a symbiotic bacterial interpretation of mitochondria be antagonistic to established factors in the theory of evolution?

Osborne ('17) has stated a clear and logical conception of evolution on the earth. His conception includes a chemical evolution preceding and leading up to the establishment of definite organisms. The first organisms in this conception were of a bacterial nature. These primordial bacteria or bacteria-like organisms were able to subsist on inorganic material. This conception of the first life is strengthened by the discoveries of Alfred Fischer ('00) and other later investigators. Fischer discovered a strain of bacteria that apparently represent the persistence of these primordial organisms, the Nitroso monas.

The presence of these primordial organisms in the beginning of evolution established the conditions essential for the development of those higher forms that require organic material for sustenance. It is not difficult to conceive the rôle that the primordial chlorophyl-bearing organisms played in the modus operandi of early evolution. Concerning themselves with the production of starch, the most important of the early food materials, they undoubtedly assumed the rôle of food factories preparing the way for the evolution of higher life.

The simplest organisms containing chlorophyl are bacteria or bacteria-like organisms. These organisms are generally called the blue-green algae. Campbell ('99), Thom ('12), Osborne ('17), and other investigators, however, maintain that they are more closely related to bacteria than to algae. Regardless of their relationship, whether with the bacteria or with the algae, the fact remains that chlorophyl is one of the oldest specialized materials of living matter.

It is in harmony with known biological behavior to conceive of chloroplasts not as organs developed in the cytoplasm of higher plants, but to look upon them as bacteria or bacteria-like organisms that accepted the leisure of a symbiotic partnership in the struggle for existence. In exchange for the nourishment supplied to it by the host plant, the invading symbiont furnishes an indispensable product to the host organism. It is obvious that if this interpretation coincides with reality, then the chloroplast is an example of *absolute symbiosis*.

The presence of chloroplasts in certain animal cells makes the foregoing hypothesis all the more alluring. It is true, that in some of these animal cells the chlorophyl-bearing bodies are unicellular algae that have been ingested by the host organism. This fact, however, serves to demonstrate the possibility that certain animal cells are so chemically constituted that algae may live within their cytoplasm with apparent impunity. The possibility also suggests itself that unicellular chlorophyl-bearing organisms may develop a symbiotic relationship with some animals and that the products of the chlorophyl-bearing organisms may be essential to the sustenance of the animal cell.

In a discussion of algae and their relationships, Campbell ('99) refers to some chlorophyl-bearing algae that have developed a symbiotic relationship to some higher plants. There is no reason to suppose that all unicellular green or blue-green algae belong to a single species. To the contrary, botanists recognize many species. The fact that some species of algae may be recognized in a symbiotic relationship with higher plants indicates the possibility that other species may have developed an absolute symbiosis with some higher plants. After an organism has developed absolute symbiosis, it is not conceivable that it is capable of an independent existence under natural conditions. It is, also, to be expected that an absolute symbiont would lose some characters and properties that the free-living progenitor possessed.

One of the best known and oldest examples of symbiosis is furnished by the lichens. This symbiosis was first described by Schwendenen in 1860, and has since been confirmed by the investigations of a host of authors. The lichens furnish not only an indisputable example of absolute symbiosis, but also varying degrees of symbiotic relationships.

In the lower lichens the algal symbiont is capable of independent existence. While it has not been demonstrated, Schneider ('97) and other investigators believe that the fungal symbiont in some lowly lichen may also be capable of independent existence. In the most highly developed lichens the symbiosis is complete or absolute; neither symbiont can live without the other. Here we have an example of symbiosis that, apparently, is identical with every detail in the relationship of chloroplast to host plant.

Between the most lowly and the most highly developed lichens, various degrees of symbiosis are represented. This gradation of symbiosis is accompanied by differences in reproduction. In the lowly lichens, where symbiosis is incomplete, the algal and fungal symbionts reproduce independently. In higher forms, but where the symbiosis is still incomplete, an accessory reproduction may take place by a type of budding in which both algal and fungal symbionts are represented. In the most highly developed lichens, where there is absolute symbiosis, both the algal and fungal

symbionts enter into the formation of the reproductive organ. This organ which is comparable to the germ cells of higher organisms was first named 'soredium' by Acharius (from Fünfstück, '07). Various types of soredia may be found in different lichens. In general, the fungal symbiont supplies a part (very much of the character of the spore organ in fungi) which arranges itself around the algal contribution (the gonidia). In other words, *the algal symbiont is carried from one generation to another in the reproductive element of the fungal symbiont.* This condition is absolute symbiosis in its highest development.

This latter reproductive phenomenon indicates the solution to a problem that will assuredly arise in connection with a symbiotic bacterial conception of mitochondria: If mitochondria are symbiotic bacteria, what is their source? Indeed, we would not seek some avenue of entrance in the adult organism nor in the embryonic body. The search would logically begin in the germ cell. The presence of mitochondria in the germ cell has been fully demonstrated. To seek the original source of a symbiotic-bacterial mitochondrium would lead us back to the dawn of evolution. Such an attempt, per se, is impossible. On the basis of biological behavior as exemplified in the absolute symbiotic lichens, there is but one logical answer to the above-stated question: *The symbiotic-bacterial-mitochondria are carried from one generation to another in the germ cells.*

Another question presents itself in connection with a symbiotic bacterial theory of mitochondria: Does such a theory harmonize with the known factors of cell activity? Altmann ('90) originally advanced the theory that the 'bioblasts' (mitochondria) are the ultimate units of life and the cytoplasm of the cells in which they are contained is lifeless. Altmann's ideas, apparently, were chiefly theoretical. Verworn ('99) and other investigators demonstrated the untenable position of such a hypothesis. Abundant evidence may be offered to prove that cytoplasm, by itself, possesses properties that are characteristic of living matter. If Altmann had limited his dissertation to a consideration of the 'bioblasts' alone, the burden of proof would have fallen on his adversaries. He misled his critics by introducing an erroneous conception of cytoplasm. This brought forth ridicule from his

critics instead of further scientific analysis. Following Altmann's dissertation, a great amount of theory has been advanced concerning mitochondria and cytoplasm. If we analyze many of these theories we find very little if any real evidence as a basis for their pronouncement.

The cell may be considered a unit chemical factory into which materials are almost continually passing and products are being eliminated. The simplest type of cell, if it may be considered a cell, is the bacterial organism. It is simple in the sense that it responds more readily to environmental conditions than do the highly differentiated cells of complex organisms. Gourney-Dixon ('20) has collected a mass of evidence in this direction. During recent years there has been a great deal of evidence submitted to show that the cells of one tissue are dependent upon the product of the cells of other tissues. Adami ('10) even goes so far as to state that every cell in a complex organism performs the function of internal secretion. Such a relationship of cells is also present among the lowly bacteria and is designated as symbiosis by bacteriologists. This principle of cell dependence is, thus, not characteristic of the cells of higher organisms, but is a primitive adjustment. Further, it is highly probable that this principle of cell dependence has been a fundamental factor in organic evolution.

The symbiotic bacterial conception of mitochondria, apparently, is not antagonistic to any known principles of cell activity. On the other hand, such a conception only extends the 'cell dependence principle' to include an intimate dependence of all highly organized cells on the activities of simple cells. The Bacillus radicicola in the root-nodules of Leguminosae is incontrovertible testimony that such a functional and morphological relationship does exist.

The dependence of higher life on bacteria in a different sense has been embodied into the biological conceptions of all students in biology. The principle of the 'nitrogen cycle' stands sponsor to these conceptions. It is within the domain of logic to extend this well-established principle of dependence of higher life on bacteria to include the more intimate dependence of life processes on bacteria.

GENERAL RÉSUMÉ

From the evidence that has been submitted in this study, including sections I and II in a former paper, the author believes that he has demonstrated the following facts:

There is no fundamental difference in the staining reactions of mitochondria and bacteria.

There is no fundamental difference in the reactions of bacteria and mitochondria to certain chemicals that have been used in attempting to determine the chemical nature of mitochondria.

Mitochondria may be demonstrated by bacteriological methods.

Bacteria may be demonstrated by mitochondrial methods.

Mitochondria vary in fragility.

Bacteria vary in fragility.

Mitochondrial substance is apparently miscible with the cytoplasm of the host cell.

The author, further, believes that the following apparent facts and biological principles support a bacterial nature of mitochondria:

Similarity of form.

Similarity in staining reaction.

Similarity in chemical ractions.

Similarity in physical properties (fragility).

Similarity in functional properties (synthesis).

In harmony with principle of biological behavior as exemplified in the 'struggle for existence' resulting in symbiosis.

In harmony with known factors of evolution.

In harmony with known factors of cell activity.

In harmony with 'principle of cell dependence.'

In harmony with the principle of analogy (Bacillus radicicola, lichens).

CONCLUSIONS

In a 'balance sheet' of favorable and unfavorable evidence in behalf of a bacterial nature of mitochondria, it appears to the author that the 'unfavorable' side of the 'sheet' lacks entries. After careful analysis, the author is convinced that no property of mitochondria has been recorded in the literature that is not equally applicable to bacteria.

From the evidence that has been recorded in these studies, together with the evidence that may be found in mitochondrial literature, the author can arrive at no other conclusion than, that *mitochondria are symbiotic bacteria in the cytoplasm of the cells of all higher organisms whose symbiotic existence had its inception at the dawn of phylogenetic evolution.* The conception embodied in this conclusion presupposes that the establishment of new symbiotic complexes is coexistent with the development of new species.

These studies have been pursued by the author independently. The conceptions and principles stated in this article have been formulated entirely on the basis of evidence that the author has gathered. Portier ('18) has arrived at a similar conception of the nature of mitochondria. His treatise 'Les Symbiotes' has not as yet been read by the author. A critical analysis of Portier's book will be undertaken by the author in the near future.[3]

[3] After this article had been received by the publishers I found a reference to the spherical forms of Bacillus radicicola. Löhnis (1921 Studies upon the life cycles of bacteria. Pt. 1. Memoirs of the National Academy of Sciences, Vol. XVI, 2nd memoir. Wash.) has described spherical forms of this organism. The spherical forms are of different sizes and, according to Löhnis, represent morphogenic stages in the life cycle of Bacillus radicicola.

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EXPLANATION OF PLATES

The figures were made with the aid of the camera lucida. The lenses used were: 2-mm. apoch. oil.-immer. obj., comp. ocular no. 8. The figures in plate 1 are reduced one-half from the original. The figures in plate 2 are reduced only very little from the original.

PLATE 1

EXPLANATION OF FIGURES

1 Mature forms of Bacillus radicicola in the cytoplasm of root-nodule cells. Bensley's stain.

2 Senile forms of Bacillus radicicola in the cytoplasm of root-nodule cells. Bensley's stain.

3 Juvenile forms of Bacillus radicicola in the cytoplasm of root-nodule cells. Bensley's stain.

4 Mitochondria in the leaf stem of the white clover. Bensley's stain.

5 Mitochondria in a young unfolded leaf of the white clover. Bensley's stain.

6 Rabbit pancreas smear after pyronin-methyl green staining.

7 Rabbit liver smear after pyronin-methyl-green staining.

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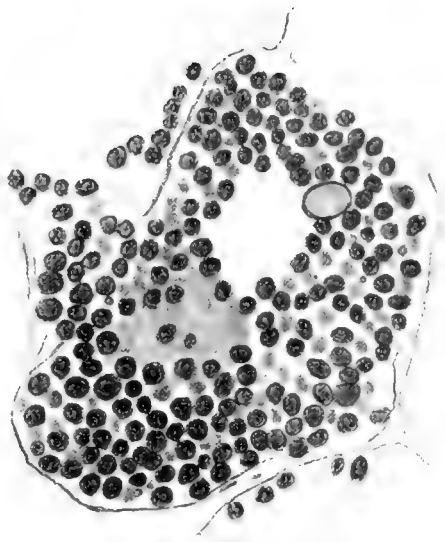

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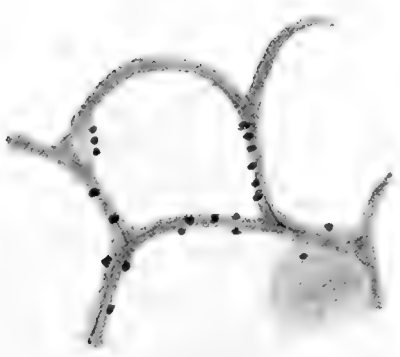

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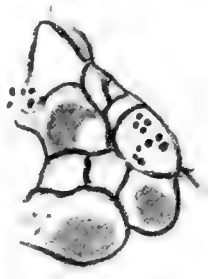

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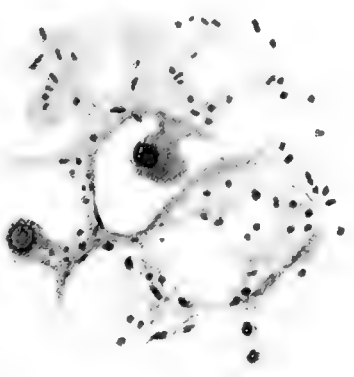

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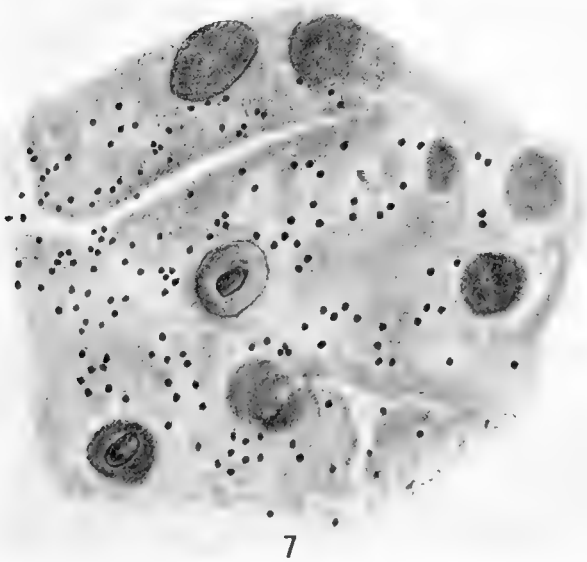

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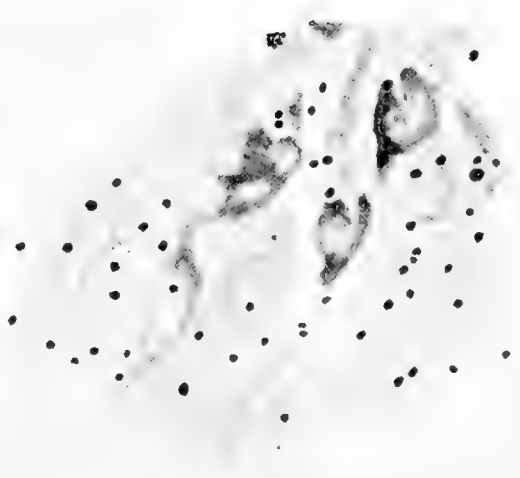

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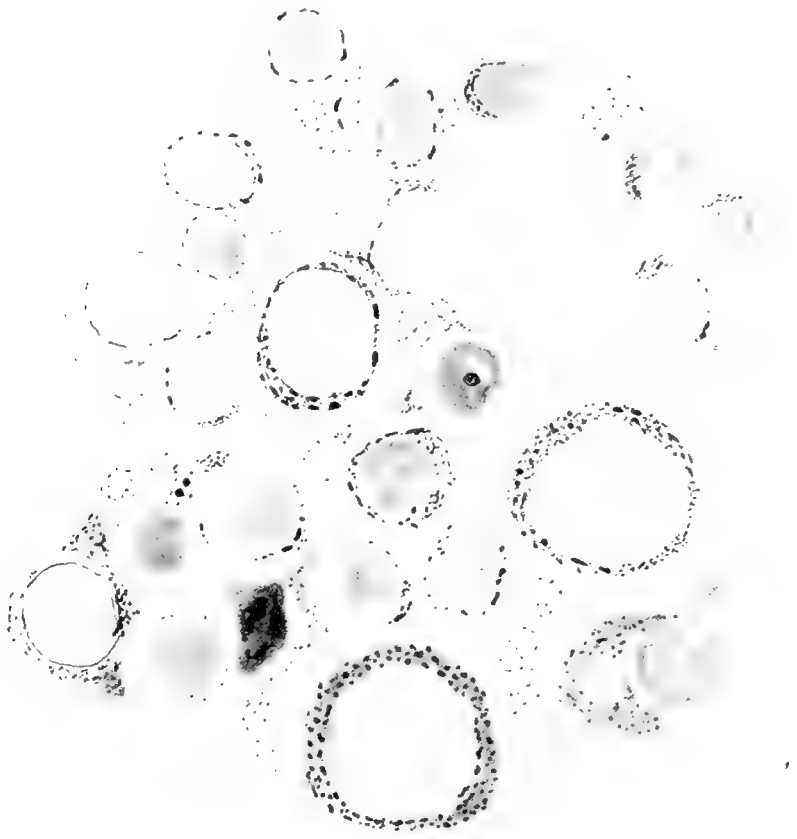
PLATE 2

EXPLANATION OF FIGURES

8 Rabbit kidney smear after pyronin-methyl-green staining.
9 Rabbit lymph-node smear after pyronin-methyl-green staining.



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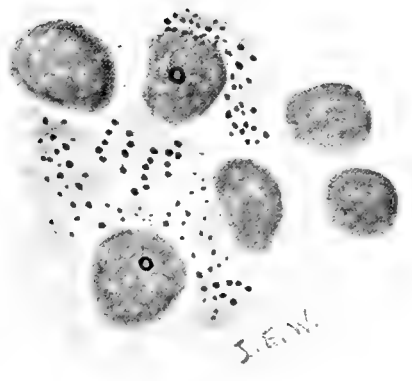

8

Resumen por el autor, A. W. Bellamy.

La susceptibilidad diferencial como base para la modificación y regulación del desarrollo de la rana.

II. Tipos de modificación observados en estados más tardíos del desarrollo.

I. El óvulo y el embrión de la rana exhiben una susceptibilidad diferencial a una gran varidad de condiciones que trastornan los procesos del desarrollo. 2. Según el tratamiento experimental y la condición fisiológica del organismo en el momento de la exposición, las modificaciones inducidas caen naturalmente dentro de clases de inhibición diferencial, aceleraciones diferenciales, aclimataciones diferenciales o vueltas a la normalidad diferenciales. 3. Estas modificaciones diferenciales son consecuencias obvias y necesarias de la susceptibilidad diferencial del organismo a los agentes empleados en la teratología experimental. 4. La respuesta de desarrollo del óvulo y embrión de la rana a una variedad de agentes externos es primariamente cuantitativa y no específica. 5. Los gradientes en susceptibilidad son paralelos a los ejes de simetría. Las regiones que se diferencian más temprano y crecen más rápidamente son las que experimentan mayor desplazamiento bajo las condiciones que trastornan los procesos del desarrollo. El grado y dirección del desplazamiento dependen en su mayor parte de la severidad del tratamiento y la condición fisiológica del organismo. 6. La existencia de gradientes de susceptibilidad, demostrada en una gran variedad de organismos, necesariamente implica la existencia de un gradiente o gradientes en los aspectos estructurales y funcionales del protoplasma en general. 7. El autor cree que el desarrollo teratológico puede explicarse más racionalmente en términos de este órden graduado de las relaciones dinámicas y actividades del protoplasma: los gradientes fisiológicos.

Translation by José F. Nonidez
Cornell Medical College, New York

AUTHOR'S ABSTRACT OF THIS PAPER ISSUED
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DIFFERENTIAL SUSCEPTIBILITY AS A BASIS FOR MODIFICATION AND CONTROL OF DEVELOPMENT IN THE FROG

II. TYPES OF MODIFICATION SEEN IN LATER DEVELOPMENTAL STAGES

A. W. BELLAMY

Hull Zoological Laboratory, The University of Chicago

SEVENTY FIGURES

INTRODUCTION

The fact that it is possible, within limits, to modify and control the developmental processes of a wide variety of organisms lends weight to the conception that the organism represents a complex or 'pattern' which, while it is characteristic of the species, entails a variety of ultimate ontogenetic possibilities. This or that aspect of development is revealed according as the developing organisms is exposed to this or that constellation of environmental factors. Viewed in this light, teratological development is simply a consequence of a deviation from the usual trend of development. Now, development may be made to depart from its usual trend in a variety of ways, and the degree or extent of the deviation from the usual course of development seems to be limited, for the most part, by two general factors. The primary limiting factor is of course the nature of the 'organismic pattern,' as Child ('20, p. 148) termed it. Since a wide variety of environmental factors has been used successfully to produce essentially similar abnormalities or deviations from the 'normal,' the second limiting factor would seem to be the intensity[1] of action of these agents rather than any specific action that might be ascribed to them.

[1] The word 'intensity' is used here in the sense of, e.g., concentration with reference to a chemical substance in solution.

Teratological developments certainly have a number of features in common regardless of how or in what organisms these are induced. One of these features is differential susceptibility, a differential that bears a very definite relation to the 'physiological axes' of the organism. This means simply that different regions of the physiological axes of organisms show different degrees of susceptibility to certain concentrations of action of a variety of physical and chemical agents. Observations by Child and his students on some two hundred species of organisms, including representatives from all the principal animal phyla and a considerable number of plants, agree in showing, at least in the simpler organisms and in the early stages of development of higher forms, that those regions of the egg or embryo that differentiate earliest and grow most rapidly, or simply the most active regions of the organism, are most susceptible to conditions that seriously affect developmental or functional processes. Several methods are available for demonstrating these differences in susceptibility,[2] the differential appearing, according to experimental conditions, as a differential disintegration gradient associated with death, as differential inhibition, as differential acceleration, or as differential acclimation or recovery in development.

The fact that such a wide variety of organisms, both plant and animal, characteristically exhibits a differential susceptibility to a considerable number of external agents and conditions (cyanides, anesthetics, acids, alkalies, certain electrolytes, several alkaloids, extremes of temperature, lack of oxygen, et al.) indicates at once that there is something fundamentally similar in the organismic pattern existing in these different protoplasms. It must mean that organismic pattern, as distinguished from the

[2] Differences in susceptibility along the axes of organisms are demonstrable as: 1) differences in survival time of one region of the organism as compared with other regions, under conditions that kill slowly without permitting acclimation to occur; 2) as differences in the degree of inhibition of growth and development, or in certain cases as differences in the degree of acceleration of these processes; 3) as differences in the rate or degree of acclimation to a certain range of less severe conditions; 4) as differences in the rate or degree of recovery after temporary exposures that inhibit development. See also Child, '20, p. 154.

specific protoplasmic constitution of each particular type or species, represents in at least certain fundamental respects a non-specific or quantitative factor in organization characteristic of at least all axiate organisms and transcending the differences in details which constitute specific or type differences in organisms. To this fundamental feature of organismic pattern, Child ('20, p. 152) has applied the descriptive term, 'physiological gradient.' Differential susceptibility or, in other words, a gradient in susceptibility, then, is merely one expression of underlying graded differences in the rate of fundamental activities and dynamic relations in protoplasm. A summary and discussion of the evidence demonstrating the existence of physiological gradients in organisms is given in the above citation so that further discussion of this principle is unnecessary. I wish merely here to indicate the usefulness of the conception in the interpretation of teratological development. Indeed, since the various types of experimentally produced terata in the frog have been produced time and again through the action of almost every agent and device known to experimental teratology, there would be little excuse for going over the ground again except for two facts which now appear very clearly. First, the degree and direction of teratological modification of development evidently depend primarily upon quantitative rather than specific factors in the action of the agents used; and, second, on this basis, the data attain considerable added significance and can be so satisfactorily accounted for in terms of the conception mentioned above.

In 1919 I presented data to show: 1) that the frog egg and embryo exhibit a differential susceptibility to such agents as KNC, CH_2O, $KMnO_4$, LiCl, HCl, NaOH, and C_2H_5OH. Those regions of the egg which differentiate earliest and in which growth is most rapid (apical and dorsal regions—in the early stages of development; in later stages any rapidly proliferating or physiologically active region) die soonest in concentrations of external agents that are lethal within a relatively short time; are most inhibited by somewhat lower concentrations, and acclimate or recover first in concentrations or treatments permitting acclima-

tion and recovery. 2) In general it was found that any type of abnormality could be produced by, *a*) regulating the concentration or intensity of action of a given agent; *b*) regulating the length of exposure of the egg to the agent—in connection with *a* and *c*; *c*) varying the stage in development at which the egg or embryo is exposed to the experimental conditions. 3) It was further emphasized that, fundamentally, the frog egg and embryo reacted to the agent or conditions used not specifically, but quantitatively. In other words, the end result is primarily a function of the intensity of action of the agent or condition used, the length of exposure, and the physiological condition of the eggs at the time of exposure to the experimental conditions.

The previous report dealt primarily with experimentally produced modifications seen in the earlier stages of development—up to and including gastrulation. This report is concerned for the most part with the modifications seen in the later stages of development—gastrulation to the time of hatching or a little later.

I am under obligation to Prof. C. M. Child, in whose laboratory the work was begun in 1916, for aid in the way of suggestion and criticism.

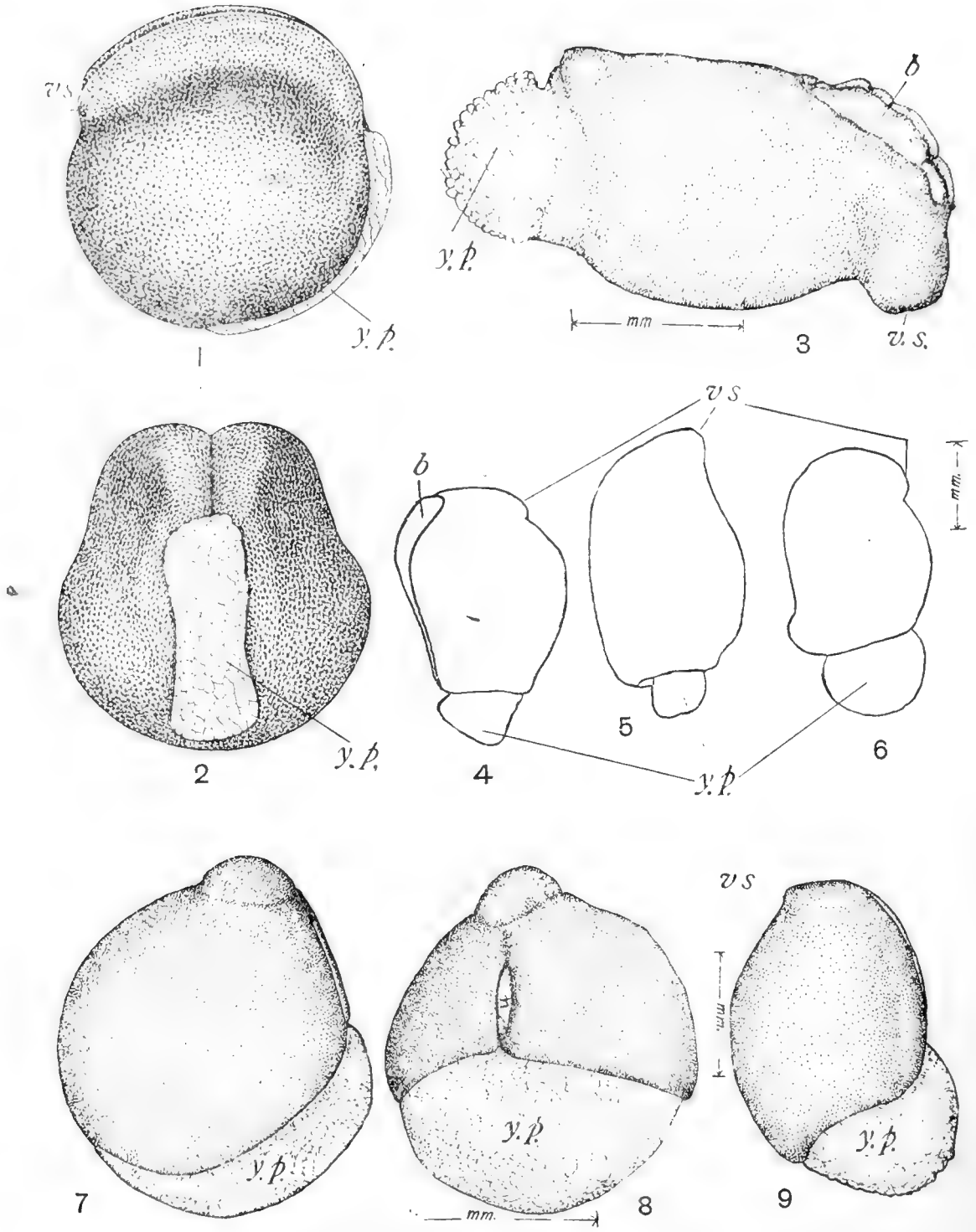
To facilitate the presentation of the experimental data and to give a brief sketch of the results of the experiments, the characteristic features of the different types of modified development are described below, together with a statement of the general experimental conditions under which the different types of modifications may be expected to appear.

TYPES OF MODIFIED DEVELOPMENT

In spite of the almost endless variety of abnormal forms that appear in experiments of this nature, the types of abnormalities obtained fall readily into four general categories, according as development has been inhibited or accelerated, or according as the egg or embryo acclimates to the experimental conditions, or, on removal to water, recovers. Furthermore, however different in appearance embryos of these different types may be, there is at least one characteristic feature common to all of them. The response of the egg or embryo is differential, whether the condi-

tions be inhibiting, accelerating, or such that the embryo acclimates to them, or, on removal to normal conditions, recovers. Hence one finds the distortion in development, regardless of the type, whether inhibition, acceleration, acclimation or recovery, appearing in relation to the principal axes or planes of symmetry of early developmental stages—anteroposterior, dorsoventral, and mediolateral—and to the secondary axes of special organs arising later in development. In early development before regional differentiation has begun, anterior, dorsal, and medial regions which are physiologically more active than their opposites, are more affected by disturbing conditions of the four sorts mentioned above than the physiologically less active posterior, ventral, and lateral regions. This is the typical condition. With the origin of special structures, however, optic vesicles, posterior growing region, limb buds, e.g., or in other words, with the origin of the secondary physiological gradients of special organs, the typical condition may be considerably modified, at least temporarily. Such special structures, which, it may be mentioned in passing, arise in an orderly relation to the primary axes, may 'flare up' with a high initial rate of metabolism that may persist through the period of most rapid proliferation and differentiation. The optic vesicles, e.g., for a time represent the most susceptible region of the anterior end of the embryo. Similarly, the tail bud, which terminates the posterior growing region, is at the time of its origin quite as or may be even more susceptible to disturbing conditions than more apical regions. These constantly changing relations in the developing embryo between the primary physiological gradients and the gradients of special organs arising later in development, together with the individual variation in the physiological condition of the embryo as a whole, make absolute uniformity among the modified embryos, whether inhibitions, accelerations, acclimations, or recoveries, highly improbable, to say the least. Great variation is to be expected in every case.

The four types of modifications obtained are characterized as: differential inhibitions, differential accelerations, differential acclimations, and differential recoveries. They are described in the order mentioned. It is hardly practicable or even desirable here

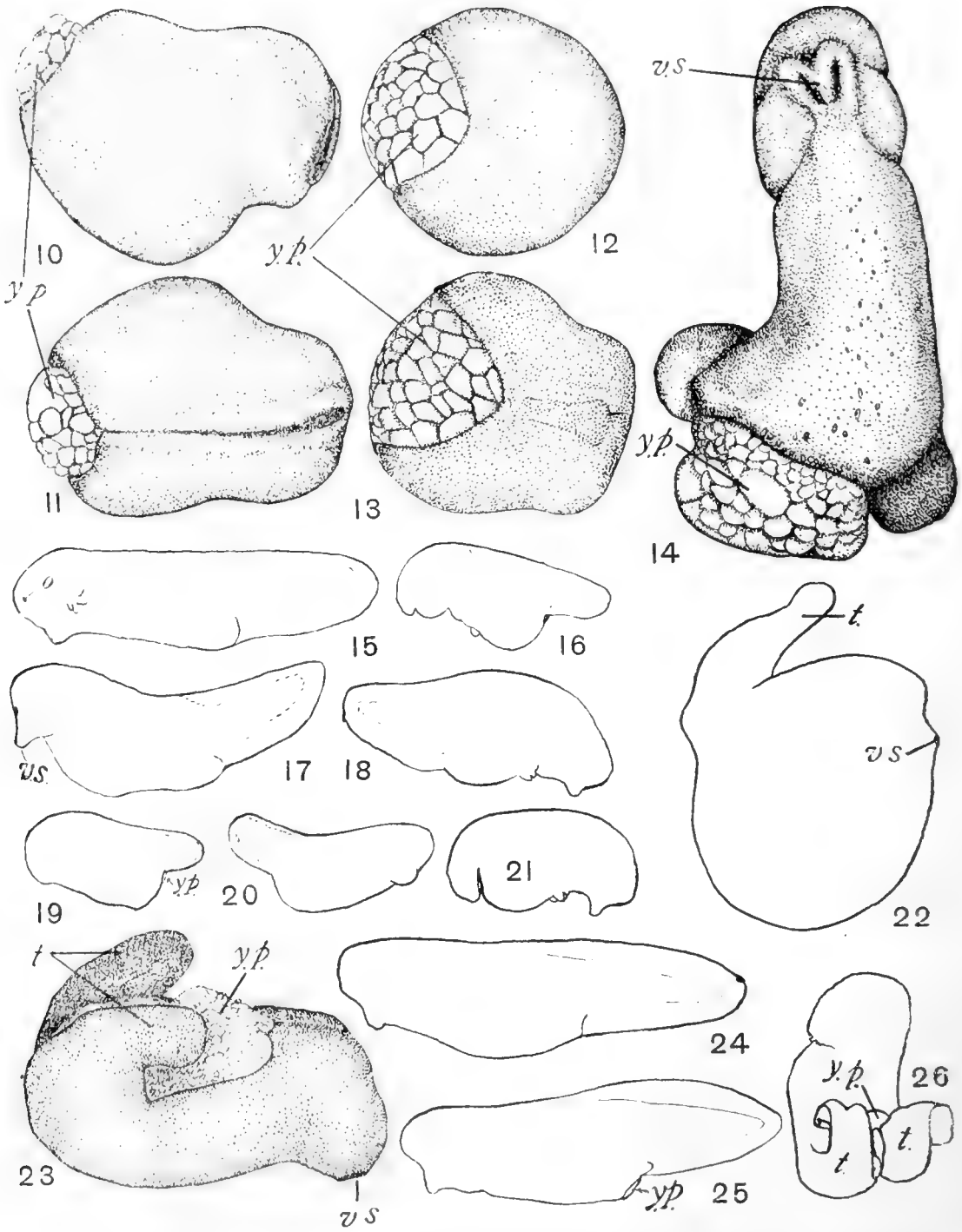


Figs. 1 to 9 Differential inhibition forms. 1, lateral; 2, dorsal view of one embryo. Eggs in late cleavage stages when placed in the solution. Treatment: 53 hours in m/10 LiCl. Note the elongated yolk plug which appears to result primarily from the relatively greater retardation of the dorsal lip region. Two outline views of the same embryo made sixteen and one-half hours later are shown in figures 52, 53. 3, same as above, but after 93 hours in m/10 LiCl. Ventral suckers, *v.s.*, are 'fused.' 4, 5, 6, three embryos from same lot as the one shown in 3. 7, lateral, 8, dorsal view of one embryo, and 9, lateral view of a second embryo. Eggs unsegmented when placed in the solution. Treatment: 76 hours in m/10.62 LiCl + 20 hours in water. *b*, open brain region; *y.p.*, yolk plug; *v.s.*, ventral sucker. The anterior end is toward the top of the page in all figures, save 3. Figures 1 to 6, from Exp. IV 59; 7 to 9, from Exp. IV 22; 4, 5, 6, drawn to slightly smaller scale.

to attempt any detailed description of the great variety of forms belonging to any of the four types. Hence, in the following four sections only the characteristic general features of the types will be pointed out. The conditions under which the different types occur may be seen from the legends to the figures and by reference to the protocols of experiments.

Differential inhibition. The modified types that fall into this class, while highly variable, have the following common characteristics: Physiologically more active regions suffer relatively greater retardation than less active regions. Among the more conspicuous external features of differential inhibition forms seen in postgastrula stages are these: Embryos with an elongated persistent yolk plug and retarded apical region (figs. 1, 2), or with a very marked retardation of apical dorsal parts and showing an equatorial blastopore with a persistent yolk plug (figs. 7, 8, 9). The later type grades back, in more completely inhibited forms, to a condition where apical differentiation does not proceed and the embryo dies as an equatorial gastrula. Where development proceeds further from such a condition, the embryo appears to elongate in the direction of the old egg axis, resulting in types that may appear to be radially symmetrical or may proceed to the formation of embryos similar to those shown in figures 3, 4, 5, 6. In somewhat later stages one characteristically finds microcephalic or even anencephalic embryos with such structures as optic vesicle, olfactory pits, and ventral suckers, when they appear at all, in various degrees of approximation to complete 'fusion' (figs. 10 to 32). In the absence of suitable conditions or opportunity for recovery, such embryos are usually dorsally concave, with the tail in some cases extending dorsalward at an angle of 90° or less with the body (fig. 22). The yolk plug may or may not be persistent, but the blastopore always, when it closes at all, does so relatively much later than in normal embryos. Spina bifida forms are of common occurrence (figs. 23, 26).

These various types are easily referable to the relatively greater retardation of physiologically more active regions, such as apical, medial, and dorsal regions, and later, the blastopore lips, primordia of optic vesicle, olfactory pits, ventral suckers, etc.



Figs. 10 to 26 Differential inhibition forms. 10, lateral and, 11, dorsal view of one embryo. Eggs in two-cell stage when placed in the solution. Treatment: 4 days in KNC + 2 days in water. 12, 13, dorsal views of two embryos. Eggs four to eight-cell stages when placed in the solution. Treatment: 48 hours in 0.0075 per cent formaldehyde. 14, ventral view of an embryo from the same lot as 12, 13, but after 72 hours in 0.0075 per cent formaldehyde. This figure illustrates a typical spina bifida condition with the two tails projecting dorsally from either side of the yolk plug. 15 to 21, eggs early gastrula stages when

Differential acceleration. Those types of deviation from the usual trend of development that are characterized as differential acceleration involve the same regions that are distorted in inhibited development, but the changes are opposite in direction. Regions of the egg, normally most active, become, under conditions that markedly accelerate development, even more precocious. As seen in later development, the conspicuous feature of accelerated development is a megacephalic condition of the embryo in which the gill plates and ventral suckers are unusually prominent. There is also a tendency toward a dorsal convexity.

It may be remarked in passing that differential acceleration forms do not deviate from the usual trend of development to anything like the extent seen in inhibited development—which, from the nature of the case, could hardly be otherwise.

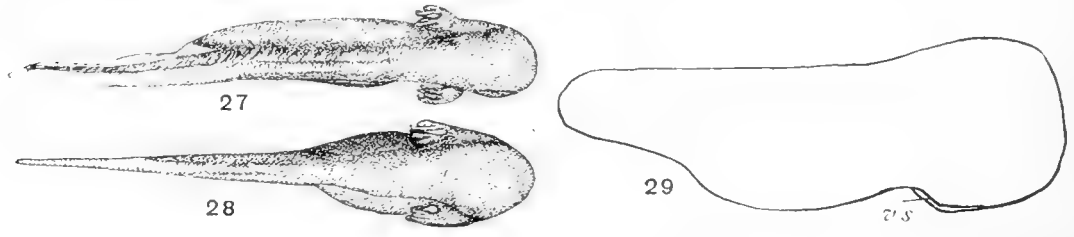
Differential acceleration differs from acclimation and recovery in that the embryos develop more rapidly than the controls—a circumstance that is especially noticeable during the early cleavage stages when the susceptibility is still relatively low. Not infrequently eggs may be accelerated during early cleavage stages and severely inhibited or killed in the same solution later in development. In mercuric chloride, e.g., eggs may be considerably accelerated during the first two or three cleavages in m/10,000 and killed during gastrula stages in m/2,000,000.

Acceleration is seen to a greater or lesser extent in low concentrations of all of the substances used, but no special efforts were made to determine the exact conditions for its appearance with

placed in the solution. Treatment: 12 hours in HCl + 81 hours in water. The 'fused' suckers can be seen as cone-like structures protruding from the anterior ventral region. 22, lateral view of an embryo, to show the marked dorsal concavity frequently seen in differential inhibition forms. Olfactory pits, ventral suckers, and probably optic vesicles 'fused.' Eggs late cleavage when placed in the solution. Treatment: 3 hours in m/5 LiCl + 8 days in water. 23, lateral view of spina bifida embryo. Eggs two-cell stage when placed in the solution. Treatment: 4 days in m/10,000 KNC + 8 days in water. 24 to 26, eggs in early gastrula stages when placed in the solution. Treatment: 3 hours in n/300 NaOH + 6 days in water. Ventral suckers and nasal pits 'fused.' 26, dorsal view of a spina bifida form with a persistent yolk plug. *t*, tail; *y.p.*, yolk plug; *v.s.*, ventral sucker. 10, 11, 23, from *Exp. KNC-C.* 9; 12 to 14, from Exp. IV 62; 15 to 21, from Exp. 103.3; 22, from Exp. IV 58; 24 to 26, from Exp. 125.2.

any substances save the ones mentioned in connection with the figures which illustrate this type of modification—figures 27 to 38.

Differential acclimation and differential recovery. Both acclimation, which occurs while the egg or embryo is still exposed to the experimental conditions, and recovery, which occurs after the egg or embryo is removed to water, involve the same regions of the developing organism that suffer distortion in inhibition or acceleration. Both are differential. These two conditions are to be distinguished from acceleration in that embryos produced under conditions that accelerate development are always in advance of the controls, while the differential acclimations and

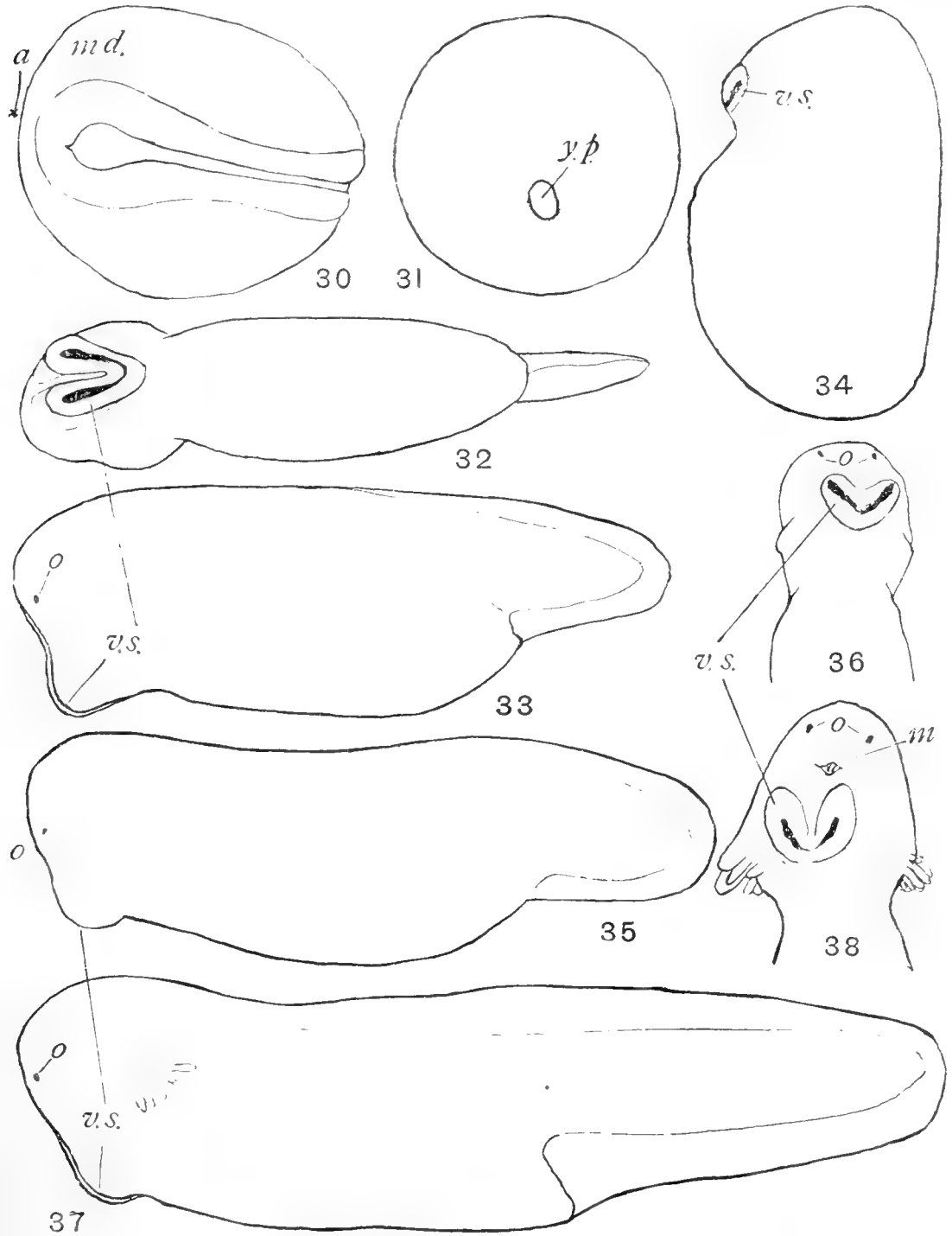


Figs. 27 to 29 Differential acceleration. 27, control; 28, embryo same age as control after 8 days' continuous exposure to n/500 NaOH (solution not changed after third day) from an early gastrula stage. Exp. NaOH 2. 29, embryo after 96 hours' exposure to m/100,000 KNC. Eggs unsegmented when placed in the solution. *Exp. KNC-C.4. v.s.*, ventral suckers.

recoveries are usually smaller than controls and differentiation has not proceeded as far. Indeed embryos that show acclimation or recovery or both may at the same time show a differential inhibition due to an earlier treatment. This is more especially true of the differential recovery forms.

As regards acclimation of the egg or embryo in the various solutions used, my data are not complete. Such data as have been obtained are brought out incidentally in connection with other experiments. A number of experiments, however, furnish conditions where recovery might be expected to take place (pp. 486, 491).

Among the more conspicuous features which mark recovery forms differentially are: relatively large tail buds on otherwise differentially inhibited embryos and a marked dorsal convexity is characteristic.



Figs. 30 to 38 Differential acceleration. n/5000 HCl. Eggs two to four-cell stages when placed in the solution. 30, embryo after 60 hours in HCl, dorsal view; 31, control at 60 hours. 32, ventral and, 33, lateral view of one embryo after 96 hours in HCl; 34, lateral view of control at 96 hours. 37, lateral and, 38, ventral view of head of one embryo after 6 days in HCl; 35, control, lateral view and, 36, ventral view of head, at 6 days. *a*, anterior; *m*, mouth; *md.*, medullary plate; *o*, olfactory pit; *v.s.*, ventral suckers; *y.p.*, yolk plug. 30 to 35, *Exp. HCl-C.1*; 35–38, *Exp. HCl-C.5*.

The conditions under which recovery will occur to a sufficient degree to reveal a marked differential are necessarily somewhat exacting. The strength of the solution (if a chemical substance is used), the length of exposure, and the physiological condition of the embryo must all be taken into consideration. If the conditions are too severe, recovery will not take place at all. Furthermore, the relation between different regions of the physiological axes of the developing organism, as regards rate or degree of activity, is continually changing. During early cleavage stages the apical pole represents the region of greatest activity as measured by rate of cell division, concentration of protoplasm, etc., and is most susceptible to a variety of external agents and conditions. Later the dorsal lip of the blastopore arises, probably by physiological isolation,[3] as a secondary region of high susceptibility.[4] In fact, this secondary or posterior growing region is at the time of its appearance and for some time afterward quite as or even more susceptible than the apical region. Apparently it represents during this time the most actively growing region of the embryo. Hence the physiological condition of the embryo at the time of exposure to the inhibiting conditions and at the time of removal from them, as well as the severity of the conditions, must play an important part in determining the rate and extent of development of those regions emphasized in recovery after removal from the inhibiting conditions.

It is usually a great satisfaction to the reader to have before him the complete records of experiments of this nature, but since the chief purpose of this report is to direct attention to the fundamental similarity of teratological forms however produced and to offer a physiological interpretation of the data, and especially since many of the essential facts of abnormal development in the frog have been recorded by a considerable number of

[3] See Bellamy, '19, pp. 349, 350.

[4] The at least temporary independence of the dorsal lip region is further indicated by certain of Spemann's ('18) recently published experiments. Taking two triton eggs at the beginning of gastrulation, he removed and exchanged between the two eggs the entire animal pole cap of cells, at the same time rotating them through 90°. In these cases he found the anterior end of the medullary plate developing in apposition to the posterior end of the lower half of the egg.

investigators, it has seemed unnecessary to burden the reader with a detailed account of all of the experiments done in this laboratory. That the results are consistent and repeatable is apparent at once from the literature of the subject. I have carried out some two hundred series of experiments, involving at least one repetition for each experiment. Those involving lithium chloride, potassium cyanide, mercuric chloride, hydrochloric acid, sodium hydrate, and formaldehyde were repeated anywhere from three to six or eight times. The experiments described are given usually in protocol form and involve mostly agents not hitherto used in experimental teratology (frog). Several experiments with LiCl are described in some detail because of the attention that, in the past, has been directed to it as having a 'specific' effect in the production of abnormal forms.

It should be mentioned that of the four types of modified development recognized here, three, viz., differential inhibition, acclimation, and recovery, are more or less the same thing that Stockard ('21) refers to in Fundulus as 'developmental arrests,' or, as we would put it, differential developmental arrests. Acclimations and recoveries are of course primarily differential inhibition forms that have, under a change in the experimental conditions undergone a remodification that is opposite in direction to the previous inhibition. The type characterized here as differential accleration is in addition to the one general type of modified development recognized by Stockard for Fundulus.

EXPERIMENTAL DATA

Experiments with lithium chloride. Eggs unsegmented at the beginning of the experiment. In this series the concentrations of LiCl used were: m/7 (experiment IV 20); m/8.5 (experiment IV 21), and m/10.62 (experiment IV 22).[5] These concentrations in percentages are approximately, 0.6, 0.5, and 0.4, respectively. Eggs from a single female, one and one-half hours after deposition, were divided into four lots, 5 cc. of egg mass to each. Three lots were placed, each into a liter of the above solutions, the fourth constituting the control. The time at which eggs were examined is measured from the time the eggs were placed in the solutions. The temperature where the experiments were carried on was maintained at 17 ± 1°C.

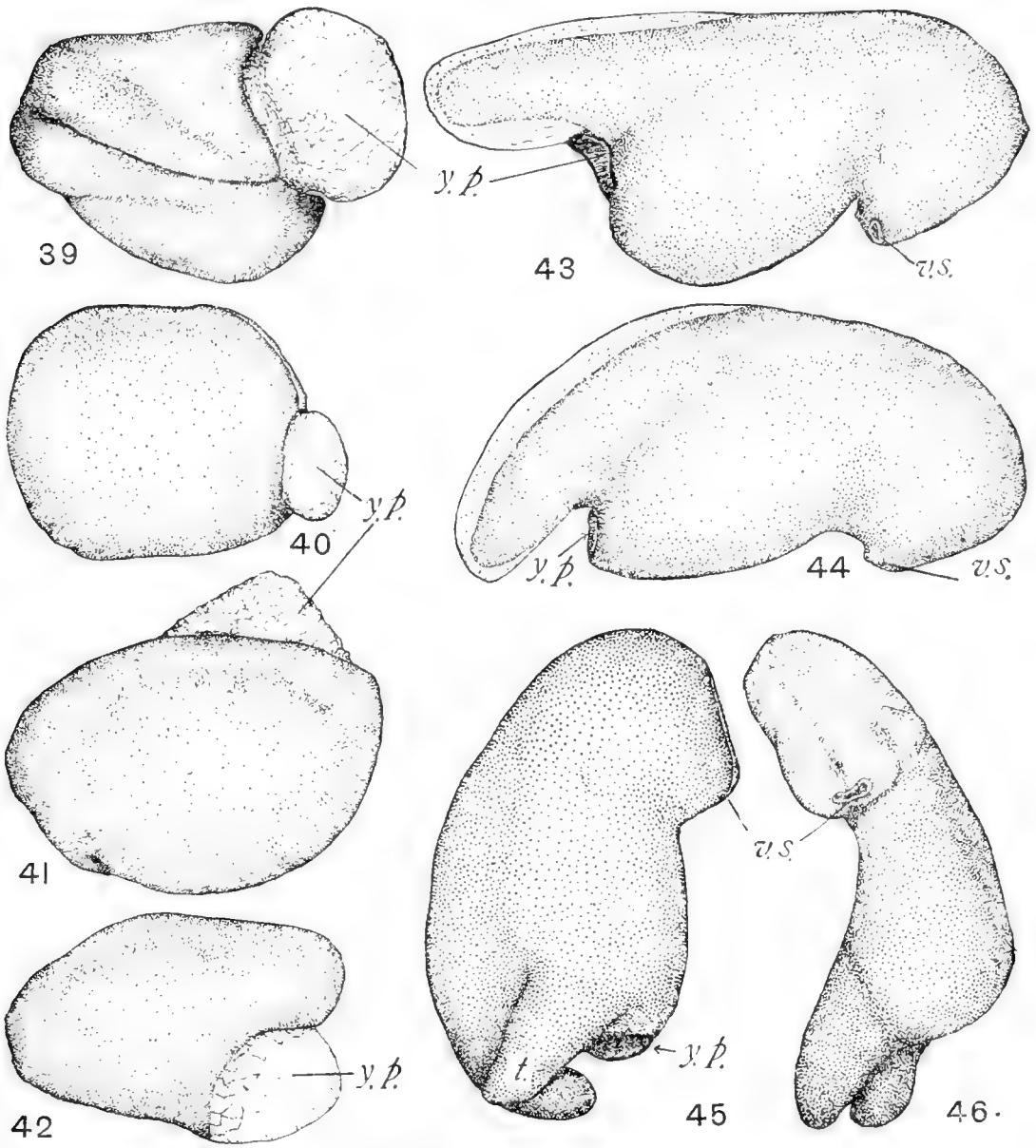
[5] Certain phases of these experiments were discussed in the previous report (Bellamy, '19, pp. 339-340). Through a typographical error, experiment IV 20, middle page 339, appears as IV 21.

In m/7 LiCl development comes to a standstill within about six hours after the eggs are exposed to the solution, although they are not killed outright, as shown by the fact that even after twenty-one hours' exposure development proceeds somewhat further when the eggs are returned to water. In m/8.5 LiCl eggs, when placed in the solution before segmentation begins, do not develop much beyond late cleavage stages unless returned to water, when they go ahead to form equatorial gastrulae.

Of the differentially inhibited forms produced in m/10.62 LiCl, those of especial interest here are the ones seen after forty eight hours' exposure to the solution plus twenty hours in water; forty-eight hours in LiCl plus forty-eight hours in water, and those exposed seventy-six hours to LiCl then removed to water for twenty hours or more. Figures 39 to 46; 7 to 9, will show more clearly than any amount of description the differential nature of the inhibition suffered under this treatment. Figures 39, 40 illustrate two embryos after forty-eight hours' exposure to m/10.62 LiCl plus the subsequent twenty-eight hours in water. Figure 39 represents a dorsal view showing a permanent yolk plug, shifted somewhat asymmetrically toward the right; small head region, with the neural groove still open anteriorly. Figure 40 is a lateral view of another embryo from the same lot as the above. Figures 41, 42 represent two embryos from the same lot as the above after seventy-three hours in water following a previous exposure of forty-eight hours to m/10.62 LiCl. The embryo 41 presents a permanent yolk plug projecting dorsally and showing practically no recovery of the posterior growing region. The head region is relatively small, but the ventral suckers are only slightly reduced. The embryo shown in 42 is markedly microcephalic, with no trace of the ventral suckers, the yolk plug projecting more ventrally and showing a considerable recovery of the posterior growing region (tail bud). While the majority of embryos from this experiment approximated the type shown in figure 42, there were a number similar to the others illustrated in figures 39 to 41, as well as practically all intermediate stages between the two types. These variations in the degree and type of recovery under these severe inhibiting conditions represent in all probability simply individual differences in susceptibility. Furthermore, the concentration used is of such severity that it lies near the border-line between conditions where recovery will not take place at all and conditions where it occurs readily. Consequently, considerable variation is to be expected (p. 484).

Figures 43 to 46 illustrate three embryos from experiment IV 22a. The treatment was: forty-eight hours in m/10.62 LiCl and subsequently eighty-three hours in water. Figures 45 and 46 are lateral and ventral views, respectively, of the same embryo. All of the embryos are microcephalic, ventral suckers partially or completely 'fused,' and with yolk plugs still protruding. A considerable number of forms with spina bifida appear. Differential recovery is evident in a number of them (fig. 44), as evidenced by the dorsal convexity and relatively large tail buds.

Figures 7 to 9 illustrate two embryos from the same lot of eggs, but exposed seventy-six hours to the solution and returned to water for twenty hours. Differential inhibition is more marked. Many of the embryos approach an anencephalic condition, and the bilateral sense organs of the head are much reduced or are not apparent at all. The



Figs. 39 to 46 Differential inhibition. 39, dorsal, 40, 41, 42, lateral views of four embryos after exposure to m/10.62 LiCl. Eggs unsegmented when placed in the solution. Treatment: 39, 40, 48 hours in LiCl + 28 hours in water; 41, 42, 48 hours in LiCl + 73 hours in water. Exp. IV 22. *y.p.*, yolk plug; *t*, tail; *v.s.*, ventral sucker. 43, 44, lateral views of two embryos and, 45, lateral, 46, ventral view of a spina bifida embryo from same experiment as those shown in figures 39 to 42, but after an exposure of 48 hours in LiCl + 83 hours in water. The embryos 43 to 46 show some differential recovery.

neural groove rarely closes and the posterior growing region is almost completely inhibited. After this treatment usually not more than half of the eggs continue development at all after return to water. In a number of embryos no external indications of bilaterality or dorsoventrality were apparent. The embryos approximated a radial type of symmetry. 'Differentiation' proceeded from an equatorial gastrula condition, merely as an apical proliferation of the pigmented cells and an elongation, as nearly as one could judge, in the direction of the original polar axis. One of these embryos was illustrated in the previous report (fig. 20).

Lithium chloride

Eggs in late cleavage stages at the beginning of the experiment (experiments IV 58 and IV 59). Experiment IV 58. Eggs in late cleavage stages (26 hours after deposition) were placed in m/5 LiCl. At intervals of $1\frac{1}{2}$, 3, 5, and 18 hours, eggs were removed to water.

Lot A. (a) 9 hours in LiCl. None of the eggs have begun gastrulation. Control: early gastrula.

(b) 18 hours in LiCl. Not much advance. About 5 per cent show slight wrinkling about midway between equatorial region and the apical pole. Many dead or dying. Control in late gastrula and early yolk plug stages.

Lot B. (a) $1\frac{1}{2}$ hours in LiCl plus $7\frac{1}{2}$ hours in water. Some of the eggs show slight indications of equatorial gastrulation (deep wrinkling in equatorial region of the egg).

(b) $1\frac{1}{2}$ hours in LiCl, plus 51 hours in water. Embryos beginning to elongate. Behind control.

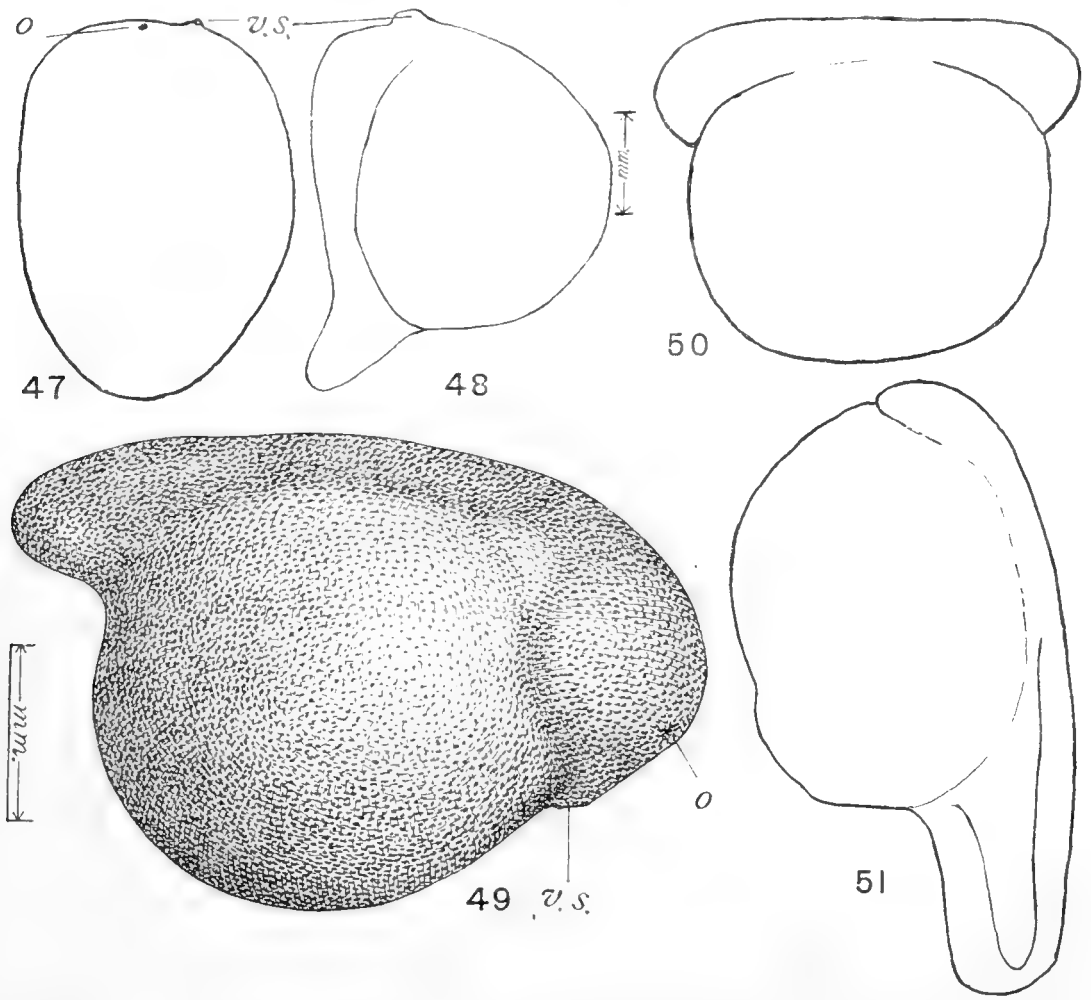
Lot C. (a) 3 hours in LiCl, plus 6 hours in water. The eggs are in approximately the same condition as those in B (a).

(b) 3 hours in LiCl, plus $49\frac{1}{2}$ hours in water. All of the eggs show marked differential inhibition. Neural fold stages with large and (probably) permanent yolk plugs.

(c) 3 hours in LiCl, plus 79 hours in water. Microcephalic to anencephalic embryos with 'fused' suckers and olfactory pits. There are probably some cyclopic embryos among these. Figure 47 illustrates one of the more extreme types obtained under these conditions. There is a single olfactory pit and the ventral suckers are reduced to a single, small, cone-like protuberance.

(d) 3 hours in LiCl, plus 103 hours in water. Development has continued and the inhibiting effects of the treatment are more marked. All of the embryos are now much distended ventrally by an accumulation of fluid in the coelome or possibly in the pericardial sac. If punctured ventrally with a needle some of the fluid comes away and the swelling recedes slightly for an hour or so when, shortly, the distention becomes as great as ever. Figures 48, 49 illustrate two of these embryos. In a number of cases (probably one-fourth), while the formation of an embryo has proceeded to some extent, one does not readily distinguish, macroscopically at least, between anterior and

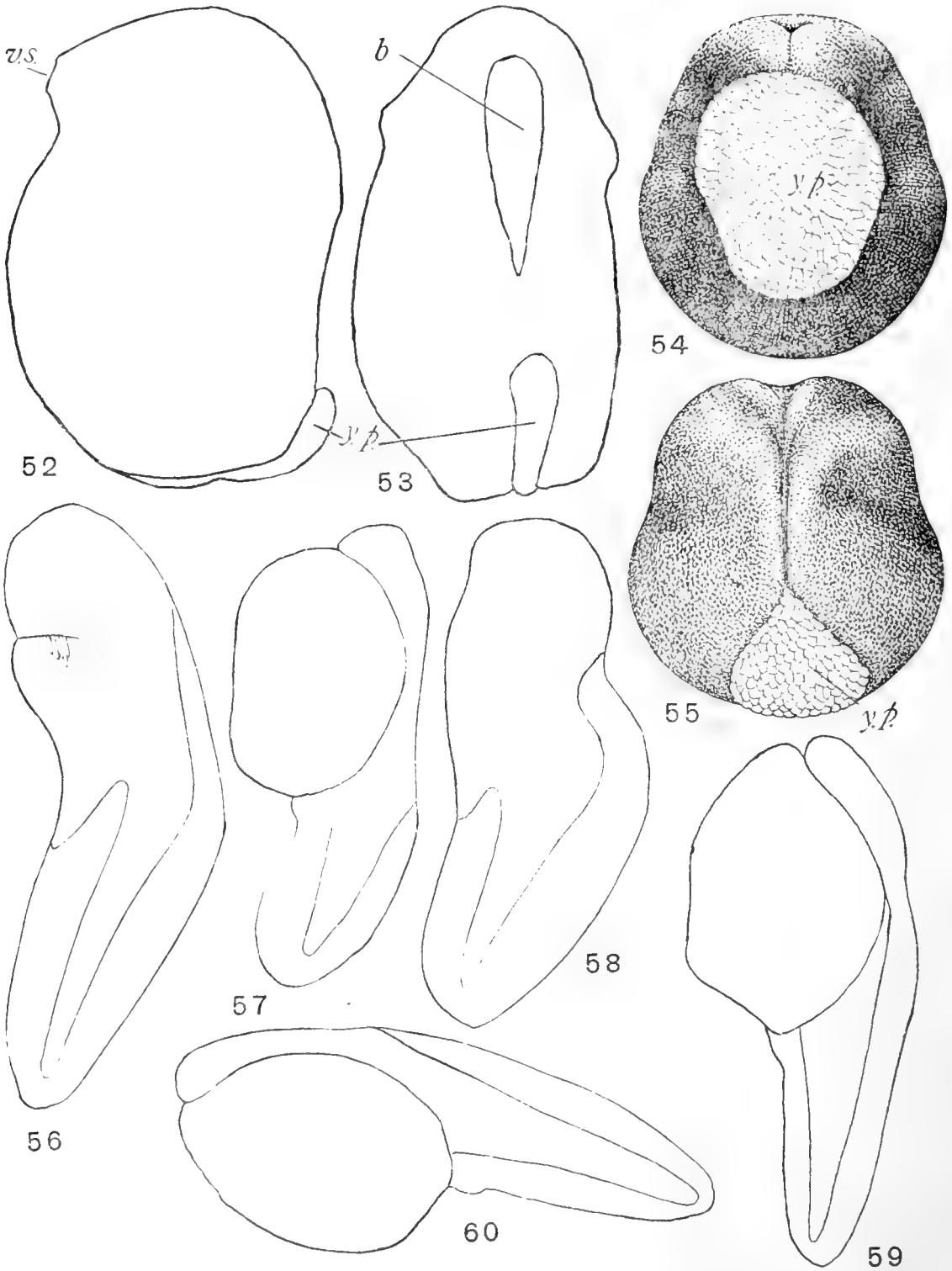
posterior ends. Except for this apparent lack of anteroposterior differentiation, they remind one just a little of a teleost egg. One of these peculiar embryos was isolated (fig. 50) because it had, at the time, the appearance of being bipolar, i.e., possessing either two heads or two tails, one at either end of the 'embryonic area.' Several days later, however, it developed into an almost anencephalic embryo with a more or less characteristic tail bud (fig. 51).



Figs. 47 to 51 Differential inhibition. m/5 LiCl. Eggs late cleavage stage when placed in the solution. Treatments: 47, 3 hours in LiCl + 79 hours in water. Lateral view (anterior end at top) of an extreme type showing little regional differentiation. 48, 49, 3 hours in LiCl + 103 hours in water. Shows some recovery. 50, 51, see text, page 489. Exp. IV 58. *o.p.*, olfactory pit; *v.s.*, ventral sucker.

Lot D. (a) 5 hours in LiCl, plus 4 hours in water. The eggs are in much the same condition as those in lot B (a).

(b) 5 hours in LiCl, plus 47 hours in water. Large yolk plug stages or equatorial gastrulae and in many cases showing a secondary invagination above the equatorial blastopore. An egg of this sort was figured (fig. 18) in the previous report.



Figs. 52 to 60 Differential inhibition. 52, 53, outline views of same embryo shown in figures 1, 2, 16 1/2 hours later. 54, 55, dorsal views of two embryos and, 56 to 60, lateral views of five embryos after treatment in m/5 LiCl. Eggs late cleavage when placed in the solution. Treatments: 54, 55, 53 hours in LiCl. 56 to 60, 53 1/2 hours in LiCl + 10 days in water. Exp. IV 59. *b*, open brain region; *v.s.*, ventral sucker; *y.p.*, yolk plug.

(c) 5 hours in LiCl, plus 77 hours in water. Generally no advance—dead or dying as equatorial gastrulae.

Lot E. (a) 18 hours in LiCl, plus 34 1/2 hours in water. No advance over Lot A (a).

Experiment IV 59. Eggs from the same batch and of the same age as those used in experiment IV 58 were placed in m/10 LiCl. Eggs removed to water at intervals of 1 1/2, 3, 5, 24, and 53 1/2 hours.

Lot A. (a) 18 hours in LiCl. Late, inhibited gastrulae to early yolk plug stages.

(b) 53 hours in LiCl. Early neural fold stages, most of which have a long narrow yolk plug—a consequence of the relatively greater inhibition of the dorsal lip region. Figure 1, dorsal and 2, lateral views of a single embryo, shows an oblong yolk plug extending over approximately 90°. Figures 52, 53 show in outline two views of the same embryo 16 1/2 hours later. The embryo is microcephalic, ventral suckers approximated but not entirely 'fused' and with the neural groove open through most of its length. Figures 54, 55 show two similar embryos from the same lot.

(c) 93 hours in LiCl. All degrees of approximation to complete 'fusion' of ventral suckers and olfactory pits. All of the embryos have permanent yolk plugs. Many are nearly or quite anencephalic. Others show the anterior part of the neural groove open and undergoing disintegration (figs. 3, 4, 5, 6).

(d) 107 hours n LiCl. No further advance. Almost all dead.

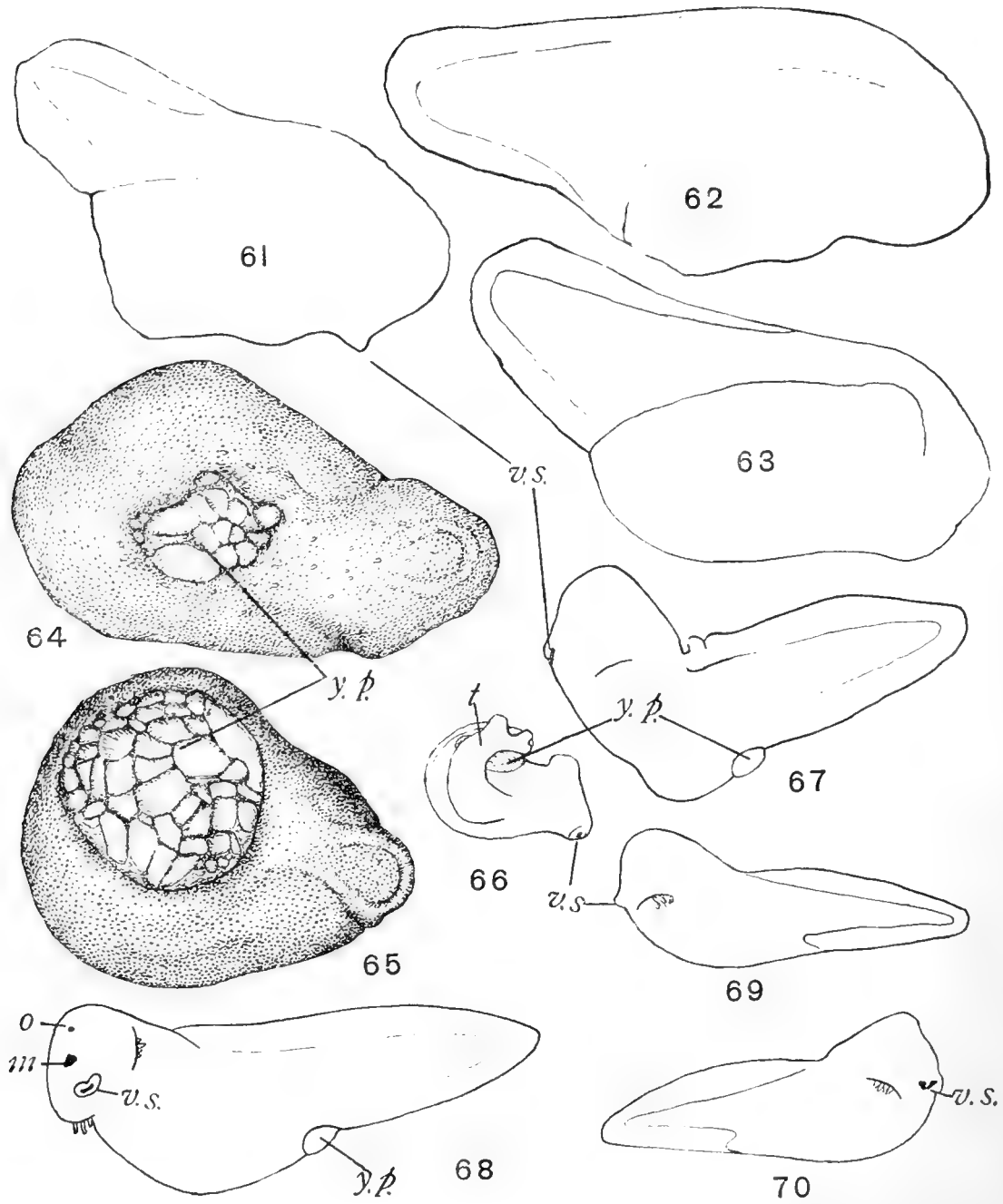
Lots B, C, D. Eggs exposed 1 1/2, 3 and 5 hours to this solution and then returned to water, hatched simultaneously or slightly behind the control, showing no marked effects of the treatment.

Lot E. (a) 24 hours in LiCl, plus 28 1/2 hours in water. Some differential inhibition as shown by the relatively greater retardation of the dorsal lip region. Neural folds just closing. A small yolk plug is still present.

(b) 24 hours in LiCl, plus 57 1/2 hours in water. Hatching slightly behind the control. Almost complete recovery. Tails seem relatively a little larger than in control.

Lot F. (a) 53 1/2 hours in LiCl, plus 28 hours in water. Marked differential inhibition. The embryos show all degrees of approximation to complete 'fusion' of ventral suckers and olfactory pits.

(b) 53 1/2 hours in LiCl, plus 114 hours in water. Microcephalic embryos with ventral suckers, olfactory pits, and optic vesicle approximated or 'fused.' All of the embryos show varying degrees of differential recovery as indicated by the dorsal convexity and disproportionately large tails. Figures 56 to 60 show five of the embryos after 10 days in water.



Figs. 61 to 70 Differential inhibition. 61 to 63, lateral views of three embryos after 1½ hours in m/5 LiCl and 5 days in water. Eggs in early gastrula stages when placed in the solution. The greater inhibition here appears to be a consequence of the higher susceptibility of the eggs at the time of gastrulation. Exp. 121.1 E. 64, 65, dorsal views of two embryos after 24 hours in m/5000 KNC and 5 days in water. Eggs in two-cell stage when placed in the solution. Microcephalic with shallow neural groove and persistent yolk plug. Exp. KNC-C.8a. 66, a spina bifida and 67, 68, two other embryos after 4 days in m/10,000 KNC and 8 days in water. Eggs in two-cell stage when placed in the solution. Same experiment as embryo shown in figure 23. 68 is shown with the anterior end twisted somewhat to one side, to show the almost completely united ventral suckers. 69, 70, two embryos after 4 days in KCN + 8 days in water. Less than 1 per cent of the embryos in this experiment showed this extreme inhibition. Exp. KNC-C.10. *m*, mouth; *o*, olfactory pit; *t*, tail; *v.s.*, ventral sucker; *y.p.*, yolk plug.

Potassium cyanide

Eggs in a two-cell stage at the beginning of the experiment (experiment *KNC-C.9*[6]). The eggs were divided into two lots and treated as follows in m/10,000 KNC:

Lot A. (a) 48 hours in KNC. Equatorial gastrulation beginning in a few eggs. Control eggs show advanced yolk plug stages.

(b) 4 days in KNC. More than 90 per cent of the eggs show a circular blastopore following the equatorial region (equatorial gastrulae). The controls are now elongating embryos with heads formed.

(c) 5 days in KNC. Little if any change. The eggs continued in this solution without further advance in development until the eighth day, when they began dying in considerable numbers.

Lot B. (a) 4 days in KNC, plus 2 days in water. Mostly neural fold stages with relatively small head region and showing a persistent yolk plug. Figures 10, 11 illustrate one of the less extreme embryos of this type. Most of the embryos show a much larger yolk plug and situated more dorsally and also showing greater inhibition of the head region. Comparison with figure 55 reveals a striking resemblance in the type of abnormality produced with the aid of two widely different chemical agents—potassium cyanide and lithium chloride.

(b) 4 days in KNC, plus 5 days in water. Mostly microcephalic forms showing spina bifida and with persistent yolk plugs. All of the embryos show various degrees of approximation to complete 'fusion' of ventral suckers and olfactory pits. Figure 23 illustrates one of these embryos. It will be noticed that the yolk plug is situated dorsally and that the two tails curve upward with the distal ends directed toward the anterior end.

(c) 2 days in KNC, plus 8 days in water. Spina bifida of various sorts. Figure 66 illustrates one of these, showing in addition a microcephalic condition and a single ventral sucker. This is representative of the lot at this age and resembles somewhat the embryo illustrated in figure 23. Figures 67, 68 are later stages of the few less extremely modified embryos drawn to the same scale. Figure 68 is a ventrolateral view of one showing the ventral suckers almost completely 'fused' and a single olfactory pit. Probably 5 per cent of the embryos in this lot were similar to 67, 68, the remaining embryos approximating the type shown in figure 66.

This experiment is typical of those furnishing conditions mentioned at the beginning of the description of the experiment. The range of concentrations of KNC that will produce considerable deviations from the normal and still permit development to proceed beyond gastrula stages is rather limited. In m/1000 development stops in mid-cleavage stages with the pigmented and unpigmented cells approximating the same size. Likewise in m/5000 development stops during cleavage

[6] Citations of experiments in italics refer to those done by Professor Child in 1916.

stages—usually late cleavage stages. The eggs have much the same appearance as those treated in m/1000 KNC. The eggs will continue development, however, if they are removed to water or to lower concentrations of cyanide. For instance, in experiment *KNC-C.6*, where eggs undergoing the first cleavage were exposed to the following concentrations of KNC: m/5000 24 hours, m/10,000 24 hours, m/20,000 24 hours, m/50,000 12 hours, reached late yolk plug stages. The yolk plugs were large and protruded considerably. In many cases the yolk plugs were elongated in the median plane, i.e., elliptical in outline. Even with the preliminary treatment of 24 hours in m/1000 KNC, then 24 hours in m/20,000, 12 hours in m/50,000, and 24 hours in m/100,000 (experiment KNC H6—eggs from same lot as above), equatorial gastrulae are formed somewhat similar to those produced in m/500,000 $HgCl_2$—24 hours in the solution and 24 hours in water,[7] and to those produced in LiCl—48 hours' exposure to m/10. A few eggs from this lot were similar to the 'secondary invagination' forms obtained frequently in m/10 LiCl after 48 hours' exposure.[8] In m/20,000 KNC development is retarded and the inhibition is differential, but very few extreme modifications are seen.

Potassium cyanide

Experiment *KNC-C.* 10. Eggs from the same female and in two-cell stage were divided into two lots and treated as follows in m/20,000 KNC:

Lot A. (a) 48 hours in KNC. Mostly advanced yolk plug stages, much like control. There is, however, a slight retardation of the dorsal lip region.

(b) 4 days in KNC. The embryos range all the way from late blastula and early gastrula stages to closed blastopore and early neural fold stages—mostly the latter. A few embryos of the spina bifida type. Control: elongated embryos with distinct heads.

(c) 6 days in KNC. Blastopore has closed in most of the embryos. Body still round or only beginning to elongate. A few spina bifida present.

(d) 8 days in KNC. Nearly normal in form, though developing slower than control. Many dying after hatching. Ten to 20 per cent of the embryos spina bifida.

(e) 9 days in KNC. Little or no further advance. Sixty to 70 per cent dead or dying. Heads range from about normal to distinctly small or retarded.

Lot B. (a) 4 days in KNC, plus 2 days in water. Nearly all of the embryos elongating; head becoming distinct. All less advanced than control. Many of the embryos show differential recovery distinctly as shown by the relatively advanced condition of the head and in some case of the tail buds.

[7] See figure 14, previous report.

[8] See previous report, figure 18.

(b) 4 days in KNC, plus 4 days in water. Seventy per cent hatched, others dead in membranes without elongating. The hatched animals seem to have rather large heads.

(c) 4 days in KNC, plus 7 days in water. Actively swimming embryos assuming the regular tadpole form. Smaller than control, but with relatively larger heads—showing a differential recovery.

(d) 4 days in KNC, plus 8 days in water. Nearly all normal tadpoles except that the heads are relatively larger. A few spina bifida and microcephalic forms remain alive. Figures 69, 70 illustrate two of the very few—about 1 per cent—extremely modified embryos. They are microcephalic and bent dorsally. In one, shown in 80, the ventral suckers are completely 'fused,' but only partly fused in the other, 81.

If the eggs are placed in m/10,000 KNC in late gastrula stages, development proceeds slowly for several days and ceases with the embryos dying in early neural fold stages.

In m/50,000 and m/100,000 KNC there is usually some acceleration of development and many of the embryos appear somewhat megacephalic. The acceleration is not as great nor is the differential nature of the acceleration as marked as in certain concentrations of HCl (p. 497). Figure 29 shows a camera-lucida outline of one of the more extreme megacephalic types.

Experiments with formaldehyde

Experiment IV 62. Eggs in four to eight-cell stages were treated as follows in 0.0075 per cent formaldehyde.

12 hours. Early cleavage stages with pigmented and unpigmented cells approximating the same size.

24 hours. Early inhibited gastrulae.

48 hours. A few early neural fold stages and these with very shallow neural grooves and with persistent yolk plugs. Figures 12, 13 illustrate two of these.

72 hours. 95 per cent spina bifida. Ventral suckers approximated (fig. 14). There appears to be some acclimation especially in the head region.

133 hours. Not much change. Many dying.

A number of concentrations of formaldehyde were tried ranging from 0.01 to 0.001 per cent, but the concentration used in experiment IV 62 was the only one that gave any considerable number of markedly modified embryos in the later stages of development. The percentage strength was calculated on the basis of 40 per cent commercial formalin. For example, the 1 per cent (approximate) stock solution from which the other strengths were made up was made by adding 1 cc. of the commercial product to 39 cc. of water.

Experiments with HCl

In all of the experiments involving HCl and NaOH the solutions were made up from freshly prepared (in the case of HCL) n/10. These stock solutions were diluted with an amount of well-water that would give, theoretically, the strength of acid or alkali stated for each experiment. And while, in the case of HCl the concentration of acid was certainly lower than that stated due to the presence of carbonates and salts in the well-water, the solutions were always prepared in the same way, so that, regardless of the actual concentration or of the hydrogen ion-concentration, the figures representing the strength of acid are sufficient for purposes of comparison. It will be understood, then, that the concentration given, e.g., n/5000, is relative, indicating merely the amount of n/10 stock solution added to a given quantity of well-water necessary to furnish a theoretical n/5000 solution. We hope later to report a series of experiments similar to these in which the actual hydrogen-ion concentration is known. In all experiments liter Erlenmeyer flasks were used. They were filled with the solution and stoppered. Controls were run parallel in stoppered flasks of well-water. Solutions were changed daily unless otherwise noted.

Figures 15 to 21 illustrate seven embryos from experiment 130.3. The eggs when in early gastrula stages were placed in n/750 HCl and after twelve hours were removed to water. The figures show their condition eighty-one hours later. They show just about the same abnormalities and the same range of variation in type as is seen after treatment with a great variety of other agents and conditions. Embryos of much this same type were obtained in three other series of HCl experiments, furnishing approximately the conditions noted above.

One curious consequence of the HCl treatment in low concentrations was a very marked acceleration of development. Without attempting to decide at present whether the acceleration is due directly or indirectly to the HCl as such, the results are perfectly clear cut and definite. Both Professor Child and myself have obtained these acceleration forms independently and repeatedly. Furthermore, there is a tendency for the embryos to become megacephalic under such conditions and often the tails, ventral suckers, and gills are unusually prominent. In other words, the acceleration is more or less differential. The embryo apparently does not simply develop more rapidly as a whole, but certain regions are more accelerated than others, viz., the same regions that are most inhibited under conditions that markedly retard developmental processes.

Experiment HCl-C. 1. n/5000 HCl. Eggs two to four-cell stage at beginning of the experiment.

30 hours in HCl. Early gastrula. In advance of control.

60 hours in HCl. Neural fold stages. Control, small yolk plug stages (figs. 30, 31).

72 hours in HCl. Blastopore closed and embryos beginning to elongate. None of the control embryos elongating yet.

96 hours in HCl. Embryos in advance of control. Heads seem relatively larger than in normal embryos (figs. 32, 33, 34).

Experiment HCl-C. 5. n/5000 HCl. Eggs two to four-cell stage at the beginning of the experiment.

48 hours in HCl. Distinctly accelerated. Blastopore closed and medullary folds distinct. Control: advanced yolk plug stages.

96 hours in HCl. Many hatched. Heads seem relatively large. None of the control embryos hatched as yet.

6 days in HCl. Much accelerated. Tails long and broad. Figures 35, 36, 37 and 38. The heads of these embryos appear relatively longer and broader than controls and the gills are further developed and widespread. Actively swimming.

Experiment HCl-C. 6. n/20,000 HCl. Eggs two to four-cell stage at the beginning of the experiment. Control same as for the experiments just described.

48 hours in HCl. Development accelerated. Blastopore closed. Medullary folds distinct and beginning to close. Dorsal region elongating.

96 hours in HCl. Embryos are more accelerated than those in n/5000. Nearly all hatched.

9 days in HCl. Heads relatively large and gills unusually prominent. The embryos remind somewhat of Amblystoma tadpoles.

The experiments with NaOH were carried out in the same way as those with HCl, and in most cases were ran parallel with eggs from the same female and one control for the two series. Save for the slightly different concentrations used, there was no particular difference in the experiments with the two agents either as regards method or results. A single example will suffice here. Eggs in early gastrula stages exposed from 1 1/2 to 12 hours to n/300 NaOH and returned to water show after several days microcephalic embryos with the bilateral sense organs and ventral suckers approximated or 'fused' and numerous spina bifida forms are present. Figures 24 to 26 represent three embryos from experiment 125.2 E. The eggs—early gastrula at the start of the experiment—were exposed 3 hours to n/300 NaOH and removed to water. The camera-lucida sketches were made six days later.

If eggs are taken when the susceptibility is low—early cleavage or unsegmented—and somewhat lower concentrations of NaOH used, n/400, n/500—n/1000, development is accelerated in much the same way as in the HCl experiments described above. Figures 27, 28 represent two sister embryos of the same age. Figure 27 is the control and 28 an embryo exposed 8 days to n/500 NaOH. In this case the solution was not changed after the third day. The megacephalic condition—differential acceleration—is apparent at once.

As regard acclimation of the egg or embryo in the various solutions my data are incomplete, but a number of the experiments furnished conditions where recovery might be expected to take place. Potassium permanganate seems to be a favorable agent for the purpose. While

quite toxic in concentrations of m /5000 or higher, the toxicity appears gradually to grow less, due to the formation of the insoluble oxide.

Eggs in an eight-cell stage were placed in m/5000 potassium permanganate, and after 48 hours' exposure a series of stages was present, varying from nearly normal yolk plug stages to equatorial gastrula stages. The controls at this time were in neural fold stages. After 72 hours most of the embryos were of the type designated as differential recovery forms. The heads are relatively very large and the ventral suckers and gill plates are prominent. At this time there still remained alive a few equatorial gastrulae and several spina bifida forms, microcephalic and with the persistent yolk plug situated dorsally. Variations of this sort can hardly be avoided in material such as the frog egg for reasons already set forth. Moreover, the thickness of the gelatinous membranes varies somewhat, especially after the large egg masses have been cut apart into smaller masses containing ten or a dozen eggs, as was done in all of the experiments. In this particular experiment approximately 75 or 80 per cent of the embryos were of the differential recovery type, so that on the whole the results are perfectly clear-cut and definite.

Figures 41, 42, which are described on page 486, represent what appear to be variations in recovery for which the factors mentioned above are, I believe, in part responsible. In addition, the conditions under which these embryos were produced were severe enough that recovery rarely occurs at all. Consequently, some variation is to be expected. But whatever the extent of the variation may be, the significant point is that for the most part, recovery following a previous inhibition is differential. Figure 44 illustrates differential recovery following a previous treatment with lithium chloride.

It is not always possible to distinguish between acclimation and recovery, especially under conditions like the permanganate experiment cited above. In this case the strength of the solution is gradually changing, due to the reduction of the permanganate (the solution was not changed during the course of the experiment). But little or no acclimation occurs under the conditions which produced some of the embryos. Hence, in this case the results are in all probability a consequence of the earlier inhibition and later recovery. Many of the embryos were convex dorsally and the tail buds were relatively large and conspicuous. Similar embryos appeared after the following treatment: 2 days in water (blastopore closed and medullary folds appearing at this time), plus 2 days in m/10,000 KNC, plus 2 days in water. The differential was even more marked in these embryos (experiment *KNC-C*. 13).

Recovery of the embryo, then, is differential in the same sense that inhibition is differential. Those regions of the embryo that are normally most active, recover soonest when the developing animal is removed from the inhibiting conditions.

THE INTERPRETATION OF ABNORMAL DEVELOPMENT

Quite apart from any interpretation that may be given to the facts, the egg and embryo of the frog exhibit a differential susceptibility to various factors of the environment. That is, those regions of the egg or embryo that are functionally most active are most susceptible to agents or conditions that at all seriously disturb developmental processes. This is only another way of saying that these differences in susceptibility bear a direct relation to the principal axes of symmetry of the egg and embryo. The different methods of demonstrating differential susceptibility have already been discussed.

Given what may be called gradients in susceptibility which is demonstrated, it follows as a necessary consequence that any considerable disturbance of development, experimentally or otherwise induced, save certain mechanical disturbances of course, will appear as a differential. Hence, in the absence of specific effects of particular agents or conditions on particular regions of the egg or embryo, inhibition, acceleration, acclimation, or recovery in development must be differential. As a matter of fact, inhibition that is at all marked is differential, regardless of how it is induced. The same is true to a considerable extent of accelerated development and in those cases where the egg or embryo acclimate to or recover from some disturbing condition.

If additional evidence is needed to convince one of the differential nature of inhibited development in the frog, it can be had by examining the data of previous workers in this field—Gurwitsch, '96; Hertwig, '92, '95; Bataillon, '01; Morgan, '03; Jenkinson, '06; these are a few of the representative papers. The great majority of modifications in the development of the frog obtained by these other workers in the field are so obviously differential inhibition forms that a detailed discussion of them seems quite unnecessary.

It is significant that practically all, certainly the most common types of abnormalities and especially those experimentally induced not only in the frog, but also in organisms generally, fall naturally into the categories mentioned earlier in the paper, viz., differential inhibition forms, or more rarely, since the conditions

for their appearance are less often realized, abnormalities showing differential acclimation or recovery, or differential acceleration.

Often the embryo shows a combination of inhibition and acclimation or recovery or both. The failure to distinguish these different types when seen in the same embryo may lead to some confusion in attempting to interpret abnormal development. I do not, of course, intend to imply that all abnormalities are consequences of conditions which, operating relatively early in development, produce differential inhibitions, accelerations, acclimations, or recoveries. Mechanical disturbances and the like and probably other disturbances coming near the end of development belong to quite a different category. With the great majority of teratological forms falling readily into the classes mentioned above or combinations of them which in turn must be consequences of differential susceptibility, the task of interpreting teratological development resolves itself largely into a matter of accounting satisfactorily for differential susceptibility.

The reasons for regarding differential susceptibility as one expression of underlying graded differences in the dynamic relations and activities of protoplasm—physiological gradients—have been presented elsewhere (Child, '20; Bellamy, '19).

Given the organismic pattern as a gradient or gradients in fundamental functional and structural relationships in a specific protoplasm, and there is abundant experimental evidence to support this idea, teratological development becomes understandable as a disturbance of this underlying order. Since this order is a graded one paralleling the axes of symmetry of the organism, any considerable disturbance of it must also entail a parallel and differential modification in the development of the embryo.

SUMMARY

1. The egg and embryo of the frog exhibit a differential susceptibility to a great variety of conditions that disturb developmental processes.

2. According to the experimental treatment and the physiological condition of the organism at the time of exposure, the induced modifications fall naturally into classes of differential

inhibitions, differential accelerations, differential acclimations, or differential recoveries.

3. These differential modifications, are obvious and necessary consequences of the differential susceptibility of the organism to the agents used in experimental teratology.

4. The modifications produced are perfectly characteristic, not of a particular agent or condition, but of a particular concentration or 'intensity' of action of that agent. In other words, the developmental response is primarily quantitative and non-specific.

5. Differences in susceptibility parallel the axes of symmetry. Those regions which usually differentiate earliest and grow most rapidly are most distorted, the degree and direction of the distortion depending largely upon the severity of the treatment and physiological condition of the organism. There are, in short, gradients in susceptibility paralleling the axes of symmetry, both primary and secondary.

6. The existence of susceptibility gradients—whose existence is demonstrated in a great variety of organisms—must mean an underlying gradient or gradients in the structural and functional aspects of protoplasm generally.

7. It is in terms of disturbances in this underlying graded order in the dynamic relations and activities of protoplasm—physiological gradients—that we believe teratological development is most rationally accounted for.

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Resumen por el autor, A. L. Salazar.

Sobre una forma particular de atresia de los folículos de De Graaf (coneja) revelada por el método tano-argéntico.

El autor describe un tipo especial de atresia de los folículos graafianos en el ovario de la coneja, revelada por su método. Este tipo se observa en los pequeños folículos del ovario de tipo ovigénico, caracterizándose por la formación de un largo cordón tanofílico en los intersticios de las células de la granulosa. El autor examina la conexión de este tipo de atresia con la atresia hidrópica de los cordones ovigénicos y sus restos (nódulos epiteliales, folículos aováricos de Regaud), recientemente descritos por él, y discute la significación de los casos descritos.

Translation by José F. Nonidez
Cornell Medical College, New York

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SUR UNE FORME PARTICULIÈRE D'ATRÉSIE DES FOLLICULES DE DE GRAAF (LAPINE), RÉVÉLÉE PAR LA MÉTHODE TANNOFERRIQUE

A. L. SALAZAR

Institut d'Histologie et d'Embryologie de l'Université de Porto, Faculté de Médecine (Portugal)

CINQ FIGURES

Dans les ovaires adultes de la Lapine, appartenant au type ovigène, on trouve un certain nombre de formes atypiques d'atrésie folliculaire. Parmi ces formes, les follicules à cordon tannophile présentent un intérêt spécial à cause de leurs rapports avec l'atrésie hydropique, que nous avons signalée récemment[1] dans l'ovaire de la Lapine. Nous allons décrire ici quelques types de follicules à cordon tannophile; ensuite nous étudierons leurs rapports avec l'hydropisie atrésique des cordons ovigènes et de leurs reliquats. Cette étude est basée sur des coupes traitées par notre procédé au tannin-fer, suivant la technique que nous avons indiquée ailleurs.[2]

A. LES FOLLICULES À CORDON TANNOPHILE

Le type d'atrésie en question est caractérisé par l'existence dans les interstices entre les cellules de la granulosa ou dans la place totale que celle-ci occupait, d'un long cordon plus ou moins épais, coloré en noir intense par la réaction en question. Ce cordon présente comme caractéristiques générales l'intense tannophilie, la longueur et des sinuosités innombrables; il remplit parfois tout le follicule, dont la granulosa est invisible ou réduite à quelques cellules. Ces follicules à cordon tannophile peuvent se présenter avec quelques variations: nous représentons dans les figures 1, 2 et 3, trois spécimens. Dans les coupes traitées par la méthode tanno-ferrique ces follicules, comme d'ailleurs tous les ovisacs dans la période agonique de l'atrésie, se détachent

nettement en noir intense sur le fond blanc ou grisâtre de la préparation, où seules les formations tannophiles (pellucide, liquor folliculi, membranes de Slavjanski et des cordons, cordons ovigènes et reliquats en atrésie hydropique, etc.) se dessinent en noir.

Dans la figure 1 on voit un follicule encore peu développé. Il est limité par une membrane de Slavjanski intacte, que le tannin-fer dessine nettement, comme d'habitude, sous la forme d'un mince trait noir. Cette conservation de la membrane de Slavjanski est particulièrement remarquable étant donnée la tendance de cette membrane à s'hypertrophier dans l'atrésie typique et même dans l'atrésie des cordons ovigènes et des reliquats.[3] Au centre du follicule, teinté en gris, on trouve l'oocyte dégénéré. Dans la coupe représentée dans la figure on ne voit pas la pellucide; elle était visible dans les coupes suivantes, gonflée très tannophile, comme c'est, du reste, le cas habituel dans les périodes agoniques de l'atrésie.

De la granulosa on ne voit qu'une rangée de cellules superficielles, dont les noyaux sont à peine visibles, car le tannin-fer ne colore pas la chromatine. La portion restante de la granulosa est occupée par un cordon tannophile assez épais, très long, intensivement coloré en noir. Ce cordon présente partout sensiblement la même épaisseur, à l'exception d'un point où il se gonfle en une grosse boule noire, qu'on voit dans la figure; cette boule semble une hypertrophie locale du cordon. Celui-ci présente partout des flexuosités et des circonvolutions si nombreuses et si serrées qu'il est impossible de le suivre et de savoir s'il s'agit d'un cordon unique ou de plusieurs cordons enchevêtrés. Il s'agit probablement d'un cordon unique, car on ne voit nulle part des solutions de continuité. Nous avons taché de représenter par les différentes graduations de ton l'aspect que présente dans la coupe, assez épaisse, ce cordon capricieusement pelotonné. L'épaisseur de la coupe permetait la représentation du cordon en plusieurs plans. La rangée de cellules de la granulosa, qu'on voit dans la périphérie, sous la membrane de Slavjanski, semble intacte; mais le tannin-fer, ne colorant ni le noyau ni le cytoplasme, il est impossible de l'affirmer avec certitude. Cependant,

la forme du noyau, à peine visable par réfringence, est régulière, et il n'y existe pas des phénomènes de plasmolyse ni de caryolise. Le liquor interstitiel[2] est invisible; on aperçoit débris dans le voisinage de la pellucide dégénérée. Cette absence du liquor interstitiel peut être réelle ou apparente; car, dans ces cas atypiques, sa tannophile est parfois si faible qu'el est souvent difficilement visible. Les cellules de la granulosa qu'on voit dans la figure disparaissent dans les coupes suivantes; dans ces coupes le follicule en question est entièrement occupé, outre l'oocyte et la pellucide dégénérée, par le cordon tannophile. Nous avons examiné soigneusement les interstices entre les flexuosités du cordon, pour chercher les cellules de la granulosa ou leurs débris; nous n'avons réussi à voir rien de net, car l'enchevêtrement du cordon est si compliqué, qu'on n'aperçoit entre les flexuosités que l'ombre dense des autres régions du cordon.

La figure 2 représente un autre follicule atrésié avec cordon tannophile. Il s'agit d'un follicule un peu plus développé que le précedent. L'oocyte, excentrique, se trouve réduit à des fragments; la pellucide, gonflée, retractée, présente une bande interne très tannophile, colorée en noir intense, et une région externe colorée en gris de plus en plus pâle au fur et à mesure qu'on s'approche de la périphérie, où elle se termine par des estompés très larges. Elle présente une striation concentrique un peu vague. C'est, en somme, un des aspects habituels de cet élément dans les follicules atrésiés: elle se trouve en pleine deliquescence atrésique. Le follicule est limité, comme le précédent, par une membrane de Slavjanski dessinée sous la forme d'un mince trait noir. Ce trait noir, trés net et indépendant en certains points, s'estompe en d'autres, où il se confond tantôt avec le dessin du liquor interstitiel, tantôt avec le conjonctif environant, tantôt enfin avec l'un et l'autre à la fois.

La granulosa est visible en partie. On voit des plages occupées par des cellules, dont on aperçoit vaguement les noyaux et nettement les contours dessinés par le liquor interstitiel. La plus grande partie de la granulosa a été substituée par un trés long cordon tannophile, qui ondule partout. Ce cordon présente une portion qui dessine à la périphérie du follicule des spires de cou-

leuvre; la portion restante, la plus longue, présente des sinuosités si sérrées, qu'elles se touchent par leur dos. L'aspect n'est si enchevêtré que dans le follicule précédent, car les sinuosités suivent presque toujours la ligne du cordon et cette ligne présente dans son ensemble des ondulations très larges. Ici, comme dans le cas présédent, le cordon semble continu, car on n'y voit pas de solutions de continuité. Dans les coupes on voyait plusieurs follicules atrésiés présentant un cordon moins long, en général fragmenté, passant par des transitions insensibles à la substance tannophile qui s'accumule en lacs dans les interstices entre les cellules de la granulosa atrésiée. On pourrait échelonner toute une serie d'exemplaires depuis ceux qui présentent un cordon très long, comme celui de la figure 1, jusqu'à ceux qui n'en possédent que quelques fragments. Ces follicules à cordon plus réduit et fragmentaire occupent le centre de f. c. j. en formation, au contraire de ceux que nous venons de décrire. En effet, ceux-ci ne sont pas suivis de la formation de corps jaunes atrétiques ce qui est, du reste, le cas habituel dans l'atrésie des follicules très petits. Les follicules atrésiés avec cordon petit, irrégulier, fragmenté, existent d'ailleurs dans les ovaires du type interstitiel, atrésique-interstitiel, etc., au contraire des formes précédentes, que nous n'avons rencontrées jusqu'ici que dans l'ovaire du type ovigène. Nous n'insisterons pas pour le moment sur ces formes attenuées, c'est-à-dire à petit cordon fragmenté, qui établissent une transition entre les formes atypiques (ou mieux rares) et l'aspect du follicule agonique coloré par le tannin-fer; nous y reviendrons avec plus de détails dans un travail sur les moments agoniques de l'atrésie du follicule et la formation du corps jaune atrétique. Nous voulons simplement faire remarquer qu'entre les formes les plus atypiques représentées dans les figures 1, 2 et 3 et les cas typiques habituels il existe une série graduelle de formes de transition.

Le cas représenté dans la figure 3 appartient encore à la catégorie des précédents, mais il s'en distingue par quelques caractères particuliers. Le follicule présente la forme d'une grosse poire. La portion grosse représente le follicule proprement dit; le pédicule est un fragment du cordon ovigène resté en connexion avec

l'ovisac. Ce fait est très fréquent, surtout dans les ovaires du type ovigène, mais il présente dans ce cas un aspect curieux. D'abord, l'appendice est constitué par une seule rangée de cellules; puis, il est si long, que nous n'avons pu le représenter qu'en partie dans le dessin; nous n'avons pu d'ailleurs le suivre jusqu'à son terminus, tant il était long. Un examen plus détaillé montre le suivant. La portion grosse, c'est-à-dire le follicule proprement dit, présente deux pellucides en déliquescence atrésique. Le centre des deux pellucides est occupé par des débris de l'oocyte respectif. Les deux pellucides présentent une portion interne homogène, très tannophile, et une région externe, plus étendue, plus pâle; cette région externe est, du reste, commun aux pellucides, ce qui est dû à la liquéfaction. D'ailleurs, les régions internes, tannophiles, se confondent en partie, par la même raison. À la périphérie on ne voit plus la membrane de Slavjanski; on en aperçoit les débris, à gauche, sous la forme d'un petit cordon spiralé, un peu épais. Cependant, les limites du follicule sont bien visibles, car la transition entre les différentes régions du follicule et le tissu conjonctif environnant est trés nette. La portion restante du follicule, correspondant à la granulosa, est occupée par une substance colorée en gris pâle, qui se confond insensiblement avec la région externe, pâle, des deux pellucides déliquescentes, dont les limites sont encore visibles, à cause de la couronne de vacuoles qu'y a déterminé le fixateur. Dans la substance pâle qui occupe le lieu de la granulosa on ne voit plus de cellules; nous ne voulons pas dire qu'elles n'y puissent exister, car la méthode tanno-ferrique n'est pas apropriée pour les mettre en relief. Cependant, soit par leur réfringence, soit par une légère teinte gris-pâle, on voit d'habitude les noyaux: par exemple, ils sont visibles dans l'appendice annexé au follicule. Par contre, on voit dans la granulosa des filaments tannophiles irréguliers, ici un peu épais, là très fins, parfois isolés, d'autres fois liés en réticulum. Ces filaments n'ont rien d'atypique; on les voit plus développés et plus définis dans le moment agonique de l'atrésie des follicules; nous les étudierons plus tard, dans un autre travail. Outre ces filaments, on voit encore dans la masse pâle qui occupe la place de la granulosa des coagula plus colorés. Ces coagula

prennent dans certains points la forme de cordon flexueux, dans d'autres celle de coagula diffus, à contours estompés; dans d'autres régions encore on les voit présenter un aspect réticulé, car les masses en question y sont criblées de vacuoles.

Nous ne savons pas ce qui représente la masse pâle qui occupe la place de la granulosa. Elle peut résulter de la déliquescence des cellules de la granulosa ou de l'épanchement au dehors de la substance qui constitue la région externe, pâle, des pellucides. Cette derniére hypothése semble d'abord la plus vraisemblable; mais si l'on remarque que les limites externes des pellucides sont encore marqués, grâce à la couronne de vacuoles artificiels; que ces vacuoles ne sont pas visibles dans la masse grisâtre qui occupe la granulosa; qu'il n'existe dans la zone pâle des follicules les filaments fins caractéristiques de la granulosa agonique (excepté à gauche, entre les deux pellucides, où les limites ne sont pas nets), on penche surtout vers la première hypothéses. L'identité de coloration ne suffit pas d'ailleurs pour établir l'identité de substance. Il se pourrait encore que ces deux hypothèses fussent réelles, les deux causes ayant determiné la formation de la masse pâle. Les masses plus tannophiles, qu'on voit dans la figure et que nous avons décrites plus haut, représentent les fragments d'un cordon tannophile. La forme en cordon, comme nous l'avons dit, est encore visible en certains points; dans d'autres on ne la reconnait plus. La tannophilie de ces fragments est beaucoup moins intense que celle des cordons que nous avons décrits dans les autres follicules. Il semble, en résumé, qu'il s'agit soit de petits cordons, soit d'un cordon fragmenté, en deliquescence.

Le follicule n'aurait, en résumé, rien de bien particulier, si n'était pas la disposition extrêmement bizarre qu'on voit dans la base de l'appendice.

Comme le montre la figure 3, un long cordon trés epais, très tannophile, occupe cette région. Il décrit constamment des flexuosités très serrés, tout en serpentant autour de la région où l'appendice s'implante sur le follicule, région qu'il cache. Ensuite, conservant toujours sa coloration intensivement noire, il diminue graduellement d'épaisseur et rampe autour de l'appendice,

toujours ondulant et serpentant: puis il disparaît avec l'appendice, dont nous n'avons pu voir l'extrémité, comme nous l'avons dit. Dans la base de l'appendice le cordon, toujours flexueux, ondule à la superficie du follicule. Une partie du cordon semble logé dans l'intérieur du follicule et dans la base de l'appendice, une autre partie semble extérieure. Un peu au dessus, dans la région où l'appendice sort du follicule, en s'amincissant, tout le cordon semble extérieur à l'appendice; plus loin, quand le cordon, plus mince, rampe adossé à l'appendice, il est toujours extérieur; s'il existe, à la base de l'appendice, des tronçons qui semblent logés dans l'intérieur du follicule et dans la base de l'appendice, il faut avouer que l'absence de membrane de Slavjanski et l'aspect embrouillé de la préparation dans cette région laissent l'esprit dans le doute. Quoiqu'il en soit, la plus grande partie du cordon, du moins, est nettement extérieure; cela fait penser tout de suite que ce cordon n'est que la membrane de Slavjanski et la membrane propre de l'appendice devenues hypertrophiques. En effet, nous avons démontré à l'aide du tannin-fer que les cordons ovigènes possèdent une membrane propre,[3] ce que Paladino avait toujours nié; cette membrane existe également dans les reliquats des cordons, soit qu'ils restent libres, soit qu'ils demeurent annexés au follicules. Nous avons montré que ces membranes propres peuvent s'hypertrophier et se transformer en cordons flexueux tannophiles dans l'atrésie des cordons et de leurs reliquats. Comme d'habitude il arrive la même chose pour la membrane de Slavjanski (qui n'est pas autre chose, comme nous l'avons montre,[3] qu'un fragment de la membrane du cordon ovigène), on comprend aisément que l'aspect représenté dans la figure 3 puisse être determiné par l'hypertrophie simultanée de la membrane du follicule (gonflée dans le voisinage de l'appendice, disparue dans la portion restante) et de la membrane de l'appendice. Mais cette explication est immédiatement démentie si l'on observe ce fait déconcertant, à savoir, que l'appendice conserve intacte sa membrane propre. Dans la figure on peut la voir dans toute l'extension du long appendice, sous la forme de deux traits minces qui le limitent des deux côtés. Ces traits disparaissent ici et là sous les flexuosités du cordon, divergent dans

la région où l'appendice sort du follicule et disparaissent plus loin. Donc, le cordon tannophile ne semble pas provenir de l'hypertrophie atrésique des membranes limitantes en question; d'où vient-il alors? Est-ce que le cordon, d'abord intérieur, logé dans la granulosa, a émigré vers l'extérieur? Cette hypothèse est très peu probable. Est-ce que, grâce aux constantes remaniements de l'ovaire, le follicule en question a été ficelé par un cordon provenant d'un follicule disparu, le cordon ayant resté, sous forme de débris, dans le strome, comme il arrive souvent? L'intimité des rapports existants entre le cordon, l'appendice et le follicule, rend cette hypothèse aussi improbable que les autres. Faute de mieux, nous reviendrons ainse à la première hypothèse. Mais comment harmoniser cette interprétation avec ce fait paradoxal, la conservation de la membrane dans l'appendice? On peut le faire de deux manières. En effet, si l'on suppose que la membrane de Slavjanski a subi l'hypertrophie habituelle dans la région voisine de l'implantation de l'appendice et que la membrane, ainse hypertrophiée, rampe, au fur et à mesure de sa croissance, au long de l'appendice, grâce à une attraction chimio-taxique quelconque, on aurait l'image représentée dans la figure. Mais on peut encore admettre que le cordon tannophile provient en réalité de l'hypertorphie simultanée d'une partie de la membrane folliculaire, celle qui voisine la base de l'appendice, et de la membrane propre de celui-ci. Car, si l'on suppose que la membrane propre des cordons ovigènes et de leurs reliquats n'est pas un élément homogène, simple, mais une formation complexe, on peut tout de suite admettre une dissociation atrésique de ses éléments, les uns subissant l'hypertrophie, les autres non. La membrane pourrait être, par exemple, d'origine épithéliale et conjonctive à la fois, mais nous verrons plus loin que cette hypothèse est peu admissible. En admettant qu'elle est seulement d'origine épithéliale, on peut supposer que le produit élaboré se différentie ensuite, cette différentiation étant l'index soit d'une structure complexe, soit d'une substance qui se présente avec des étapes de maturation diverses, progressant vers l'extérieur. Les choses peuvent même être encore plus simples. Car les traits qui délimitent l'appendice ne représentent peut-être autre

chose qu'une sorte d'exoplasme ou de cuticule de la cellule, normalement caché sous la membrane propre adhérente. Cette dernière explication nous semble la plus probable, car nous avons observé des faits semblables dans la membrane des follicules de De Graaf. On y voit parfois, au début de l'hypertrophie atrésique de la membrane de Slavjanski, partir de la portion déjà épaissie un cordon plus ou moins long, que ondule à périphérie du follicule; ce cordon suit la membrane qui reste encore visible. Pour rendre plus claire notre idée, nous nous servirons d'une comparaison. Dans les coupes colorées par le tannin-fer on ne peut distinguer la pellucide de la membrane vitelline; dans ces coupes la pellcuide, ou mieux, certains types de pellucide y apparaissent sous la forme d'une bande homogène colorée en noir intense. Cet anneau noir, par son bord interne, délimite nettement l'oocyte, de sorte que la membrane vitelline y apparaît noyée dans la tannophilie intense de la pellucide. Si quelque chose d'analogue existe dans les membranes des cordons ovigènes, de leurs reliquats et de la membrane de Slavjanski, le cas de la figure 3 serait d'une explication plus facile.

B. RAPPORTS DE CE TYPE D'ATRÉSIE AVEC L'ATRÉSIE HYDROPIQUE DES CORDONS OVIGÉNES ET DE LEURS RELIQUATS

Donc, quoique l'aspect de ces follicules atrésiés soit très bizarre, seule l'existence du cordon caractérise ces follicules: l'oocyte et la pellucide présentent des aspects qu'on touve partout dans les follicules atrésiés. Quelle est l'origine et la signification de ce cordon? Les faits, que nous avons signalés dans l'atrésie des cordons ovigènes et de leurs reliquats,[1] rendent assez facile l'explication de ces cas. Ces faits, signalés dans une note trop succinte, seront décrits en détail et largement illustrés dans un mémoire. Nous rappellerons ici les faits indispensables pour la discussion du cas présent: nous renvoyons le lecteur, pour la description compléte au mémoire illustré, qui paraîtra prochainement. Dans les interstices entre les cellules des cordons ovigènes ou de leurs reliquats quand les coupes, fixées dans le liquide de Bouin, ont été colorées par notre procédé au tannin-fer, on voit souvent s'accumuler une substance liquide ou pâteuse, à figure

de coagulation homogène et très tannophile (fig. 4). Cette substance, en s'accumulant dans les interstices entre les cellules épithéliales, dissocie et désagrège le cordon ovigène ou le reliquat; c'est ce que nous avons appelé *l'atrésie hydropique*, car elle consiste dans l'accumulation pour ainsi dire monstrueuse d'une substance liquide ou semi-liquide dans l'intérieur du reliquat ou du cordon ovigène. Cette hydropisie tannophile est très fréquente dans les ovaires du type ovigène, mais on la rencontre dans les ovaires du type folliculaire, atrésique et même interstitiel; seulement, dans ces derniers types on ne voit plus des cordons ovigénes hydropiques mais uniquement des reliquats. Cette hydropisie est, en effet, la seule forme de dégénérescence connue des reliquats, grands ou petits (les follicules anovulaires de Regaud, par exemple).

Or, dans quelques reliquats, et parfois aussi dans les cordons ovigènes, on ne voit plus un magma hydropique à l'intérieur, mais un cordon tannophile flexueux, parfaitement analogue à ceux que nous venons de décrire (fig. 5). Ce cordon tannophile se loge dans les interstices entre les cellules du reliquat, tout comme la substance tannophile hydropique; il ne doit pas être confondu avec un cordon également tannophile et flexueux, qu'on voit parfois à la périphérie du reliquat; ce dernier n'est autre chose que l'hypertrophie atrésique de la membrane du cordon ovigène ou du reliquat.

Entre les reliquats qui sont en dégénérescence hydropique typique et ceux qui présentent un cordon tannophile à l'intérieur il existe toutes les passages. Le cordon est constitué par une substance plus dense, modelée, présentant une forme définie et indépendante, tandis que dans l'hydropisie tannophile typique nous voyons une substance plus fluide, qui ne possède pas de forme propre et emprunte celle des cavités et interstices où elle se trouve logée. Nous ne voulons pas dire que le cordon tannophile soit une étape plus avancée de l'hydropisie, car, s'il était ainsi, nous trouverions toujours un cordon tannophile dans les reliquats atrésiques des ovaires du type interstitiel tandis qu'on y trouve, au contraire, l'atrésie hydropique typique. La formation du cordon tannophile représente ainsi simplement une

modalité spéciale de l'atrésie hydropique: les deux formations ont, donc, la même origine et le même processus génétique. Or, l'origine de la substance hydropique n'est pas difficile à établir. Dans les interstices entre les cellules des cordons ovigènes et de leurs reliquats il existe un liquide qui est homologue du liquor interstitiel et du liquor de l'antrum des follicules (fig. 5). Ce liquor interstitiel des cordons et de leurs reliquats est mis en évidence par le tannin-fer, qui laisse les cellules en blanc, tout comme dans le granulosa des follicules de Graaf: seulement la coloration est ici beaucoup plus faible et plus insconstante. La substance hydropique semble résulter d'une hypertrophie atrésique de ce liquor interstitiel; elle est homologue de l'hypertrophie du liquor interstitiel, qu'on voit souvent, quoique d'une forme plus discrète et moins dominante, dans la période post-chromatolytique des follicules de De Graaf atrésiés, quand ils sont colorées par le tannin-fer. De sorte que le cordon tanophile doit être considéré ainsi comme une modification atrésique du liquor interstitiel des reliquats.

Cette modification peut être interprété de deux façons: ou il s'agit d'une altération directe de ce liquor ou d'une altération indirecte. Dans le premier cas, on doit supposer que le liquor s'altère grâce à un déséquilibre quelconque de la dynamique métabolique du reliquat; dans le second cas, on peut admettre que les cellules du reliquat, ayant ségrégué d'abord le liquor interstitiel quand elles étaient encore normales, produisent ensuite une nouvelle substance hydropique qui, passant dans les interstices, s'y accumule et forme tantôt la substance hydropique, tantôt le cordon tannophile; c'est-à-dire, il s'agit dans cette dernière hypothése d'une altération atrésique de la dynamique cellulaire.

Quoiqu'il en soit, nous voyons que l'existence d'un cordon tannophile n'est nullement exclusive des follicules que nous venons de décrire. En effet, le cordon tannophile de ces follicules est absolument analogue à celui qui se forme dans les reliquats épithéliaux. Il présente la même tannophilie intense, les mêmes flexuosités, la même localisation. La seule différence, d'importance nulle, se rapporte à la longueur du cordon, beau-

coup plus grande dans les follicules que dans les reliquats. Un fait important à propos de cette homologie est le suivant. Nous avons dit plus haut qu'il existe des ovisacs avec cordon tannophile qui remplit presque tout le follicule, comme dans la figure 1, d'autres où le cordon est plus court, d'autres encore où il est constitué par des tronçons fragmentés, en partie confondus avec les magma atrésiques. Ces derniers existent das les ovaires du type ovigène et non en tous: jusqu'ici, nous avons trouvé ces follicules à cordon long seulement dans l'ovaire caractérisé par l'xistence d'une grande poussée de cordons ovigènes. Or, c'est précisément dans ces ovaires qu'on trouve les reliquats avec cordon tannophile analogue. D'un autre côté, parmi les follicules à cordon ce sont les plus petits qui présentent rélativement le plus grand cordon (comparez figs. 1 et 2). Ceux qui sont presque entièrement remplis par le cordon sont encore très proches de l'étape primordiale; il est même possible que quelques follicules tout jeunes prennent, à cause du développement du cordon, des dimensions supérieures à celles qui leur sont habituelles. Tout cela—coexistence dans les ovaries du type ovigène de follicules à grand cordon avec des reliquats qui présentent dans leur intérieur un cordon analogue; le plus grand développement du cordon dans les follicules très petits—semble indiquer qu'il existe des rapports entre ce type spécial d'atrésie et l'existence d'une poussée ovigène très active. Il se peut que cette forme d'atrésie ne soit autre chose qu'une manifestation d'un processus caractéristique des reliquats. Cette manifestation, normale dans les reliquats, serait aberrante dans les follicules. Car le nombre de reliquats à cordon est beaucoup plus nombreux que celui des follicules qui présentent également celui-ci. Ce contraste est très net, puisque les reliquats qui restent annexés aux follicules présentent souvent (dans les ovaires ovigènes) le cordon tannophile, tandis que le follicule ne présente aucun signe de dégénérescence. Cette manifestation, aberrante dans les follicules, semble être une sorte de désorientation présentée par un processus d'habitude orienté dans un sens défini dans les cordons ovigènes et dans leur reliquats; pour rendre claire notre pensée nous dirons qu'il s'agit d'une sorte de mouvement des cordons

ovigènes et des reliquats qui s'est propagé par inertie jusqu'aux follicules. Ce retentissement, très marqué quand le follicule dégénère dès le premier âge, s'estompe plus tard, quand l'ovisac n'est atteint par l'atrésie qu'après avoir parcouru une certaine portion de sa trajectoire évolutive. Il est même possible que ce retentissement, plus ou moins accentué, concoure pour que le follicule dégénère plus précocement. L'étude de ces influences sur l'atrésie des follicules de De Graaf nous semble d'une grande importance pour expliquer certains types d'atrésie, qui s'écartent du processus atrésique typique de l'ovaire adulte, qui le type chromatolytique. D'une manière générale, les processus atrésiques embryonnaires, c'est-à-dire ceux qui frappent la première et la deuxième prolifération et les follicules de l'ovaire embryonnaire, dont la connaissance est due surtout aux travaux de Winiwarter et Sainmont, contrastent nettement avec l'atrésie typique de l'ovaire adulte. Dans l'atrésie embryonnaire nous voyons l'activité dissociante du conjonctif, la fragmentation et la dissociation des ovisacs, etc., mais rien qui rappelle, même dans les follicules, les phénomènes décrits dans ce que nous avons appelé les périodes pré-chromatolytique, chromatolytiqùe et post-chromatolytique.[4] Mais, si nous étudions un ovaire adulte du type ovigène, nous voyons ce contraste s'attenuer. Car nous voyons, dans l'atrésie des cordons, à côté des processus spécifiques, d'autres qui ne le sont pas: des phénomènes de chromatolyse discrète; dans l'atrésie des follicules, à côté des processus chromatolytiques typiques, nous voyons d'autres qui ne le sont pas: par exemple, la pénétration et la dissociation conjonctive, très atténuée. Nous y voyons encore des processus spécifiques, comme celui qui caractérise les follicules à cordon tannophile, que nous venons de décrire. De Winiwater et Sainmont[5] ont d'ailleurs insisté déjà sur que les processus d'atrésie se substituent lentement les uns aux autres. Or, l'ovaire à type ovigène semble représenter une étape de transition, placée au début de l'âge adulte. On y voit les derniers vestiges des processus atrésiques embryonnaires, des processus atrésiques spécifiques et des processus atrésiques adultes. Cette coexistence semble indiquer un changement important dans la dynamique des processus

atrésiques, car dans les ovaires du type ovigène, où la poussée n'est plus en pleine vigueur, et dans ceux qu'ont déjà presque atteint le type folliculaire où elle est éteinte, on voit disparaître entièrement les vestiges des processus atrésiques embryonnaires, s'atténuer ou disparaître les processus spécifiques du type ovigène bien caractérisé, à poussée ovigène intense, et, par contre, se généraliser les processus atrésiques habituels du type adulte. D'un autre côté, si nous examinons les processus atrésiques qu'on voit dans l'ovaire du type folliculaire, du type atrésique et du type interstitiel, on remarquera que les follicules dégénèrent par atrésie chromatolytique seulement après qu'ils ont atteint une certaine grosseur, les petits follicules, au contraire, dégénérant en général d'une manière différente. Ces processus atrésiques des petits follicules, encore mal étudiés, ne sont peut-être que des reliquats de processus dégénératifs caractéristiques des étapes antécédentes. Tout cela, il faut le dire, est basé sur que les ovaires du type ovigène, folliculaire, atrésique, interstitiel,[8] représentent l'évolution de l'ovaire comme organe, mais ceci n'est qu'une hypothèse fondée sur la comparaison et les rapports de ces types; l'évolution de l'ovaire adulte de la Lapine est encore aujourd'hui absolument inconnue, car les recherches de Winiwarter[5] n'ont été encore prolongées, jusqu'à l'âge sénile, par aucun biologiste. Quoiqu'il en soit, il n'est pas moins vrai que le type d'atrésie des follicules à cordon tannophile doit être considéré comme une répercussion des processus atrésiques spécifiques des cordons ovigènes et de leurs reliquats, dans les ovaires adultes du type ovigène.

Une autre question est la suivante. Le cordon tannophile des follicules et celui des reliquats, nous le savons déjà, sont des formations analogues; donc, ce que nous avons dit à propos de la formation du cordon dans les reliquats s'applique à celui des follicules. Cependant, il y a, à ce propos, un point à discuter, d'importance capitale. En effet, nous avons attribué la formation du cordon tannophile à une aberration des processus sécrétoires des cellules épithéliales, soit du reliquat, soit du follicule. Mais la formation du liquor folliculi aux dépens des cellules de la granulosa n'est pas encore un fait absolument prouvé. Cette

question a été étudiée par certains auteurs, V. der Stricht, par exemple, qui n'en ont pas donné des preuves absolument décisives; elle n'a pas encore été posée pour les reliquats et les cordons ovigènes, où, que nous sachions, l'existence d'un liquor interstitiel, homologue du liquor folliculi, n'avait été pas encore signalée jusqu'à nos travaux. Nous disons homologie et non identité, car non seulement le liquor interstitiel des reliquats et celui des cordons, d'un côté, le liquor primordial[3] et leurs dérivés, de l'autre, ne présentent exactement les mêmes caractères, mais il faut remarquer encore que la formation du follicule primordial marque pour les cellules de la granulosa un changement de vie, de destinée et de fonctions qui les écartent, au point de vue fonctionnel, des cellules des reliquats, dont elles sont cependant les sœurs. Cette orientation nouvelle de leur vie est due à l'apparition de l'oocyte et elle débute en plein cordon ovigène, dès que les cellules de ce cordon se sont différenciées en oocyte et en cellules satellites; tandis que l'absence de cette différentiation déterminera la formation d'un reliquat plus ou moins volumineux, où les cellules n'ayant plus, à ce qu'il semble, de rôle à remplir, ou ayant un tout autre rôle actuellement inconnu, ne se différentient ni se multiplient plus. Elles vivent dans le reliquat une vie végétative, y évoluent parfois d'une manière désorientée et finissent les unes pour se noyer dans les magma hydropiques qui dissocient les reliquats, les autres pour tomber dans le tissu conjonctif, où leur destinée ne peut être déterminée. Mais cette différence, à certains égards fondamentale, ne possède, au point de vue qui nous occupe, qu'une importance très secondaire; et la question, qui depuis longtemps se débat à propos de la formation du liquor folliculi, peut être généralisée au liquor interstitiel des reliquats.

Or, l'application de la méthode tanno-ferrique à l'étude de cette question semble précisement fournir des preuves, qui sont contraires au rôle élaborateur des cellules épithéliales des reliquats et des follicules. Car cette méthode colore le liquor primordial et leurs dérivés (le liquor interstitiel, le liquor de l'antrum, la substance liquide des Corps de Call et Exner et la pellucide) en noir intense, mais ne colore aucun enclave dans les cellules

épithéliales en question. Le liquide séreux qui, d'après V. der Stricht,[6] est élaboré dans les cellules de la granulosa des ovisacs de la Chauve-souris et passe ensuite dans les interstices, ne se colore pas avec le tannin-fer. Donc, ce produit séreux, s'il existe, n'est pas tannophile et change de constitution quand il tombe dans le liquor interstitiel. Dans l'évolution de la granulosa, depuis l'étape caractérisée par l'existence d'une seule rangée de cellules cubiques, étape où le liquor folliculi se montre par la première fois, jusqu'à l'age mûr du follicule, jamais des enclaves tannophiles ne se montrent dans le cytoplasme des cellules épithéliales du follicule. Mais ce fait n'a, en somme, aucune importance, car il suffit de penser que la substance élaborée par la cellule peut changer de constitution quand elle est expulsée. Les cas de ce genre sont trop fréquents dans les processus de sécrétion glandulaire pour qu'il faille y insister ici. Par contre, il existe une série de faits, qui nous portent à admettre le rôle actif des cellules épithéliales du follicule dans l'élaboration du liquor. À ce propos, nous ne devons pas envisager les cellules en question d'une manière étroite et localisée, mais dans l'ensemble de leur vie, de leur évolution, de leurs changements et de leurs manifestations, soit dans le follicule normal, soit dans le follicule atrésique. Parmi ces faits, la plupart très connus, nous rappellerons ceux que nous avons dernièrement mis en relief. Le liquor folliculi se montre, comme la méthode tanno-ferrique le met en évidence, dès l'étape de follicule primordiale. Il forme alors ce que nous avons appelé le liquor primordial. Ce liquor primordial est une sorte de blastème, d'où dérivent plus tard le liquor interstitiel, le liquor de l'antrun, la substance liquide des Corps de Call et Exner et la pellucide. Or, ces différentiations progressives sont toujours acompagnées d'un remaniement des cellules épithélialis qui semblent présider et orienter la différentiation du liquor primordial. C'est ainsi, par exemple, que la figure de précipitation de la substance liquide des Corps de Call et Exner change dès que les cellules se sont disposées en rosace autour du noyau central. En résumé, la vie même de la cellule épithéliale du follicule nous fournit un ensemble de faits plus important pour définir son rôle élaborateur que la démonstration

histologique d'une substance endo-cellulaire quelconque en rapport immédiat avec ce rôle élaborateur. Nous ne voulons, d'ailleurs, nullement nier que la transsudation provenant des vaisseaux de la thèque ne puisse remplir un rôle dans l'élaboration du liquor; ce rôle, qu'il se résume en fournir à la cellule de la granulosa des des matériaux ou qu'il consiste dans une transsudation interstitielle concourant à la formation du liquor, n'exclue nullement l'activité des cellules épithéliales dans l'élaboration du liquor. Or, cela étant ainsi, on comprend aisément qu'un déséquilibre atrésique dans l'activité des cellules en question puisse produire des produits atypiques. Le cordon tannophile des follicules, que nous venons de décrire, est un produit de ce genre, d'aprés notre manière de voir. Cette aberration de l'activité des cellules de la granulosa est une répercussion dans les follicules de processus dynamiques qui sont normaux dans les reliquats, où ils aboutissent à la formation des magma tannophiles dans la dégénérescence hydropique. Nous ne voulons pas dire que ce mouvement transmis aux follicules et qu'y détermine ces formes atrésiques rares représente un cas anormal; la disparition progressive du cordon dans les follicules de plus en plus âgés, montre, au contraire, qu'il s'agit d'un phénomène systématique. Il s'agit, en somme, d'après nous, d'un cas particulier qu'il faut intégrer dans un complexus de phénomènes encore mal étudiés, que nous avons signalés plus haut, à savoir: la répercussion jusqu'aux follicules de phénomènes spécifiques aux poussées ovigènes. Ces phénomènes s'attenuent de plus en plus, au fur et à mesure que le follicule est frappé d'atrésie dans un âge plus avancé, donc plus écarté du moment ovigène; cette atténuation est acompagnée d'une évolution progressive de l'atrésie vers le type adulte, chromatolytique. Mais cette répercussion peut aller plus ou moins loin, et cela devra nous expliquer un certain nombre, du moins, de formes d'atrésie atypiques.

Nous voulons faire remarquer encore un autre fait. La ressemblance entre les cordons tannophiles des follicules et l'aspect de la membrane de Slavjanski atrésique est frappante; la même ressemblance existe entre le cordon tannophile des reliquats et la membrane hypertrophiée, qu'on voit parfois autour des reliquats

et qui est l'homologue de la membrane folliculaire atrésiée. Parfois même, dans les reliquats, le cordon tannophile intérieur se continue avec le cordon extérieur sans aucune séparation.

On voit, en somme, que toutes ces formation atrésiques sont sœurs, possédent la même origine et sont dues à des processus histo-dynamiques analogues. Or, si l'on admet que la formation du cordon tannophile dépend d'une modification atrésique du rôle sécrétoire des cellules épithéliales soit du follicule soit du reliquat, comme nous l'avons avancé plus haut, on doit admettre également la participation des mêmes cellules dans l'hypertrophie atrésique de la membrane de Slavjanski, phénomène presque constant dans la période post-chromatolytique et même dans la période agonique de presque tous les types d'atrésie.

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PLAQUE

PLAQUE 1

EXPLICATION DE FIGURES

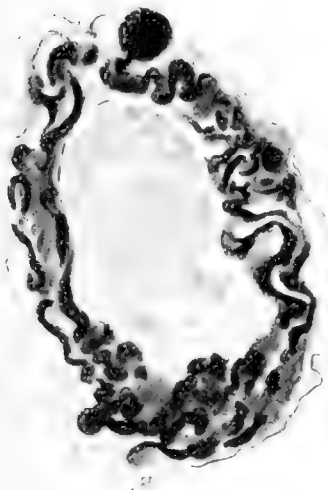
1 Follicule petit, avec très long cordon tannophile. Ovaire du type ovigène en pleine poussée. Fixation, liquide de Bouin. Méthode tanno-ferrique. Obj. $\frac{1}{12}$, oc. 2. Dessin au crayon.

2 Follicule un peu plus grand, avec cordon tannophile. Même ovaire. Fixation, liquid de Bouin. Méthode tanno-ferrique. Obj. $\frac{1}{12}$., oc. 2. Dessin au crayon.

3 Follicules à deux oöcytes, avec un long appendice annexé (reliquat du cordon ovigène). Cordon tannophile dans le follicule et dans l'appendice. Même ovaire. Fixation, liquide de Bouin. Méthode tanno-ferrique. Obj. $\frac{1}{12}$, oc. 2. Dessin au crayon.

4 Follicule anovulaire de Regaud en atrésie hydropique. Ovaire du type interstitiel. Fixation, liquide de Bouin. Méthode tanno-ferrique. Obj. $\frac{1}{12}$, oc. 2. Dessin au crayon.

5 Follicule anovulaire petit, avec cordon tannophile. Ovaire du type ovigène. Fixation, liquide de Bouin. Méthode tanno-ferrique. Obj. $\frac{1}{12}$, oc. 2. Dessin au crayon.



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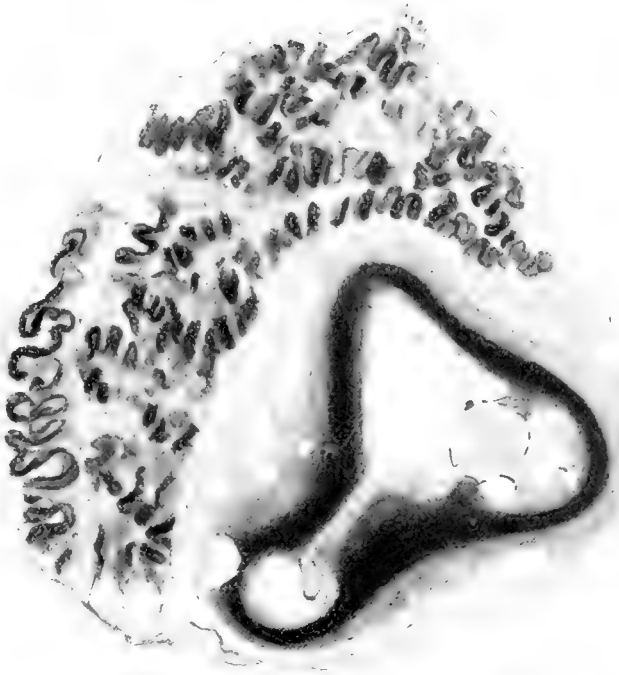

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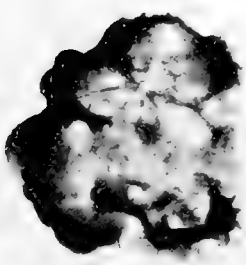

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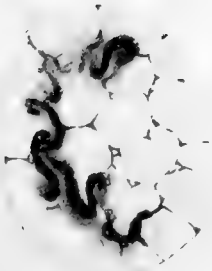

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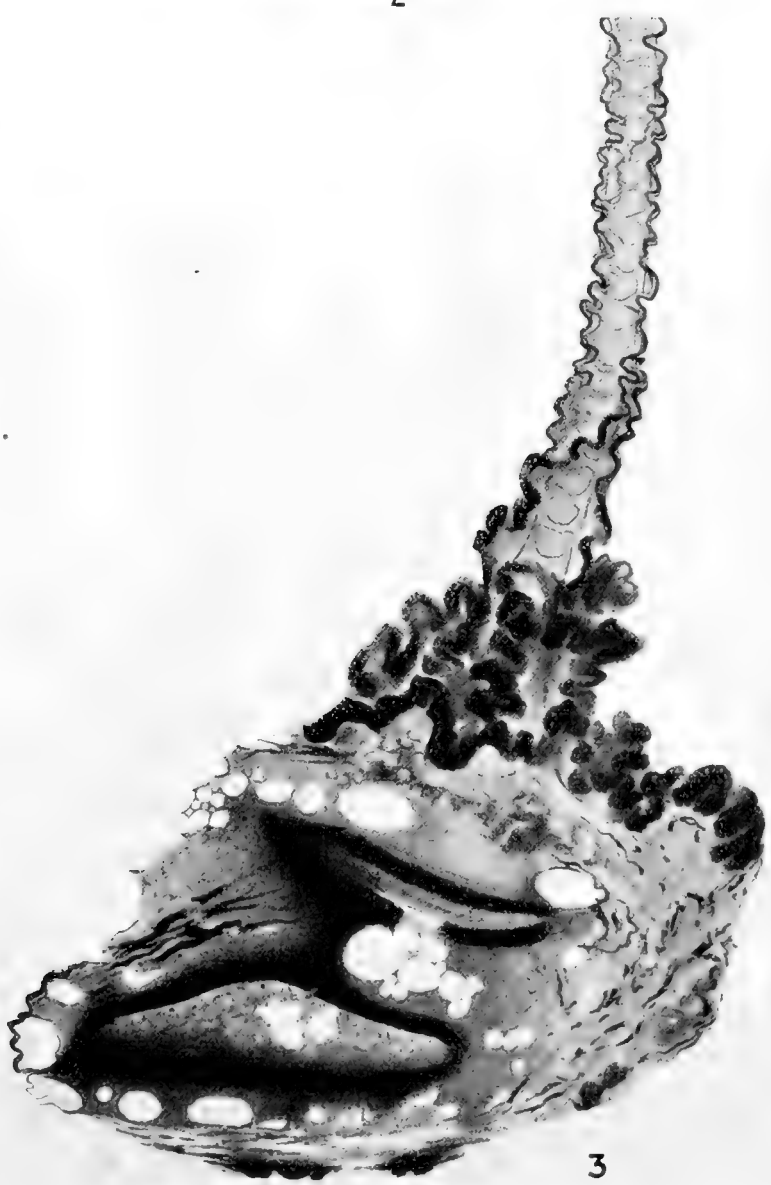

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SUBJECT AND AUTHOR INDEX