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LYMPHOCYSTIS TUMOURS IN THE RED MULLET (*MULLUS SURMULETUS* L.)

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(Plates I–IV)

A specimen of the red mullet (*Mullus surmuletus* L.), caught in Plymouth waters in October 1949, was found to have tumours projecting externally on the pectoral fins. They were spherical or elliptical in shape, the largest being 20 mm. long. On the suggestion of Mr G. A. Steven some of these outgrowths were cut out and given to the writer for microscopic examination.

When examined fresh they appear to consist of small, spherical, cyst-like bodies of various diameters, but there was no obvious clue as to their nature. After fixing in Bouin and sectioning, they are seen to be composed of cells of various sizes (Pl. I, figs. 1–4). It can be seen that these cells lie close to one another, leaving only a little space for the embedding connective tissue which is characterized by the abundance of its own small cells. The blood vessels met with in this tissue contain fish erythrocytes. The whole is covered by stratified squamous epithelium (Pl. I, fig. 2).

The cells of the tumours have large nuclei very much like those of egg cells, and this resemblance is made still greater by the presence of a distinct cell membrane. On the other hand, they show certain peculiarities which do not occur either in ova or in other cells of normal tissues, viz. their cytoplasm includes bodies in its peripheral layer which stain readily with chromatin stains. Examined under higher power these bodies appear as a network of bars of different shapes, including alveolar spaces, the peculiar arrangement of which can be better seen in tangential sections (Pl. IV, fig. 20).

Although there are certain differences in the appearance of the cells of different sizes, there can be no doubt that they all represent successive stages of development of the same elements growing from the inconspicuous size of a few microns up to giant cells of more than 400 μ in diameter. During this development critical stages must be of common occurrence as many of the cells are obviously in a state of degeneration.

From this description it is clear that these tumours are those characteristic of the lymphocystis disease known to attack various fresh-water and marine fishes, but, apparently, not yet recorded in the red mullet.

The term 'lymphocystis' is a misnomer in both components of the word and may evoke a totally wrong idea as to the nature of the disease; it originates from the time when the cells of these outgrowths were mistaken for parasitic

Sporozoa. The history of the subject is an unusual one, for the peculiar aspect of the cells gave rise to another erroneous assumption that they were eggs laid by some animal under the skin of the fish.

Weissenberg (1914) was the first to recognize that these cells were hypertrophied fibroblasts of the fish itself, and Joseph (1918) came to the same conclusion independently. To Weissenberg goes also the merit of proving experimentally, in his earliest researches on the pike perch (*Acerina cernua*), the infective nature of this disease; he was able to confirm this later on some American fishes (*Stizostedion vitreum*, *Lepomis macrochirus* and *L. gibbosus*).

The bibliography of the subject and a list of species in which the lymphocystis disease has been described are given by Nigrelli & Smith (1939); recent additions have been supplied by Weissenberg (1945). The following species from European waters are so far recorded with the disease: *Pleuronectes flesus*, *P. platessa*, *Acerina cernua* and *Sargus annularis*.

The first data on the characteristic microscopical structure of the lymphocystis tumours have been given by Woodcock (1904). The most detailed histological investigations have been made by Joseph (1918) and Weissenberg (1920). The problem of the nature of the virus responsible for the disease has been touched upon in one of the later papers by Weissenberg (1945).

Among the established characteristics of the disease attention may be drawn to the following. The tumours may grow not only on the skin but also in the internal organs (mesentery, intestine, ovary and spleen). There has been observed a certain periodicity in the development of the disease marked by the eruption of new tumours; it has also been ascertained that the infected fishes can overcome the disease after a longer or shorter period of time and that their skin may resume quite a normal appearance.

It is generally admitted that the disease is due to a virus, although positive proof by virus-test methods has apparently not yet been given. This virus has not proved to be identical in every instance, for in Weissenberg's experiments on *Acerina cernua* and *Stizostedion vitreum* only those fishes belonging to the same species could be infected and he, therefore, assumed that the virus was species-specific. This assumption was, moreover, in agreement with the observations of other investigators that other species of fishes kept for a long time in the same tank with the infected species did not develop similar tumours. However, in 1945, Weissenberg succeeded in transmitting the infection from the bluegill (*Lepomis macrochirus*) to the common sun fish (*L. gibbosus*).

Some Features of the Lymphocystis Tumours in *Mullus surmuletus*

On *macroscopic* examination alone one might hesitate to identify the tumours found in the red mullet with those observed by previous writers. In their descriptions and illustrations the lymphocystis tumours are represented as

numerous small disseminated swellings which, if united into larger bodies, distinctly show their composite structure. Raspberry-, mulberry- and cauliflower-like outgrowths are the terms used to give an idea of their shape. Weissenberg alone mentions that some of them may have a smooth surface.

In *Mullus* no such disseminated nodules were met with, and the few tumours (five in all) had rather smooth surfaces. Only one of them, situated at the corner of the mouth, was comparatively small, being 3 mm. in diameter; the dimensions of the others ranged from 9 to 20 mm.

Microscopically, the lymphocystis cells in *Mullus* show the same characteristic features already recorded in other species, and they closely resemble those of *Sargus annularis*. Many of the details are so much alike that several of Joseph's photomicrographs might have been made from my preparations of *Mullus*. In view of these similarities, it seems superfluous to describe every detail, as they have been discussed at length by Joseph and by Weissenberg. I therefore intend to concentrate only on those points which have given rise to controversy in the past, or which have apparently not been observed in the species previously investigated.

Early Stages of the Lymphocystis Cells and the Development of the Inclusion Bodies

Although in their later stages all the lymphocystis cells look alike, according to Joseph they may have a double origin: some develop from the 'basiepidermoidale Zellen', cells situated in the epidermis, others from the connective tissue elements, especially from the osteoblasts. He thought that the inclusion network originated in the former cells from a dark-staining body noticeable even in very small (*c.* 8 μ) cells; and in the latter he first observed it in the cells which had reached *c.* 30 μ in diameter, in the shape of a small 'calotte' which quickly grows around the nucleus and becomes transformed into a network, moving later into the cortical layer of the cytoplasm.

On the basis of his own observations, as well as on theoretical grounds, Weissenberg rejects the possibility of lymphocystis cells developing from the epidermis elements. He admits their origin from the connective tissue cells and gives a detailed account of the very earliest modifications of the latter as they begin their transformation into the lymphocystis cells.

The origin of the inclusion bodies in the tumour cells of *Acerina cernua* is described by him as follows: 'At first they contained no inclusion body. But about one week later, an inclusion appeared in their cytoplasm as a tiny point which increased in size to a round compact body resembling very much a Guarnieri body of a corneal epithelium cell of a rabbit after inoculation with vaccine virus. The young lymphocystis cells grew larger and larger and corresponding to their growth the inclusion body increased more and more in size

to an oval disc, then to a calotte, which became fenestrated and sprouted into a network of basophilic staining reaction.'

Later investigations by the same writer on various species confirmed his conclusions about the origin of the lymphocystis cells and their inclusion bodies. In every instance the latter were found appearing first as one, or sometimes several, small granules, which in some species could be seen in the youngest stages of the tumour cells. When, however, Nigrelli & Smith (1939) examined a lymphocystis tumour of the orange filefish (*Ceratacanthus schoepfii*) they found features to which the following interpretation was given: 'Certain early stages ($9 \times 24\,\mu$) show cells in what resembles a binuclear condition. In other cells ($20 \times 50\,\mu$) the nuclear material appears in two forms: (*a*) as a deeply pycnotic primary nucleus of the enlarged cell, and (*b*) a secondary nuclear mass, variable in size, which becomes vacuolated and reticulated. The later mass of basophilic material represents probably the forerunner of the system of inclusion bodies which eventually become distributed in the cytoplasm of the enlarged older cell ... .'

The aspect of these cells has therefore much in common with those lymphocystis cells in *Sargus annularis* which Joseph saw developing at the basis of the epidermis, and which contained, apart from the nucleus, another body of such conspicuous dimensions that Joseph said that he hesitated to decide which of these bodies might be the genuine nucleus. But while Joseph lays stress on the point, on which he and Weissenberg are quite unanimous, that the elements of the future inclusion network do not migrate from the nucleus, Nigrelli & Smith suggest that these inclusions may originate from 'one or two nuclear masses formed in certain early stages of the disease'.

My own observations have been made on material less suitable for investigating the origin of the lymphocystis cells than if it had been obtained by experimental transmission of the disease, permitting the evolution of the infected cells to be followed from the very first manifestation of the infection. Nevertheless, the tumours of the red mullet, although they were obviously of long standing, included such a variety of stages that a whole series of them could be established even in the same preparation.

The earliest stages are rather scarce, and were found as single elements, or in small groups in some sections only. The largest of the observed nests is represented in Pl. I, fig. 3.

In this connexion it may be pointed out that the occurrence of all stages of development would favour the assumption that a frequent infection of the host cells has been taking place. On the other hand, since large parts of the tumours are composed of cells of equal size markedly differing from neighbouring areas (Pl. I, fig. 1), it is obvious that they belong to the various eruptive periods of the disease. Hence it seems probable that in the *Mullus* tumours both processes were occurring, viz. a frequent infection of a few cells, and periodical exacerbations of the disease producing simultaneous growth of a greater

number of the hypertrophic elements. The smallest cells which could be distinguished with certainty as early stages of lymphocystis cells measured 9 μ in diameter (Pl. II, fig. 6*a*). They can be recognized by their rounded shape, the deeper staining of their cytoplasm, and, more certainly, by their large spherical nuclei of vesicular appearance, each containing a big nucleolus. There are even smaller cells which, compared with the fibroblasts, show modifications tending in the same direction (Pl. II, fig. 5*a*) and which may be regarded as earlier stages, but the same degree of certainty as to their character cannot be claimed.

In all preparations observed the young stages of the lymphocystis cells appear to originate from among the connective tissue elements. No evidence has been met to support Joseph's contention that the lymphocystis elements evolve from two different kinds of host cells.

The appearance of the next stages of the growing cells may be seen in Pl. II, figs. 5–11. Some of them, if cut at suitable angles, distinctly show a large area of the cytoplasm staining more lightly (called 'sphere' by Joseph).

The inclusion bodies in the young cells have the same appearance as figured by Weissenberg, i.e. of granules surrounded by a halo. The inclusions in this Guarnieri-body stage are found so often to be 3, 4 or even 5 in number that their multiple occurrence seems to be the rule in the red mullet. If one traces the evolution of these bodies retrogressively to the smallest cells, one may see the granules diminishing in size and their halos becoming very faint. In Pl. II, fig. 5*b*, five such granules are present but they are not clearly in focus in the photograph. In the smallest cells the granules are at the limits of visibility. Although there must, of course, be uncertainty as to the nature of elements of so small a size, there are reasons for believing that, even in the smallest lymphocystis cells of the red mullet, the substance of future inclusion networks is already accumulating in particles of microscopical dimensions.

The multiplicity of the inclusions in the early stages of the lymphocystis cells was observed by Weissenberg to be a constant feature in some fishes (Pleuronectidae and *Lachnolaimus*), and in them each of these granular bodies was developing into a fragment of the network; in other species investigated by the same author (*Acerina* and *Stizostedion*) additional granules appeared occasionally but remained rudimentary.

Whether or not some of the initial granules seen in the cells of *Mullus* are disintegrating is difficult to determine, and the diminution in their number may be explained as being, at least partly, brought about by the fusion of two or more into one larger mass. The pictures seen in Pl. II, figs. 8–11, may be best interpreted as the successive stages of this process. In Pl. II, fig. 11, the larger inclusion seems likely to be composed of three granules about to fuse into one. Similar lobed outlines of the inclusions can be observed only in the cells belonging to the same range of size in which the fusion of these bodies is

occurring; they cannot be regarded as the beginning of the later changes of the inclusions, since these do not set in until the cells grow a good deal bigger.

Cells with a 'secondary nuclear mass', as described by Nigrelli & Smith, have not been found. Only in some cases there could be seen a pycnotic conglomeration of the inclusions producing a body of roughly spherical shape, but these cells were unmistakably in a state of degeneration. Binucleated cells are not uncommon, but their nuclei have an identical and typical appearance, and there is not the slightest evidence that one of them may give rise to the inclusion network. I am therefore in agreement with Joseph and Weissenberg in refuting the theory of the migration of the inclusion substance from the nuclei.

The different organization of the younger stages of lymphocystis cells in the orange filefish as described by Nigrelli & Smith would appear anomalous in view of the resemblance of the later stages of the same cells in various species of fish. It seems, however, that this may be explained as follows. From all we know, the infective agent, after penetrating the host cells, will bring about two processes, viz. the hypertrophy of the cells and the development of the inclusion networks; the latter being most probably, as has been postulated by Weissenberg, the aggregation of the virus substance. In the majority of the species investigated the hypertrophy of the cells sets in before the inclusion bodies become visible; if the reverse should occur, as might be the usual course in the species observed by Nigrelli & Smith, the inclusion body would attain a more advanced stage of development in the comparatively small cell. If this suggestion holds good, it is to be expected that in the orange filefish also, if suitable stages of the disease happen to come under investigation, such Guarnieri-like bodies will be found in the small cells.

Centriols. Owing to the multiple occurrence of the inclusion bodies, their variable size and their situation in different regions of the cells, recognition of the centriols described by Joseph in *Sargus annularis* is very uncertain. Some small granules in the lighter part of the cytoplasm, Joseph's sphere, might well be centriols, but in other cases at the same places the granules are of such size that their centriol nature is highly improbable and they are, more likely, to be reckoned among the additional inclusion bodies.

Further Stages of the Development of the Lymphocystis Cells

Inclusion bodies

The development of the inclusion bodies will be considered first, not only because their changes are the most striking feature in the growing lymphocystis cells, but also because they have an obvious bearing on the organization of the cytoplasm.

The changes in the form of the inclusion bodies, which up to now have retained their characteristic appearance of Guarnieri bodies, become noticeable in the cells of *c.* 50 μ; the oval body becomes flattened and assumes the form of a calotte which starts to expand around the nucleus, undergoing at the same time a transformation into a framework made up of irregular bars. The shape of the body during this process is often far from symmetrical: the calotte may be elongated in one direction, some bars of the framework may extend towards the nucleus, others towards the periphery of the cell, so that in the sections they may be seen scattered through the whole cytoplasm. The independence of some fragments makes it probable that some of them may develop from additional inclusion bodies. With the growth of the cells a more orderly arrangement takes place, and eventually the whole inclusion body represents a hollow sphere, the walls of which are irregularly fenestrated. In the sections it appears as a ring made up of several fragments and situated between the nucleus and the periphery of the cell. As the diameter of this spherical basketwork grows faster than that of the cells, the inclusions soon reach their cortical zone, a position maintained during the subsequent life of the cells.

The development of the inclusions and their definite arrangement in *Mullus* may be defined as being of *Sargus* type, as it is much like that described by Joseph for that fish. It differs greatly from what may be called the *Pleuronectes* type which, according to Weissenberg, consists of numerous inclusion networks densely filling the whole cytoplasm; it differs also, but to a lesser degree, from those of other species investigated by the same author.

The extent of the break-up of the framework in any one cell into separate fragments is difficult to ascertain unless all the serial sections of a given cell are examined. In tangential sections one can see how thin may be the connexions between the bars and, moreover, how different the inclusions of the same cell segment may appear if the sections are made in different planes (Pl. IV, fig. 20).

From the stage at which they reach the periphery of the cells up to the time when the latter grow to their maximal dimensions, the inclusion bodies do not show marked differences in their structure. In the large cells the bars are somewhat more widely spaced, but even then their total mass in any one cell must grow as the cell increases in diameter.

In the cells at the peak of their development the inclusions often appear divided into more fragments and show certain changes: the outlines of the bars become more rounded and their structure shows less fine pattern. These changes may be considered as leading to the degeneration of the inclusion bodies which will be discussed later.

It may be mentioned that the staining reactions of the inclusions confirm Weissenberg's opinion that, besides their main basophilic constituent, some other 'ground substance' of acidophilic properties must be present in them.

Cytoplasm.

In the young lymphocystis cells the lighter portion of the cytoplasm has the regular shape of a sphere slightly invaginated by the nucleus (Pl. II, fig. 7). At the time when it is about to start its rapid development, the inclusion body is found situated at the periphery of this sphere, and often on the opposite side to the nucleus. Later on, when it assumes the form of a calotte, it expands in close contact with the 'sphere'. When the inclusion body is seen growing symmetrically around the nucleus the lighter staining cytoplasm may also encircle the nucleus, thus interposing itself between the inclusion body and the nucleus. The frequent deviations from the regular course of development of the inclusion bodies, mentioned above, show their effects on the 'spheres' also, as the latter may be pierced by the growing bars or divided into fragments.

The interpretation of the succeeding sequence of events is uncertain. For when the inclusion body passes through the transitory stage mentioned above, characterized by its irregular growth, the cytoplasm exhibits darker and lighter patches and, moreover, develops a more distinctly reticulated texture. With further growth the reticulum becomes gradually less distinct, while the areas of differently stained cytoplasm merge together into larger patches giving to the cell a chequered appearance (Pl. II, figs. 12, 13). At this time there is also noticeable a certain change in the chemical reaction of the cytoplasm, for it now stains better with the cytoplasmic dyes, whereas in the former stages it showed basophilic tendencies.

The variegated picture of this transitory stage of the lymphocystis cells may be looked upon as reflecting the major disturbances caused by the disproportionately quick growth of the virus substance; the more regular arrangement of various cell constituents in the later stages gives the impression that some equilibrium is being restored among them. Thus, when the inclusion network reaches the stage at which it assumes the form of a more regular basketwork expanding towards the periphery of the cell, the two kinds of cytoplasm begin to accumulate, one in the centre of the cell and the other nearer to the periphery. This process leads to the formation in cells of *c.* 150 μ and upwards, of a cortical ectoplasmic layer, of more homogeneous structure, and more coarsely granulated endoplasm (Pl. II, figs. 12, 13).

The difference in staining properties of the ectoplasm and endoplasm may not be seen equally well in all cells, but, in many of them, the cortical layer appears sharply delimited, exactly as in Joseph's photomicrographs. Thus, in this differentiation of the cytoplasm also, the lymphocystis cells in *Mullus surmuletus* exhibit a great resemblance to those of *Sargus annularis*.

The different appearance of the ectoplasm in which the inclusion networks are situated must obviously reflect its particular properties; the difference in the behaviour of the two cytoplasmic layers is occasionally made evident in some degenerating cells in which the ectoplasm with its inclusion bodies is partly folded and detached from the endoplasm (Pl. III, fig. 16).

The question arises: if in the youngest cells two kinds of distinctly different cytoplasm are present, could the ectoplasm and endoplasm of the older cells be considered as deriving directly one from the lighter and the other from the darker staining portions? The evidence gained from the examination of the preparations stained with different methods is not unequivocal.

In the sections stained with Azan, or haematoxylin and eosin or xylidin red as counterstain, or even with haematoxylin alone, the 'spheres' in the small cells are always lighter in colour. In the transitory stage the direct observation of their fate is uncertain, but it seems more probable that the lighter stained areas of the cytoplasm derive from these spheres. When the differently stained portions become larger and more distinct the lighter ones are moving towards the cell periphery. Hence it may be concluded that the 'sphere' develops into the ectoplasm of the larger cells (Pl. II, fig. 12).

If, however, the preparations are stained with Giemsa the results are contradictory. In the small cells the spheres stain, as with other dyes, in a markedly lighter hue; in the transitory stage the intensively stained reticular structures of the cell dominate the picture, while other cytoplasmic elements are less recognizable; in the later stages the differently stained patches of the cytoplasm become apparent and even show more details of the distribution of the two kinds of the cytoplasm, but in this case the darker staining parts are moving towards the periphery (Pl. II, fig. 13). This phenomenon casts doubt on the former assumption which, moreover, does not agree with Joseph's statement that the endoplasm derives from the sphere. The problem must therefore remain unsettled.

Nucleus

The nucleus, which is very large in the young cells, grows more slowly than the cell itself, so that its volume, in proportion to that of the cell, becomes smaller with the progressing hypertrophy of the latter. It occupies a position at the periphery of the cell, where it is situated immediately under the cell membrane. In the larger cells the original globular form may undergo some modification, becoming ellipsoidal or invaginated, but these alterations do not distort the spherical outlines of the nucleus to such a degree as described by Joseph, and in this respect the lymphocystis cells in *Mullus* appear to differ to a greater degree from those in *Sargus*.

The constitution of the nucleus has a great similarity in both these species. It is characterized by the abundance of the nuclear sap, a very fine and sparse framework, and a large nucleolus. The latter shows great variability in form; it may include vacuolar spaces, become fenestrated, or develop irregular lobes which if detached by deep constrictions are found as separated fragments. Although young cells with two nuclei may sometimes be observed, this fragmentation of the nuclear mass seems to be chiefly responsible for the occurrence of more than one nucleolus in the larger cells.

Abnormal Features in the Lymphocystis Cells

Binucleated Cells

Cells with two nuclei are not infrequently met with in younger stages. In the group of small cells, shown in Pl. II, fig. 5, two such cells are present. Both nuclei have an identical structure, and there is certainly no question of one of them belonging to the category of elements described by Nigrelli & Smith. Larger cells with two nuclei were observed in a few instances only. This rare occurrence may be partly due, as the cells grow larger, to the decreasing chances of meeting both nuclei cut in one section at a suitable angle. It is also possible that the course of development of some of these cells is unusual. An interesting example is seen in Pl. II, fig. 12 (middle right), in which a binucleated cell contains two inclusion bodies in the calotte stage, occupying diametrically opposed positions with the sphere-cytoplasm between them and the nuclei. Further development in this direction may perhaps account for the Siamese-twin monstrosity represented in Pl. III, fig. 14.

Several Cells in One Membrane

Pl. III, fig. 15, shows seven cells, two of them very small, encircled by one collapsed membrane which obviously belonged to a large degenerated cell. In this section only part of the cells included in this way can be seen, and on looking through the following sections it was found that there were fifteen in all. Such cases cannot be very uncommon, as in a second tumour a group of six cells was found similarly enclosed in a membrane. There can hardly be any doubt about the process of formation of such features, in view of the fact that the large cells in their degenerating state (which will be discussed below) are often invaded by the connective tissue elements. If now among the latter there happen to be some carrying the virus infection they must start their development into the lymphocystis cells within the membrane of the old cell, and it may be stated that, in this as in other instances, the membrane can persist a long time after all other cell elements become completely resorbed.

Degeneration of the Lymphocystis Cells

Of all previous writers, Joseph paid most attention to the processes of degeneration of the lymphocystis cells. His findings may be summarized as follows: (i) the degeneration affects exclusively the large cells which have reached the peak of development; (ii) degeneration may proceed in two ways: either the membrane of the cell becomes ruptured at some point and the elements of the surrounding connective tissue penetrate into the lymphocystis cell, or the degeneration proceeds without this perforation of the membrane; (iii) if the cells in the process of degeneration are situated near to the epidermis the latter becomes stimulated and produces buds which extend into the adjacent tissue, filling up the spaces left by the shrinking lymphocystis cells.

The first of these statements certainly does not apply to the lymphocystis cells in the red mullet, for, as has been mentioned, the process of degeneration may be observed in cells in various stages of development. As to the third point, i.e. the part played by the epidermis, nothing of this kind could be seen: the epithelium tissue is everywhere sharply delimited and does not show any tendency to grow towards the connective tissue. However, the small amount of material which I had for investigation was insufficient to enable me to conclude with certainty that no such stimulation of the epidermis would ever occur in *Mullus* tumours.

The remaining statements by Joseph, concerning the two processes of degeneration, could be fully confirmed and, owing to the abundance of the degenerating cells in the tumours of *Mullus*, various stages of their disintegration could be observed even on the same section. Sure symptoms of this process, which are more noticeable than the above-mentioned changes in the appearance of the inclusion bodies, are the irregularities of the outline of the nucleus and of the cell itself. If the membrane is ruptured it soon becomes invaginated in various ways, assuming bizarre outlines in the sections. At the same time, small cells from the surrounding connective tissue invade the opening and contribute to the disintegration of the cell contents. When the membrane remains unperforated it may also exhibit certain deformations while the cell undergoes a gradual resorption, but degenerating cells may often be seen in which the dwindling cytoplasm becomes detached from the membrane and the latter preserves for a long time its spherical outlines. In Pl. III, fig. 17, which shows many cells in different stages of degeneration in which the membranes are selectively stained, there may be seen: (*a*) empty membranes; (*b*) membranes with numerous small infiltrating cells; and (*c*) some in which the shrinking cytoplasm has become retracted from the membrane.

In the larger cells both kinds of degeneration, viz. with and without rupture of the membrane, occur. In those of medium size and in smaller ones the membrane shows more resistance, and the resorption of the cell contents goes on without its perforation.

The course of the degeneration process, the changes in the appearance of the cytoplasm, and the disintegration of the nucleus and of the inclusion bodies, can easily be followed in their various stages, and it can be said that Joseph's description of *Sargus annularis* would apply equally well for *Mullus surmuletus*. It should be added, however, that in the latter not all degenerating cells follow the same course, for there is yet another kind of change which some of the lymphocystis cells may undergo; this is characterized by a peculiar activity of the inclusion bodies, in which there is a series of transformations now to be described.

Transformations of the Inclusion Bodies in the Degenerating Cells

As has been mentioned above, the inclusions of the largest cells may show some modifications in their structure, which have been interpreted as being of a regressive nature, indicating the beginning of the disintegration of these bodies. This assumption seems to be justified, since many intermediate stages have been observed between the typical appearance of the inclusions in medium-sized 'healthy' cells (Pl. IV, fig. 20) and in the degenerating cells (Pl. IV, fig. 21). The process by which these changes are brought about may be compared to a slow melting. At first the outlines of the networks become slightly modified; later on the progressive dissolution of finer linking bars leads to the breaking off of fragments of different sizes, whose finer structure gradually becomes indistinguishable. Finally, the network is reduced to a number of irregularly shaped globular fragments, dwindling in size and number until they disappear altogether.

In some of the degenerating cells of the medium-sized group in which the membrane does not become perforated quite a different process occurs. Not only does the inclusion network not show any signs of this dissolution process, but it starts to grow, developing at the same time a more finely detailed structure. The first recognizable stage may be seen in Pl. IV, fig. 22. In the cell on the right the bars of the inclusions, compared with those of the cell on the left, are beginning to grow a little thicker, and some changes in their fine structure become noticeable. Further transformations in the same direction lead to the appearance shown in Pl. IV, fig. 23, which is markedly different from that in Pl. IV, fig. 20. Even if such 'normal' inclusion networks may sometimes exhibit a more delicate structure than seen in this figure (fig. 20), they always show the same characteristic pattern of alveolar spaces within the bars; whereas in the stage of transformations shown in Pl. IV, fig. 23, the network resembles rather a tridimensional lattice the spaces of which are more or less losing their rounded outlines. Moreover, this lattice does not grow symmetrically; parts of it may become elongated in one direction to form strands of various shapes, sometimes even arranged in lamellae (Pl. IV, fig. 24). If such strands are cut transversely the cross-sections of finer threads might be taken at first sight for separate small bodies (Pl. IV, fig. 25).

Such pictures of networks whose lattice-like structure may be fairly distinguishable on the photomicrographs are comparatively rare; more often the dense and deeply stained networks give a blurred image (Pl. IV, fig. 26).

Whether in the course of this process more inclusion material is produced, or whether that already present at the beginning of the transformations merely becomes rearranged in such a way that its dimensions are increased, is difficult to determine. Anyhow, the inclusions gradually occupy larger areas in the cells, the spreading thread-like bars of the lattice penetrating the cytoplasm

so that in the sections the meshes of the inclusions are seen to enclose the cytoplasmic substance.

At a certain stage the network apparently acquires some new property and begins to exert a lytic action on the cytoplasm. At first small spaces appear around the threads of the network, but soon the cytoplasm enclosed in its meshes breaks up into lumps, which disintegrate in their turn into smaller particles. In consequence of this process larger spaces are formed, filled with the framework of the inclusion bodies and particles of the cytoplasm (Pl. IV, figs. 27–29). As these spaces reproduce the pattern of the growing inclusions they represent a system of irregular canals, some of them ending blindly, others joining to form larger lacunae which assume various shapes in the sections (Pl. IV, figs. 30, 31).

Up to a certain stage of these transformations the inclusion network in the lacunae stains very well. Then, however, a change occurs in its elements, causing them to appear fewer in number, until, finally, they become invisible. Whether the networks are dissolved into submicroscopic particles, or whether their chemical reaction becomes changed, could not be ascertained, since the lacunae are filled with a debris of disintegrated cytoplasm. Without differential staining discrimination of the fine fragments of the inclusions, were they really present, is therefore hardly possible. Whatever may be the true nature of this change it must come about in a comparatively short time, since few cells in transitional stages occur; and there may also be seen, not uncommonly in the same cell, some of the lacunae containing a distinctly stained basophilic network, and others in which it is already disappearing. In Pl. IV, figs. 28–31, are shown cells with inclusions in various stages of this process. The differences in the staining reaction cannot be well rendered in the photographic reproduction, and only parts of the network actually present in the preparations can be focused, but in the original sections stained with contrasting nuclear and plasmatic stains the following contents of the lacunae could be well distinguished. In Pl. IV, fig. 28, there shows a very delicate basophilic network, which later, as seen in Pl. IV, fig. 29, becomes markedly reduced (the large lumps are fragments of the cytoplasm); in Pl. IV, fig. 30, at *a*, are intensely stained basophil elements; at *b* fine basophil threads similar to those in Pl. IV, fig. 29, and at *c*, no basophil elements at all. In Pl. IV, fig. 31, only a very few particles show affinity to chromatin dyes, and practically the whole contents of the lacunae stained the same colour as the surrounding cytoplasm.

As regards the behaviour of other elements of the cells in which all these transformations are taking place it should be noted that often the differences in the appearance of the two protoplasmatic layers, i.e. the ectoplasm and the endoplasm, become more accentuated at the time of formation of the lacunae and, moreover, the territory of the ectoplasm in which the lacunae are situated becomes enlarged at the expense of the receding endoplasm (Pl. IV, fig. 31). It seems, therefore, that the substance which becomes destroyed by the

inclusion bodies must be of ectoplasmic nature, but this cannot be an invariable rule, for at some places the lytic process is seen to encroach on the endoplasm also.

It may be mentioned that the ectoplasm of the cells in which the transformations take place stains more deeply than that of other lymphocystis cells. Only after the stage has been reached at which the basophil substance disappears, and even then presumably not until some further time has elapsed, do the cells lose this property and take on a distinctly lighter tone.

In some of the cells a remarkable increase of the nucleolar substance could be noticed (Pl. IV, figs. 27, 30). As, however, the nucleoli in the lymphocystis cells of *Mullus* may be of different sizes and shapes, it cannot be said for certain whether such increase in volume is a constant feature of the cells having inclusions in process of transformation.

As seen in Pl. IV, fig. 31, very large areas of the cells may be occupied by the widening lacunae, but no cells were found whose whole territory was invaded in this way. It appears, therefore, that the destructive action of the inclusion network comes to an end with the vanishing of the basophilic reaction.

In further stages the number of particles enclosed in the lacunae is diminishing, and finally many of the lacunae appear as empty or nearly empty spaces. At the same time progressive degeneration leads to distortion of the outlines of the cells and the resorption of their contents.

It is tempting to suggest that all these modifications of the inclusions have a particular significance in the life cycle of the virus of the lymphocystis disease, and that they may presumably be regarded as preparatory stages in the transformation of the virus substance into a state in which it is ready to infect new cells of the same host or of other specimens. The rearrangements of the inclusions into a more delicate network may be regarded as transition stages of the dissolution of the compact aggregations of the virus into particles of submicroscopic dimensions. It is interesting to note that transformations of the virus substance are evidently not confined to simple disintegration since, as we have seen, the relations of the virus to the host cells enter a new phase manifested by the lytic action on the cytoplasm of these cells.

Whether the disappearance of the basophilic reaction should be ascribed only to the disintegration of the virus agglomerations into submicroscopic particles, or be due to some chemical changes, is a question to which no definite answer can as yet be given.

The fact that the majority of the lymphocystis cells degenerate without showing any activity of their inclusions leads to the assumption that the transformations may find the right conditions to start in comparatively few of the cells. Indeed, in the tumours investigated only one group of such elements has been found. It appears that even when these transformations are in progress they are liable to be interrupted at any stage. Then the inclusion bodies, instead of increasing still further in size and developing a more delicate

structure, show reverse changes turning into uniformly stained bodies of rounded outlines. Their origin from those inclusions which have started their transformations may be recognized by their dimensions, and the differences in these dimensions indicate the moment at which the process of the transformations was stopped and switched into a degenerative one (Pl. III, figs. 18, 19).

If we take the above interpretation of the behaviour of the inclusion bodies for granted, we have to assume that the completion of the life cycle of the infective agent can only be achieved in a few of the growing lymphocystis cells and apparently only in some of those which have not reached their maximal dimensions; in all others it would meet its doom. The degeneration which overcomes the virus substance during its transformations, as well as all similar processes observed in the lymphocystis tumours, are evidently manifestations of the struggle of the host organism with the infection. A similar struggle must certainly occur in other diseases in which the infective agent is localized in certain cells, and the organism attacked is capable of mobilizing its defensive measures. A remarkable feature of the lymphocystis disease is that, owing to the unusual dimensions of the affected cells and the staining properties of the virus agglomerations, it affords illustrations of the various stages of this struggle.

The importance of certain transformations as essential links in the life cycle of the virus can be related to the periodicity of eruption of new tumours, which could be explained by assuming that the transformations take place at certain times and in relatively few cells only. This would tally with the occurrence of the earliest stages of the infected cells and of the transformations in the same specimen of *Mullus surmuletus*. It would agree also with the view expressed by Weissenberg (1945) that some changes in the lymphocystis virus agglomerations might possibly take place before it is ready to produce new infections.

On the other hand, although the evidence favours the above suggestion, it may be open to dispute on the grounds that similar transformations of the inclusions were not observed by those investigators who made detailed researches with much more abundant material. In the paper of Joseph illustrated by as many as ninety photomicrographs, only one (fig. 43) shows an inclusion body with a swollen appearance somewhat similar to one of the stages I have described. No reference is, however, made to this figure in the text. Further, if such transformations had not been noticed in the lymphocystis cells of *Acerina cernua* or *Stizostedion vitreum* and, nevertheless, Weissenberg's infection experiments with the same material were successful, this would tend to discount their alleged importance. Unless, therefore, one assumes that the transformation of the inclusions did not, owing to their rare occurrence, chance to turn up in the preparations from other fishes, some doubts may legitimately arise as to the interpretation of the phenomena observed in the red mullet. It is desirable, therefore, that particular attention should be given to this point in the future.

I wish to record my gratitude to Mr G. A. Steven for the material which has been worked out in the present paper. Thanks to his observation of the unusual outgrowths, and the suggestion that they would be worth examination, the number of species known to be affected by the lymphocystis disease has been increased by the addition of *Mullus surmuletus*.

I am also greatly indebted to Mr F. S. Russell, F.R.S., for his kind help in preparing the manuscript.

Summary

A case of lymphocystis disease is described in the red mullet (*Mullus surmuletus*) from Plymouth waters. The tumours produced by this disease showed typical lymphocystis cells in all stages of development up to 430 μ in diameter. Apart from minor differences the organization of the cells closely resembled that described by Joseph in *Sargus annularis*; this was especially so as regards the so-called inclusion body, a basophil staining network situated in the cytoplasm and probably representing the agglomeration of the virus agent of the disease.

The development of the lymphocystis cells which originate from the fibroblasts was observed in its earliest stages. The inclusion bodies appear in the young cells as several (up to five) granules which most probably soon merge into larger bodies. They have the characteristic appearance greatly resembling the Guarnieri bodies, as do those discovered by Weissenberg in lymphocystis cells of *Acerina cernua* and other species. Their further development follows the same lines as observed by other writers. There is no evidence in favour of the idea that the inclusion bodies may be derived from the nuclear matter of the cell.

Various forms of the degeneration of the lymphocystis cells have been observed. Special attention has been given to the activity of the inclusion bodies in certain types of degenerating cells. This is characterized by the swelling of these bodies and the rebuilding of their substance into a network exhibiting much more delicate structure and exerting a lytic action on the cytoplasm of the host cell. As these transformations progress the network becomes less distinguishable in the lacunae formed in the cells by the disintegration of the cytoplasm, until finally its elements, previously staining distinctly with chromatin dyes, cannot be discerned any more.

It is suggested that these transformations may represent those stages in the life cycle of the virus agent during which it acquires the ability to transmit the infection to other cells of the same host or other specimens.

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EXPLANATION OF PLATES

All photomicrographs have been made from preparations of the lymphocystis tumours of *Mullus surmuletus*. The material was fixed in Bouin's fluid and embedded in paraffin. The preparations represented in figs. 1–12 and 17–31 have been stained with Weigert's iron haematoxylin and various counterstains, those in figs. 13–16 with Giemsa's stain.

PLATE I

Fig. 1. Part of a lymphocystis tumour with cells in various stages of development and degeneration. The large upper portion composed of cells of nearly equal size has presumably originated at a later eruption of the disease than the remaining portion of the tumour. The degenerating elements are seen also in the middle of this younger portion.

Fig. 2. Part of the lymphocystis tumour covered by cutaneous epithelium.

Fig. 3. Group of the youngest lymphocystis cells among others advanced in their development. Note in the latter the aspect of the inclusion network cut at various angles.

Fig. 4. Group of lymphocystis cells older than those in the preceding figure with various stages of development of the inclusion bodies. Other cells greatly hypertrophied. Note the shape of the nucleolus in the large cell.

PLATE II

Figs. 5–11. Early stages of the lymphocystis cells. All photographs have the same magnification. Fig. 5: *a*, connective tissue cell showing changes most probably representing the first stages of the evolution of the lymphocystis cells; *b*, cell with five basophil granules (not well distinguishable on the photograph); *c*, cell with a 'sphere'; *d*, binucleated cells; *e*, cells with inclusions in the Guarnieri-body stage. Fig. 6: *a*, small cell with distinct characters of lymphocystis element; *bl*, blood vessel with an erythrocyte. Fig. 7: cells with distinct spheres and inclusion bodies. Fig. 8: cells with inclusion bodies of various sizes; the largest with two bodies which are going to fuse. Fig. 9: further stage of the merging of two inclusion bodies. Fig. 10: cell with three inclusions, one of them as well as the big inclusion in the second cell seemingly representing the final stage of the fusion of two granules. Fig. 11: cell with two inclusions; the larger has a lobed appearance probably caused by the outlines of three granules fusing into one.

Fig. 12. Several cells with lighter and darker portions of the cytoplasm irregularly distributed and becoming arranged, in the larger cells, as ectoplasm and endoplasm. Note on the right side of the figure a small binucleated cell with two 'spheres' and symmetrically developing inclusion bodies. Haematoxylin-eosin staining.

Fig. 13. Cells in the same stages as in the preceding figure but stained with Giemsa's. The darker staining cytoplasm assembles on the periphery of the cells. Note the distinctly delimited ectoplasm in the large cells.

PLATE III

Fig. 14. Two cells united by a cytoplasmic bridge.

Fig. 15. Several cells developing within the membrane of a degenerating cell. Other cells in this and the preceding figure are in various stages of the degeneration.

Fig. 16. Part of the lymphocystis tumour in which nearly all cells have degenerated. *a*, empty membranes; *b*, membrane with invading connective tissue elements; *c*, membrane with shrinking cell contents.

Fig. 17. Degenerating cell in which the ectoplasm containing the inclusion bodies is partly folded and detached from the endoplasm.

Figs. 18, 19. Inclusions of different appearances depending on the stage of their development interrupted by the degeneration. Fig. 18: *a*, degenerating inclusions which have not started their transformations; *b*, those which were in the early and (fig. 19) in the later stage of transformation.

PLATE IV

All photomicrographs have been made with the same magnification (oil-immersion $\frac{1}{7}$ in.).

Fig. 20. Usual 'normal' appearance of the inclusion bodies on a tangential section of a lymphocystis cell of medium size.

Fig. 21. Disintegration of the inclusion bodies in cells degenerating in the manner observed in the majority of the lymphocystic cells.

Fig. 22. First signs of transformations of the inclusion bodies seen in the cell on the right. Note the swelling of the bars and their more delicate structure as compared with the cell on the left.

Fig. 23. Rearrangement of the inclusion networks in a lattice made up of delicate threads.

Fig. 24. Arrangement of the transformed network in strands and lamellae.

Fig. 25. Similar stage as in preceding figure with several strands cut transversely.

Fig. 26. Part of a lymphocystis cell with inclusions in a transformation stage slightly older than in fig. 22, showing increasing area occupied by these bodies. In order to make the inclusions more prominent a red filter was used and therefore in figs. 20–26 the cytoplasm in which the networks are developing is rendered nearly invisible.

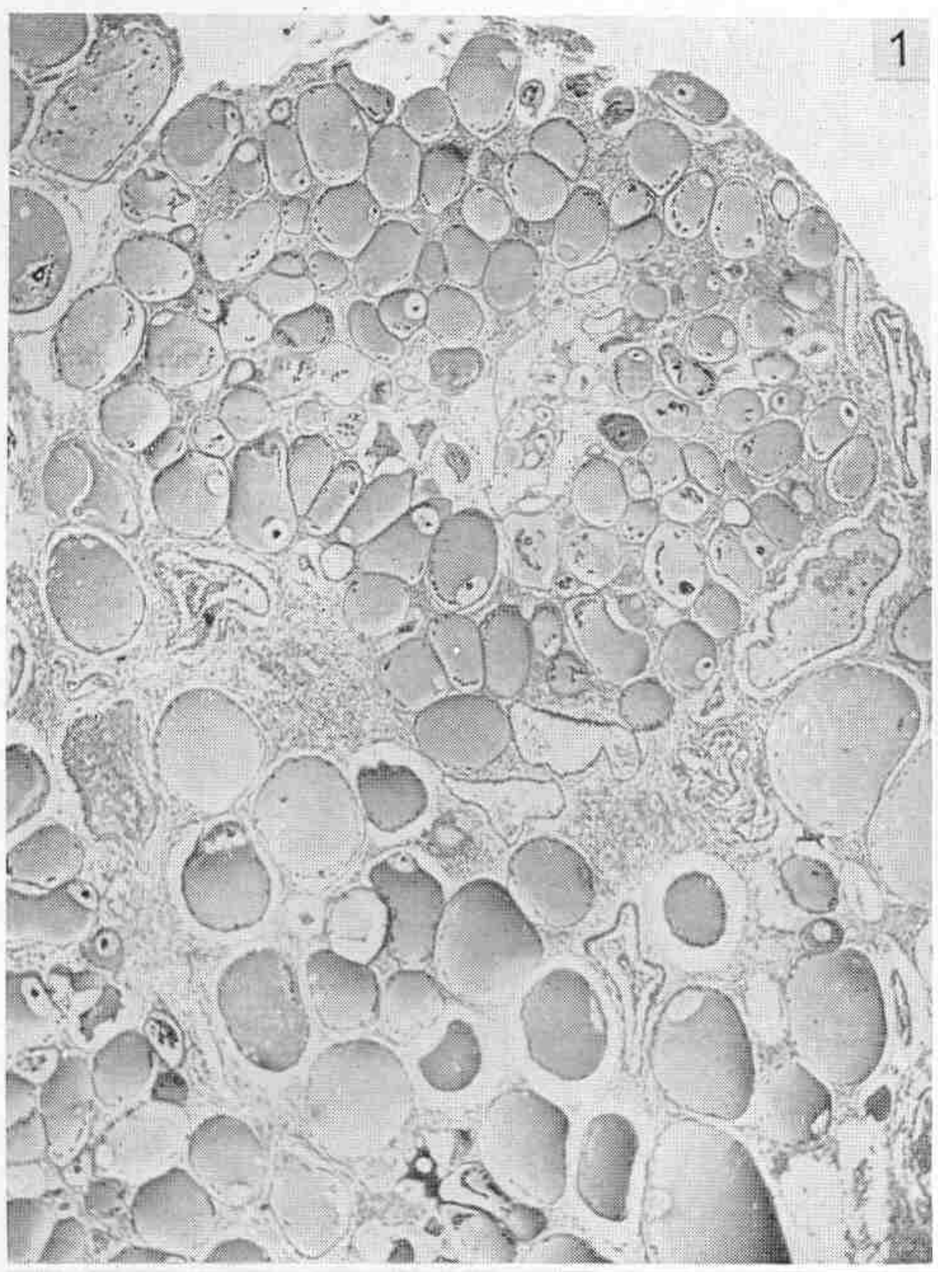
Fig. 27. Part of a lymphocystis cell with the first stages of the dissolution of the cytoplasm. *a*, cross-sections of thicker and very thin threads of the network with narrow spaces around them indicating the beginning of the dissolution seen in more advanced stages round the larger parts of the inclusions; *b*, inclusion network flattened in lamellae which, being forshortened in the picture, do not show their finer structure; *n*, degenerating nucleus with large masses of nucleolar origin.

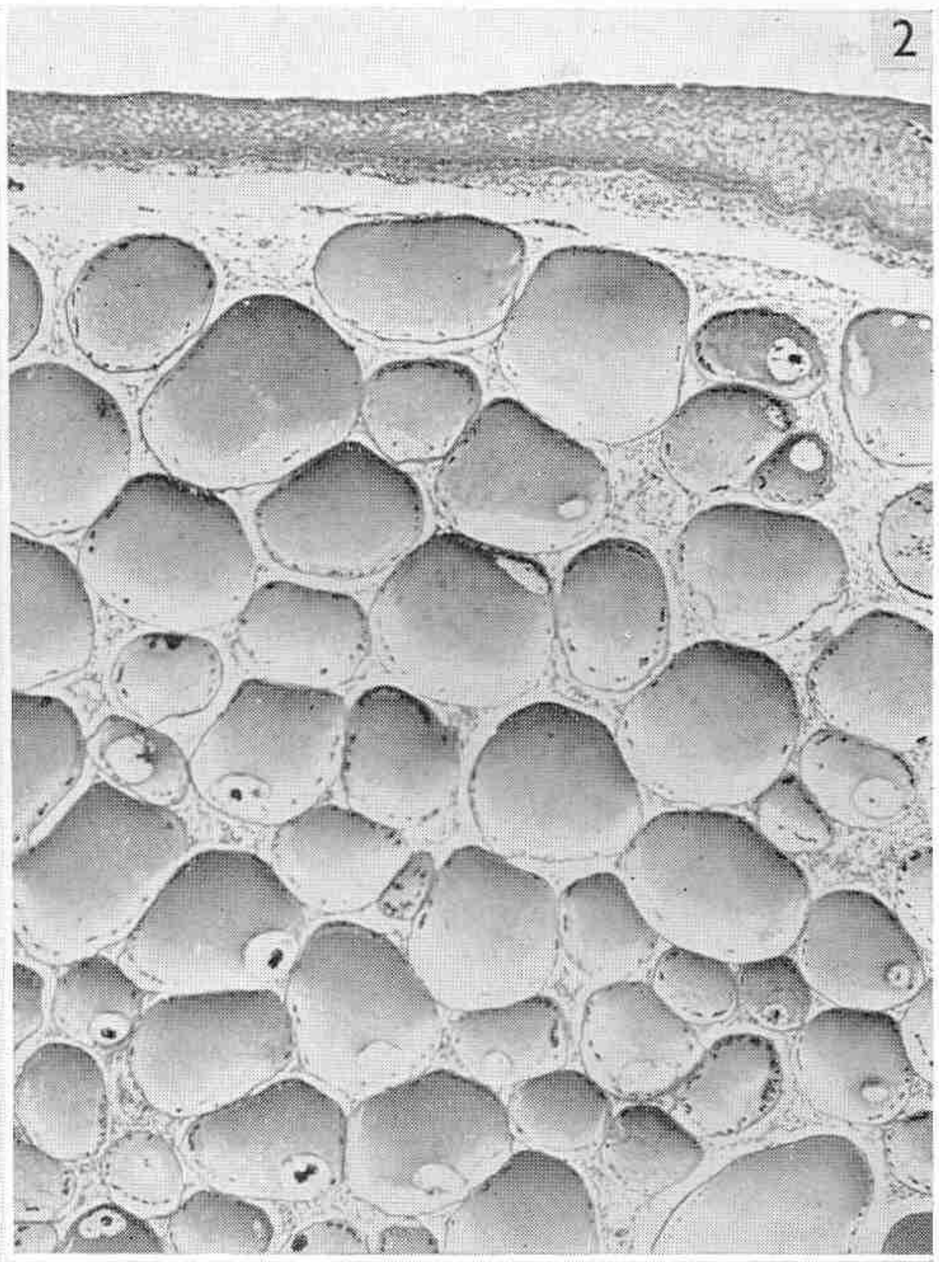
Fig. 28. Lacunae with fine threads of the inclusion network.

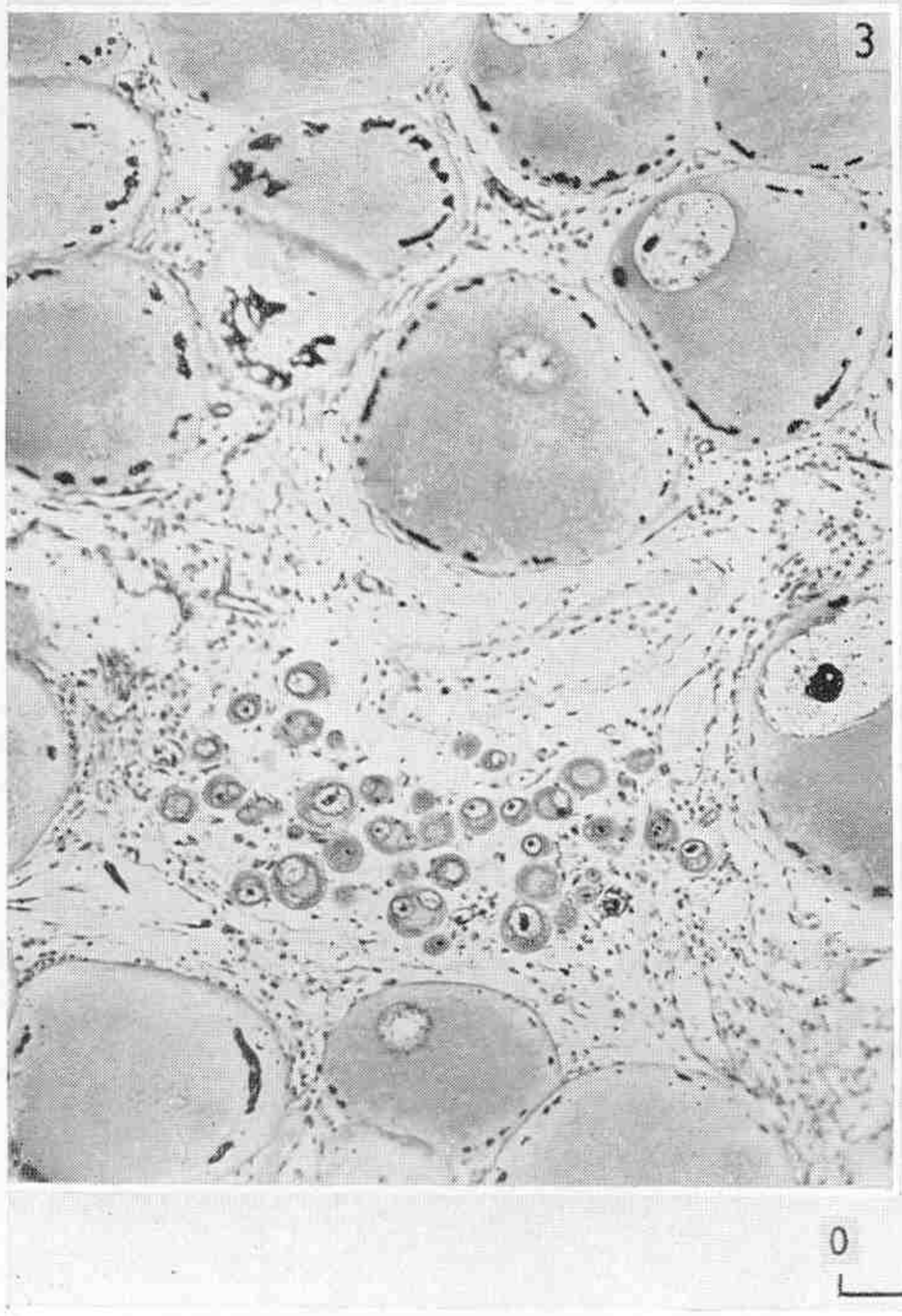
Fig. 29. Lacunae with lumps of disintegrated cytoplasm and threads of inclusion network in the stage when they are becoming less visible.

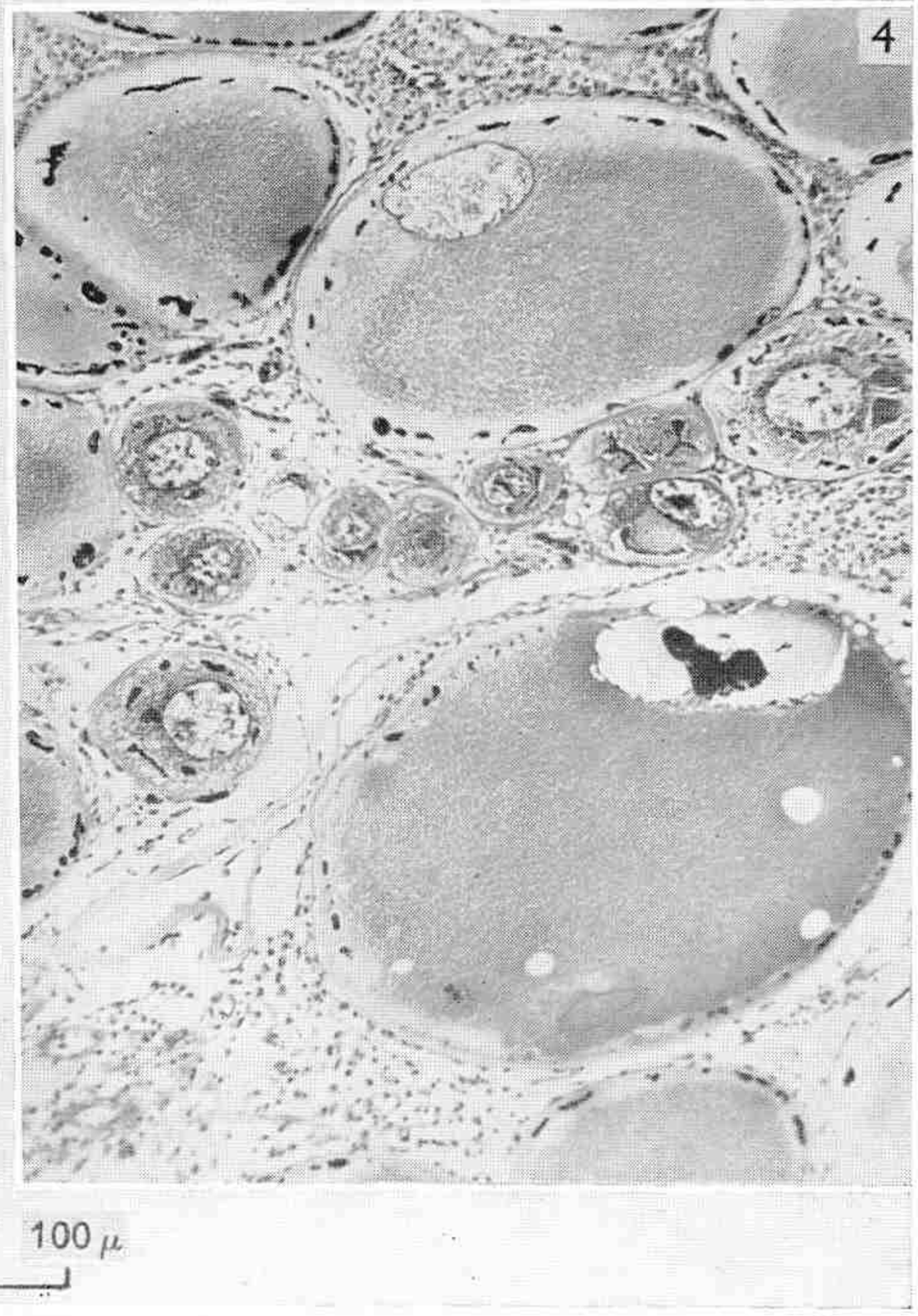
Fig. 30. Parts of three lymphocystis cells with lacunae containing in *a*, deep stained basophil elements; in *b*, lumps of cytoplasm and hardly visible basophil threads; in *c*, particles of various sizes not giving any basophil reaction. Note the shapes of the lacunae cut at various angles and the large nucleolar mass in one of the cells.

Fig. 31. Lymphocystis cell in an advanced stage of the formation of the lacunae filled up with particles not showing basophilic staining reaction. Some of the lacunae are empty. Note the enlarged territory of the ectoplasmic layer.

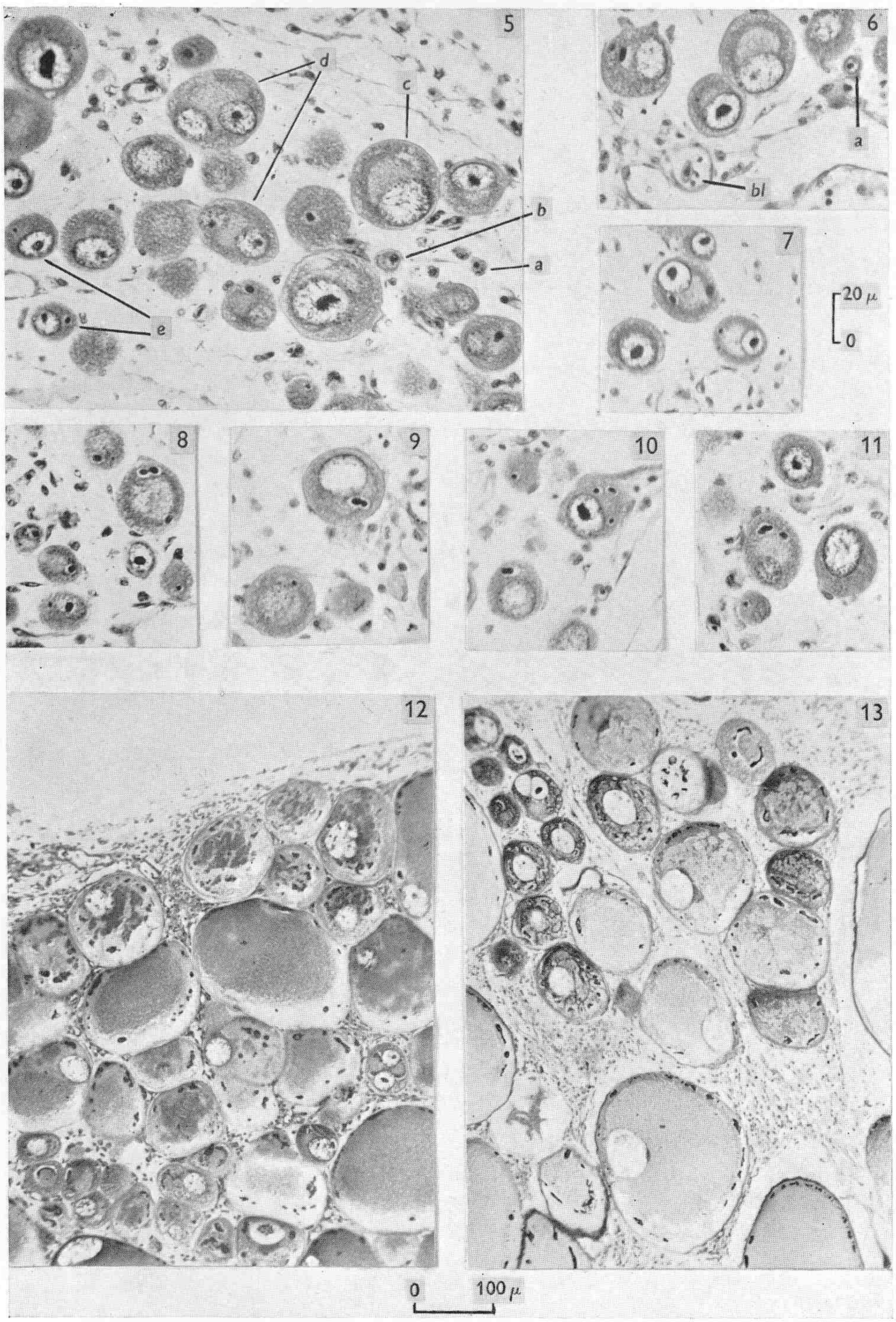




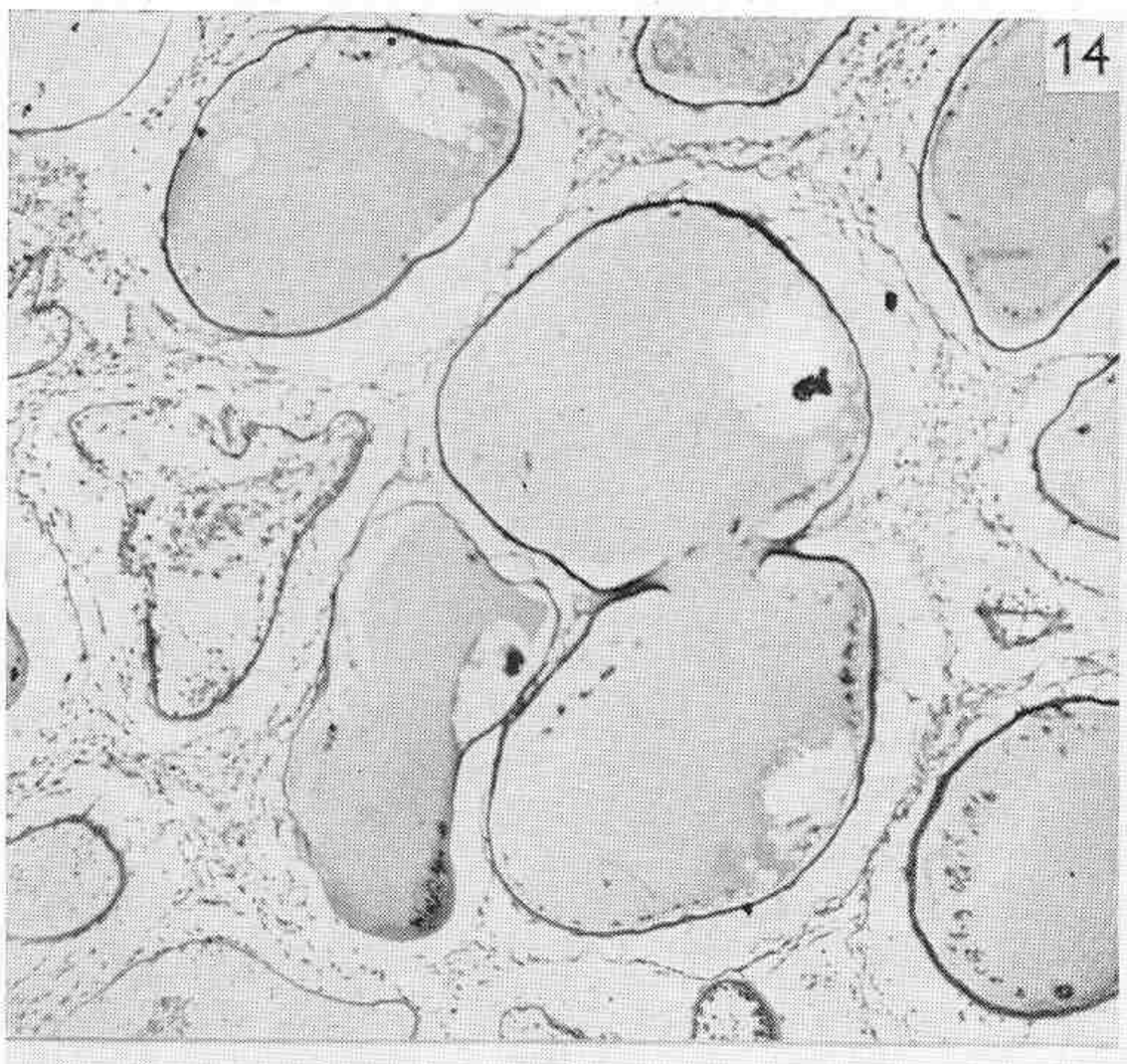


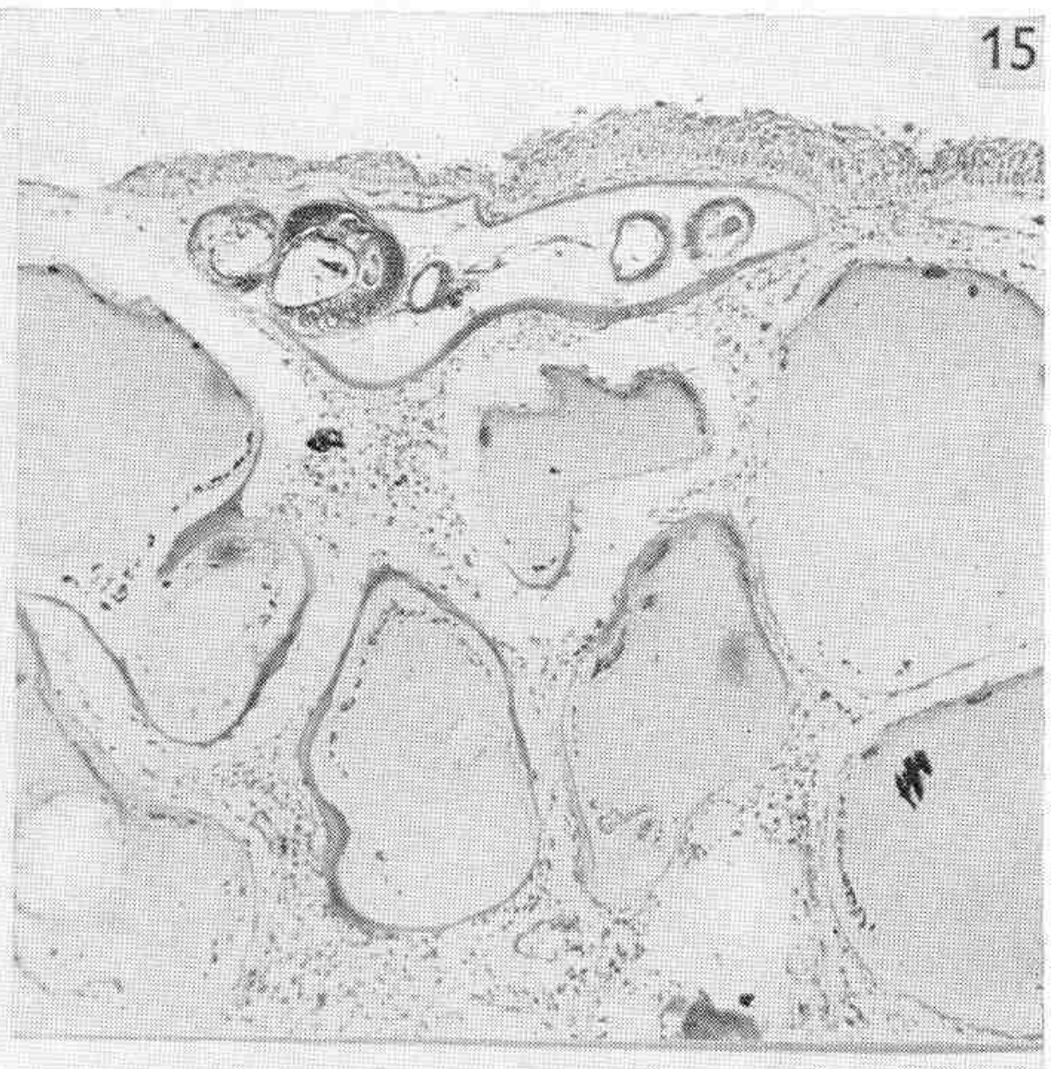


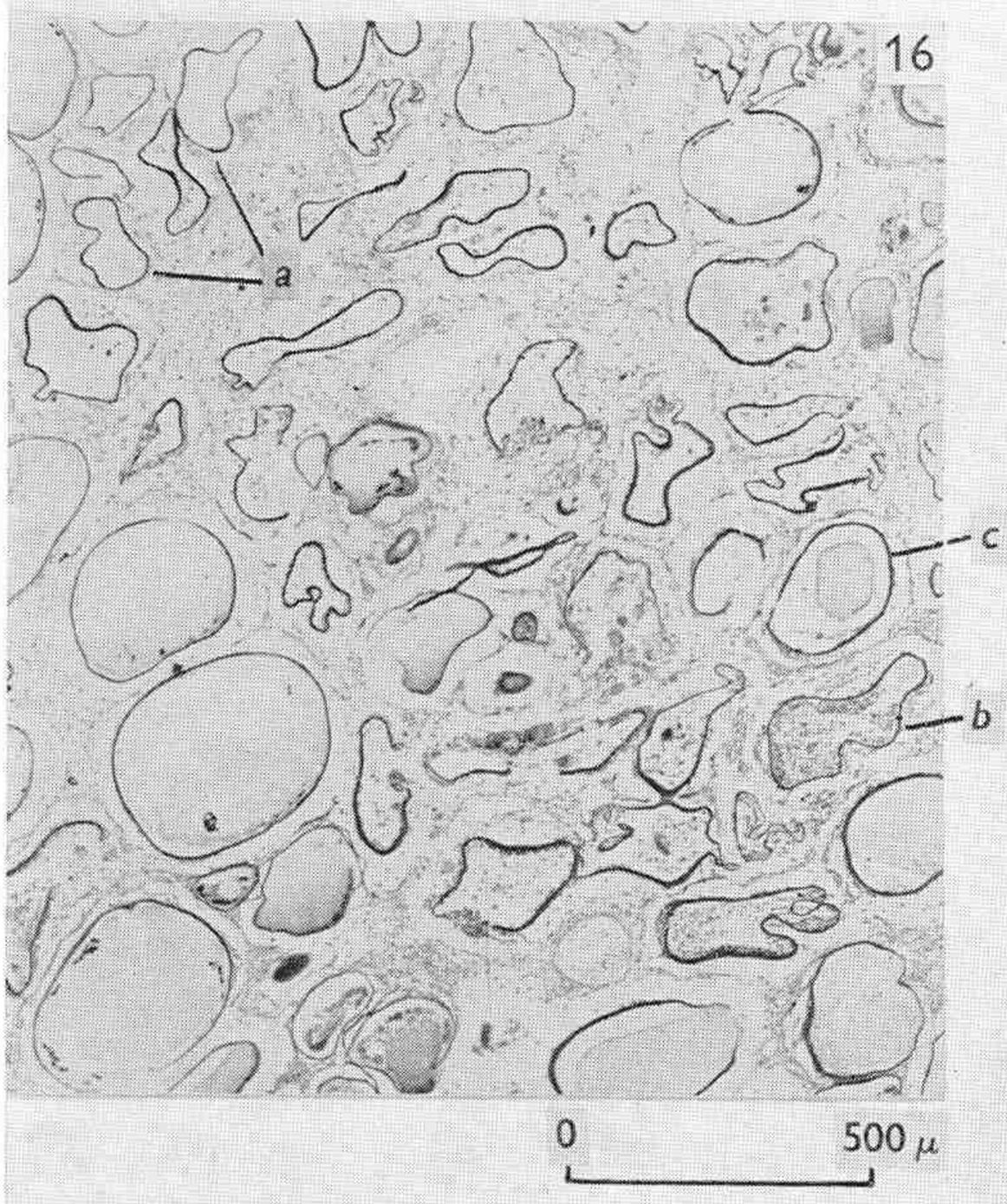
Figs. 1–4.

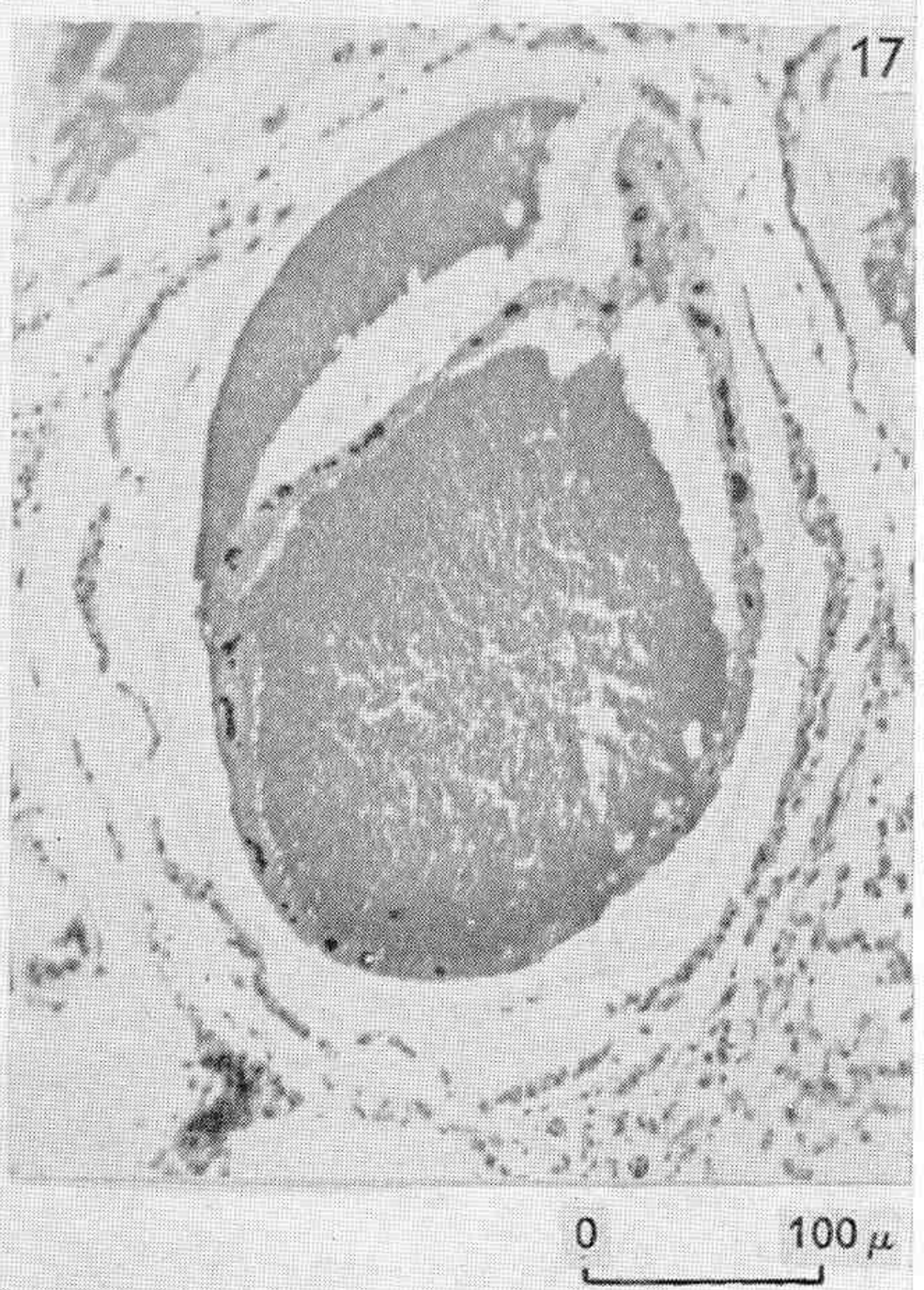


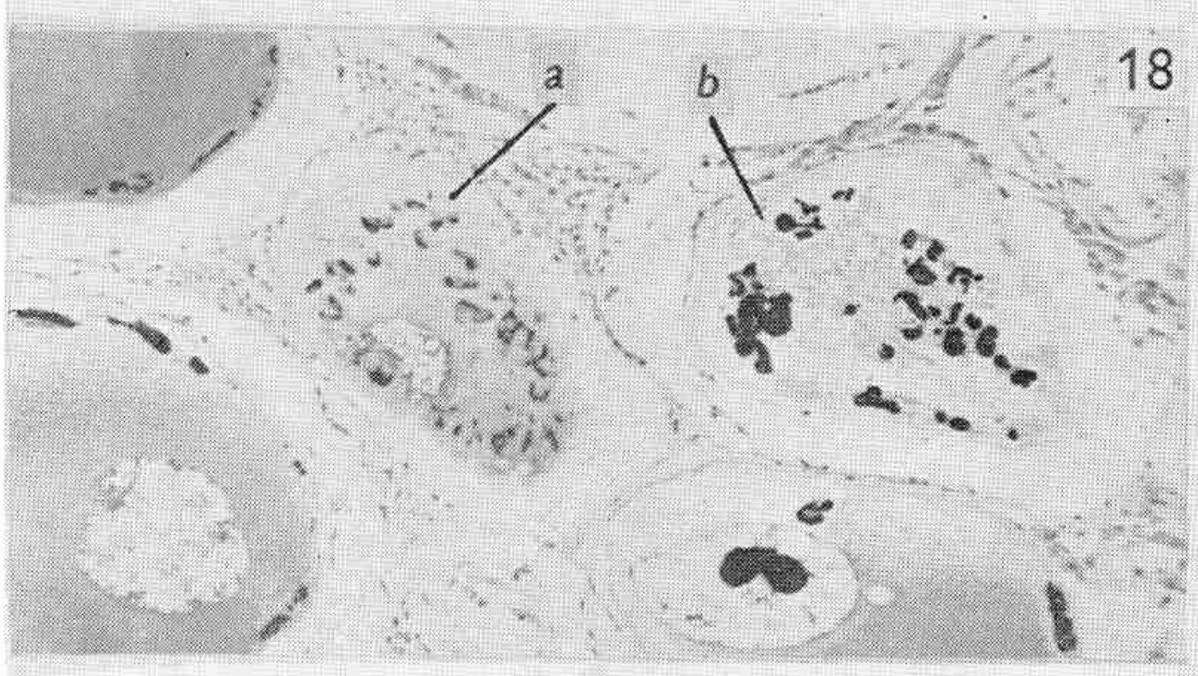
Figs. 5–13.

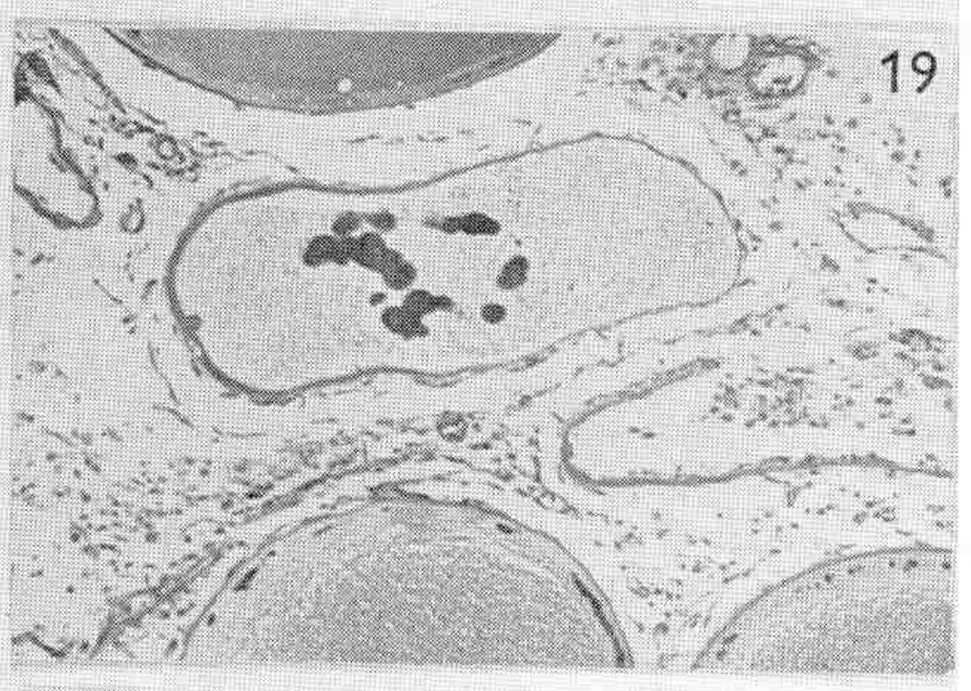












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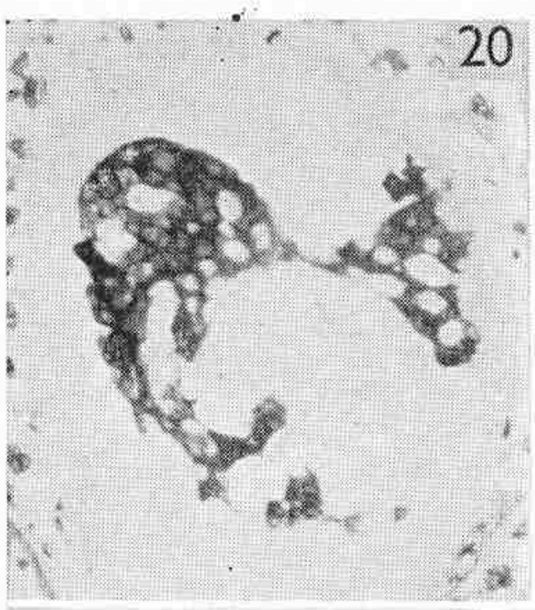
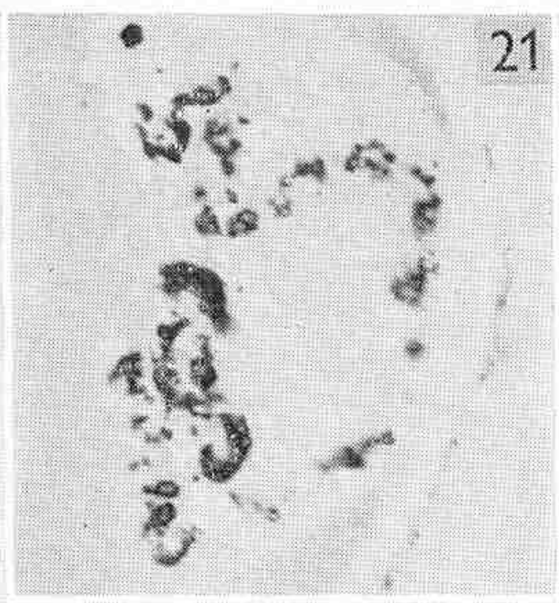
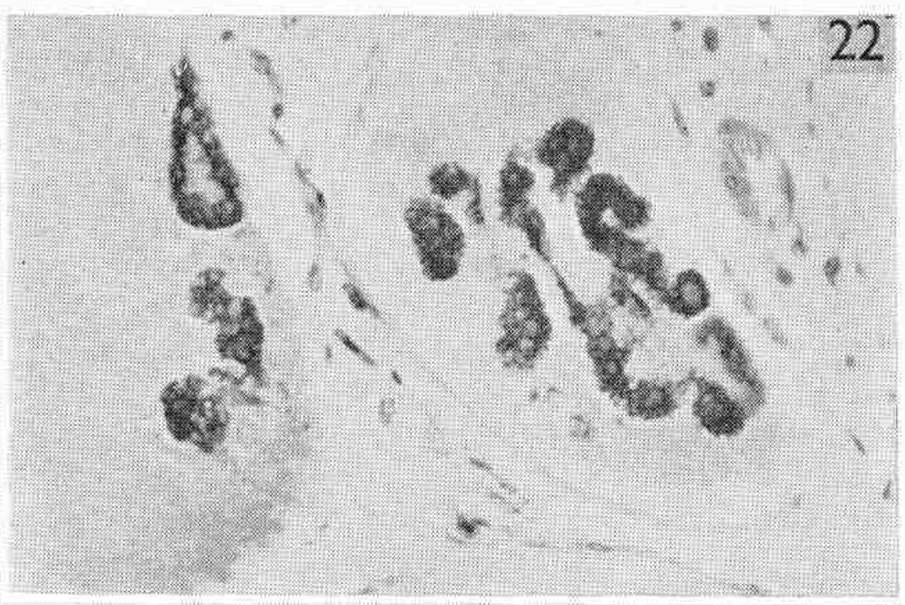
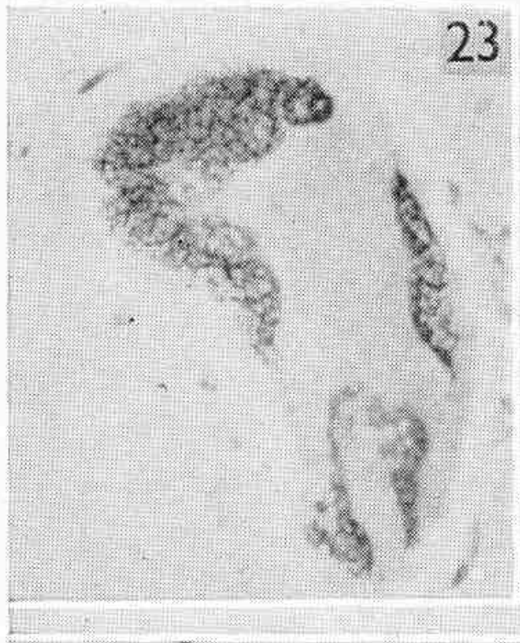
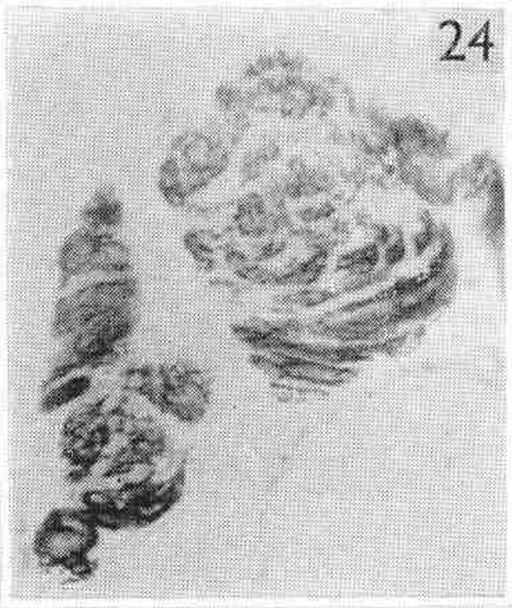
Figs. 14–19.

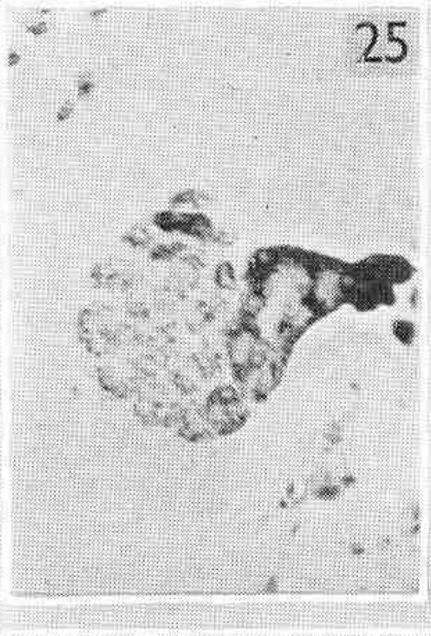


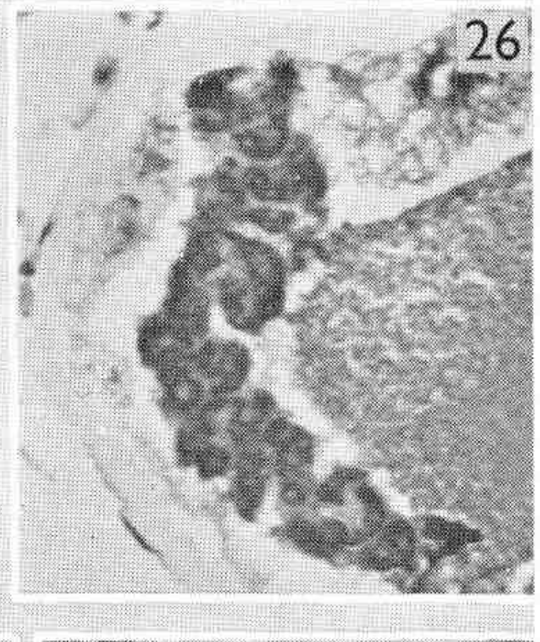


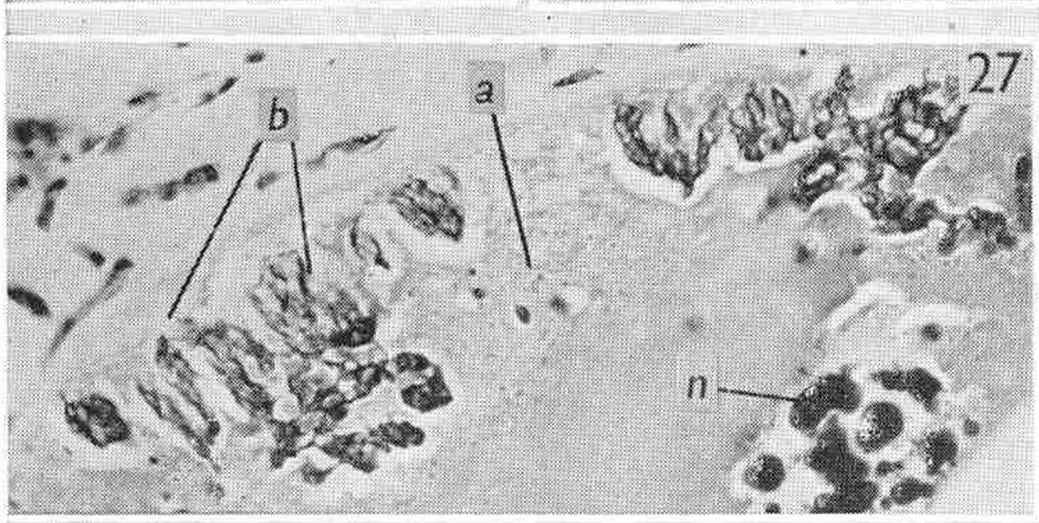


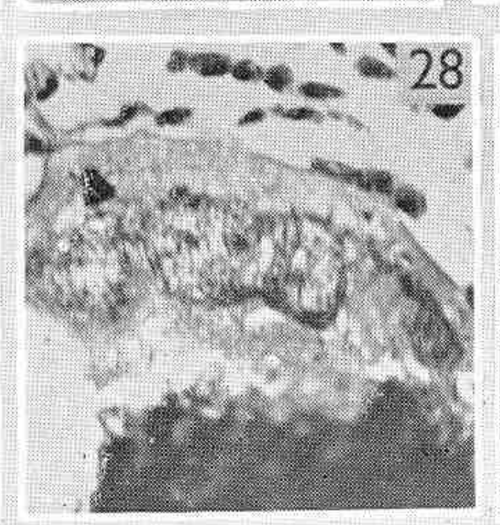


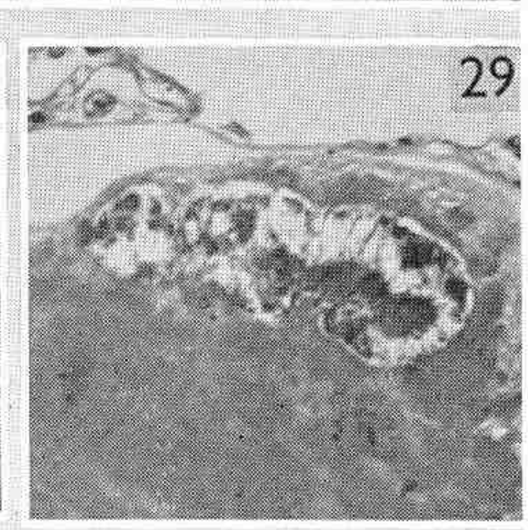


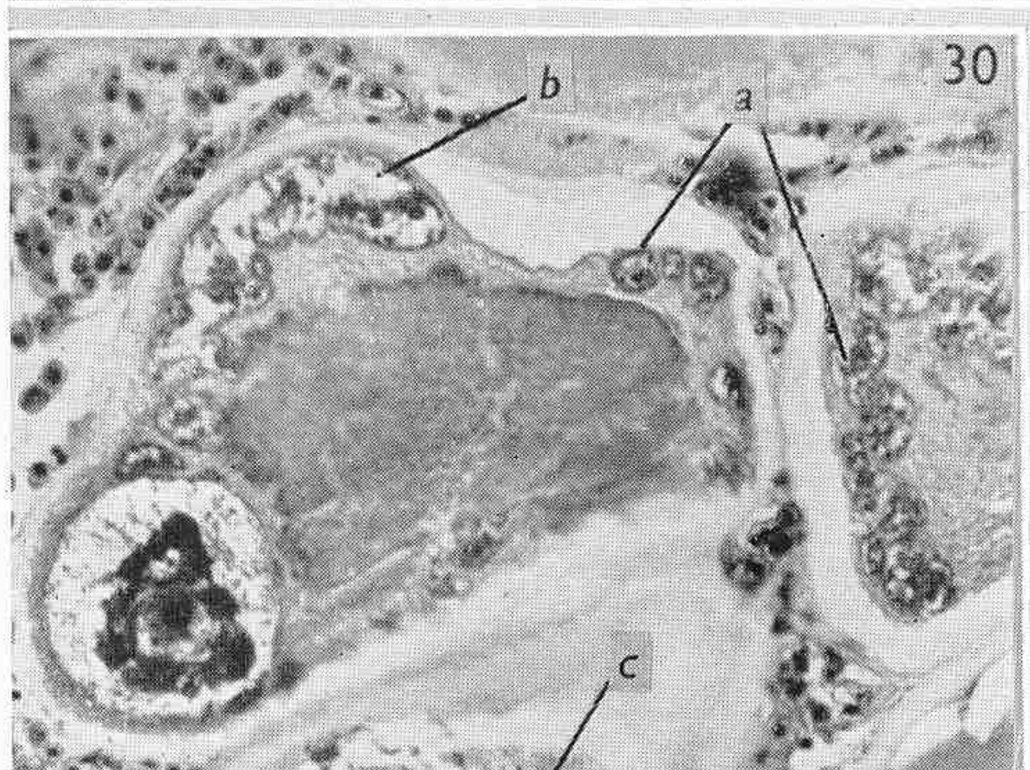


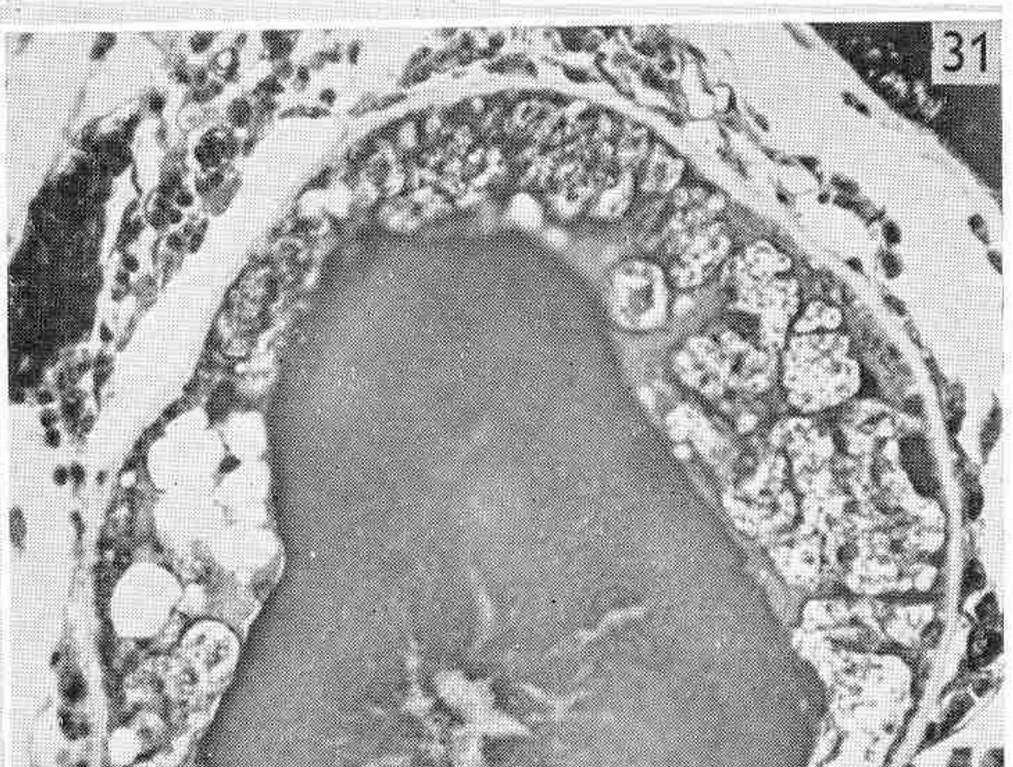












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Figs. 20–31.