EXPLANTATION EXPERIMENTS ON THE INFLUENCE OF THE CONNECTIVE TISSUE CAPSULE ON THE DEVELOPMENT OF THE EPITHELIAL PART OF THE SUBMANDIBULAR GLAND OF *MUS MUSCULUS*

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INTRODUCTION

The epithelial bud, which in the mammalian embryo represents the first rudiment of the submandibular gland, is surrounded by a zone of thickened connective tissue which is quite distinct from the neighbouring tissue; this zone is termed the 'capsule', although it forms not only the definitive capsule but also the interlobular and intralobular septa and all the connective tissue around the acini and ducts.

Some hint of the existence of a connective tissue thickening around the epithelial anlage is found in certain papers dealing with the development of the gland (Chievitz, 1885; Göppert, 1906), but Kallius (1910) seems to be the first to state explicitly that the thickening is clearly delimited from the surrounding tissue.

More accurate details about the capsule and a study of its development are given in papers by Moral on the submandibular gland of the pig (1913) and of the mouse (1915–16). According to this author the connective tissue thickening in its earliest stage is 4–5 times as large as the epithelial bud, consists of round, closely packed cells and is surrounded by a region with very few cells.

Similar observations were made on human embryos by Heidenhain (1921), Fischel (1929), Löwenkron (1930), Bertelli (1931), Clara (1933–4) and Streeter (unpublished observations personally communicated by Heuser, 1949).

Moral, Fischel and Löwenkron suggest that the embryonic capsule is formed in response to a stimulus from the epithelium. According to Moral its main function is to provide a transitory filling to occupy the place destined for the glandular epithelium, which enters and invades the capsule.

The changes in the capsule during development, consisting mainly in an arrangement of the cells along concentric lines and the appearance of fibres, were studied thoroughly by Flint (1902–3*a*, 1903) and Moral. These authors suggest that there may be a reciprocal morphogenetic action of the connective tissue and epithelium; apart, however, from the merely passive function mentioned above of filling a space, the only action which is in fact defined is the orientating influence of the epithelium on the connective tissue cells and fibres, produced by pressure or some other factor acting at a distance.

In a previous research (Borghese, 1950) it was shown that the rudiment of the submandibular gland would develop almost normally when explanted *in vitro*. I therefore decided to use the tissue-culture technique to investigate experimentally the problem of the reciprocal influence of the epithelium and connective tissue on the development of the gland.

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MATERIAL AND TECHNIQUE

For the first series of experiments described below, the glands were obtained from embryos at the 13th–14th day of gestation. At this stage the epithelial constituent is flask-shaped, with a neck which will produce the main duct, and a terminal swelling from which the adenomeres are derived. The capsule is well defined, but only a very small part of it is occupied by the epithelial anlage. Earlier gland rudiments are unsatisfactory for explantation, as the connective tissue thickening is not clearly distinguishable under the dissecting binocular microscope and the epithelium does not grow well in culture.

The second series of experiments was made with glands at a later stage of development, when the epithelium had already begun to branch. Sometimes branching begins as early as the 13th day of gestation, but in other embryos it does not appear until the 14th or 15th day. Glands taken at still later stages are not suitable for such experiments because the epithelium completely occupies the capsule.

The explants were cultivated in a medium composed of equal parts of fowl plasma and extract of 11–12-day chick embryos. In the first series of experiments two cultures were grown by the watch-glass method (Fell & Robison, 1929) and two by the hanging-drop method with the tissue embedded in the medium. All the remaining explants were grown by the hanging-drop method but were placed on the surface of the clot. Each culture was observed daily and its outline drawn by means of a camera lucida.

The cultures were fixed at various intervals in Zenker's fluid or Susa. Some were stained with carmalum and mounted whole, others were serially sectioned and stained with azan or haematoxylin and eosin.

I. EXPERIMENTS ON THE UNBRANCHED RUDIMENT

(1) *The effect of removing most of the capsule on the development of the epithelium*

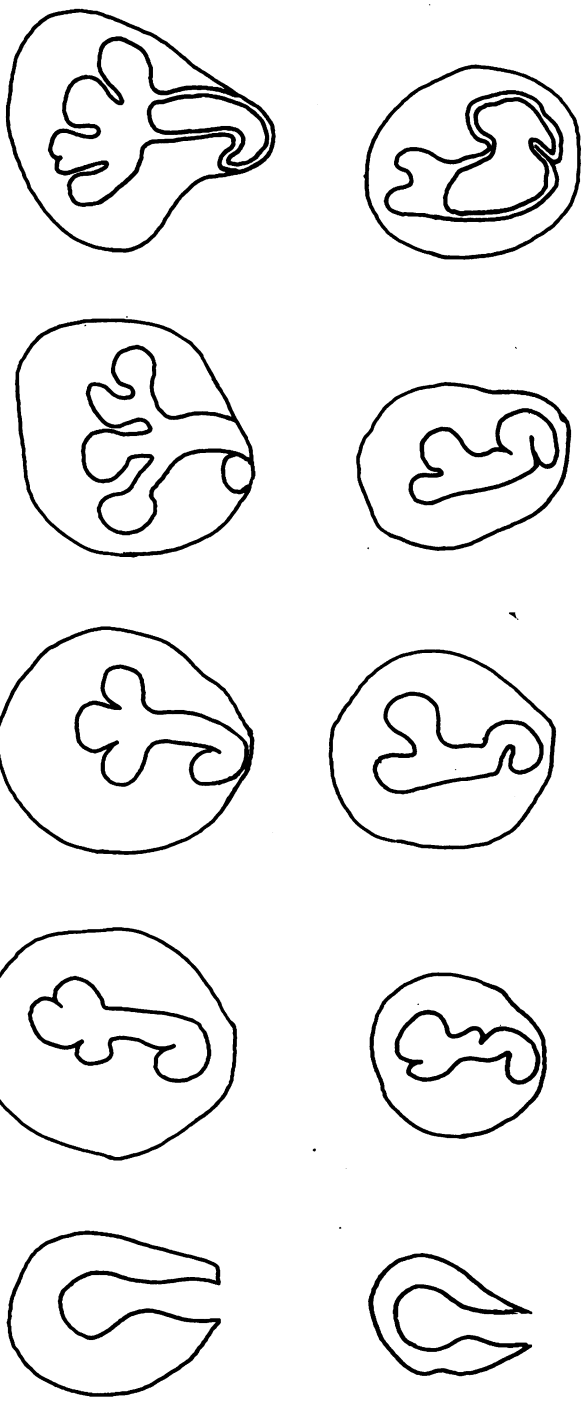
Method. Experiments were made to compare the behaviour of the epithelium in an intact gland rudiment and in one from which most of the capsule had been removed.

As stated above, in the 13–14-day gland the flask-shaped epithelial anlage extends only a short distance into the compact, oval capsule rudiment below (Pl. 1, fig. 1). Under the dissecting binocular microscope most of the capsule was removed, leaving only a thin layer of tissue around the epithelium. In the controls the capsule was left undisturbed.

The capsule was excised from twenty-five explants, but only twenty-one will be considered, because in the remaining four the controls showed early and abnormal deterioration and thus vitiated the experiment.

Results. Group 1. The cultures are divided into two groups according to the way in which the control was obtained.

Twelve cultures form the first group, in which the control consisted of the opposite gland of the same embryo grown in the same hanging-drop culture; both rudiments are at exactly the same stage in the same embryo. The material was obtained from six litters. The twelve glands and their controls were fixed after the following periods



Text-figure 1. Camera lucida drawings of the submandibular gland rudiments of the same embryo cultivated together (culture 219). The upper line of drawings shows the intact gland and the lower line the gland deprived of most of the capsule. *a*, at the time of explantation; *b*, after 1 day; *c*, after 2 days; *d*, after 3 days; *e*, after 4 days. ×52.

of cultivation: 2 days (three cultures), 3 days (two cultures), 4 days (four cultures), 5 days (two cultures), 6 days (one culture).

The behaviour of a typical pair of explants from the first group is illustrated in the text-figure. Camera lucida drawings were made at intervals of 1, 2, 3 and 4 days of two glands from the same embryo; the rudiment shown in the upper line of drawings was explanted with the capsule intact, that in the lower line was deprived of most of its capsule. The complete anlage branched regularly until by the 3rd day it had produced four buds; branching then stopped but the duct developed further and by the 4th day had acquired a medium-sized cavity. It will be seen that the duct is straight, except for a short terminal hook. The other gland, from which most of the capsule had been removed, formed only two buds which appeared later than those of the control; not only did the buds fail to divide further, but their characteristic shape had partly disappeared by the fourth day. The duct became strongly bent and by the 4th day was much dilated.

The development of the cultures is assessed according to the number of adenomeres produced at different periods of cultivation and the results are summarized in Table 1.

Table 1. *Cultures (group 1) in which the control is represented by the other gland of the same embryo*

Number of the adenomeres at different times of cultivation. Ves. = Vesicular.

			Culture period					
No.	No. of the culture		1 day	2 days	3 days	4 days	5 days	6 days
1	140	Exp.	1	1	—	—	—	—
		Control	4	5	—	—	—	—
2	143	Exp.	1	1	1	1	—	—
		Control	2	2	5	5	—	—
3	144	Exp.	1	1	Ves.	Ves.	Ves.	Ves.
		Control	1	3	3	4	8	15 (approx.)
4	145	Exp.	1	Ves.	Ves.	—	—	—
		Control	1	2	4	—	—	—
5	165	Exp.	1	2	—	—	—	—
		Control	6	6	—	—	—	—
6	167	Exp.	1	2	2	Ves.	Ves.	—
		Control	3	5	5	5	15 (approx.)	—
7	219	Exp.	2	2	2	2	—	—
		Control	3	3	4	4	—	—
8	168	Exp.	1	1	Ves.	Ves.	—	—
		Control	2	4	4	4	—	—
9	164	Exp.	1	3	3	—	—	—
		Control	2	5	5	—	—	—
10	178	Exp.	3	3	(Shapeless mass of epithelium and vesicle.)			
		Control	3	3	3	3	3	—
11	184	Exp.	2	4	—	—	—	—
		Control	3	10	—	—	—	—
12	171	Exp.	3	6	6	15–20 (approx.)	—	—
		Control	4	5	12	30–40 (approx.)	—	—

The table shows that in the first nine cultures the control glands generally produced four to five adenomeres in 2–4 days (six in one only); two, which were cultivated

longer, reached about fifteen adenomeres in 5–6 days. During the same period, five of the corresponding glands deprived of most of their capsular tissue did not ramify, three formed two adenomeres (one of these is reproduced in the text-figures) and one produced three. In all of them branching stopped early, and in several the gland subsequently lost its branches and became a small vesicle. Two cultures of this type are reproduced in Pl. 1, paired figures 2–3 and 4–5, which show the structure of the explants after 2 and 3 days' cultivation.

The remaining three cultures of this group behaved rather differently. In the 10th culture the intact control developed less than the average. In this culture both explants produced three adenomeres during the first day. After this stage the gland with the complete capsule formed only the necks of new adenomeres, and by the 4th day development stopped; the three adenomeres of the operated gland began to lose their shape on the 2nd day and deteriorated into small shapeless masses of epithelium, while the duct dilated until it was much wider than that of the control and finally formed a kind of vesicle.

In the 11th culture, the control developed abnormally well, and in 2 days formed about ten adenomeres grouped in three lobules; the operated gland, on the other hand, developed more slowly and produced only four buds.

The last pair of glands also grew to an exceptional size, probably owing to the fact that they were at a slightly more advanced stage than the others when explanted; although in each the terminal bud was still single, it showed a slight notch indicating the beginning of branching. As before, however, the control gland grew much more vigorously than its fellow from which most of the capsular tissue had been removed. In 2 days both glands formed five to six adenomeres, but by the 3rd day the intact gland had produced about twelve adenomeres, while the other showed no further development. The explants were then transplanted and both continued to grow; by the 4th day the control had produced several dozen adenomeres and a hollow but not dilated duct, while the operated rudiment had also produced a mass of adenomeres which, however, equalled a third to half the volume of the control, and a hugely dilated duct.

Group 2. The second group consists of cultures which served also as controls in other experiments described below (p. 310); for reasons which will appear later comparison could not be made with the opposite gland from the same embryo, and glands from litter-mates had to be used; since members of the same litter may not all be at the same stage of development, great care was taken to select as controls rudiments whose degree of differentiation corresponded exactly with that of the glands destined for operation.

The nine glands were fixed after the following periods of cultivation; 3 days (two cultures), 4 days (five cultures), 5 days (two cultures). The material was obtained from four litters.

The results are summarized in Table 2.

Two cultures of the first litter produced respectively five or six and ten adenomeres after 5 days' cultivation, whereas the control, although cultivated for 4 days only, had already produced about thirty to forty adenomeres. Two cultures of the second litter formed after 3 days two and seven buds, whereas three controls produced in the same time twelve, nine and four buds. Four experiments were made from the

Table 2. *Cultures (group 2) in which the control is represented by glands of littermates*

Number of adenomeres at different times of cultivation

Litter	No. of the culture		Culture period: 1 day	2 days	3 days	4 days	5 days
I	1. Exp.	172	3	4	4	5–6	5–6
	2. Exp.	173	3	5	5	10	10
	Control	171	4	5	10 (approx.)	30–40 (approx.)	—
II	3. Exp.	187	1	2	2	—	—
	4. Exp.	188	2	3	7	—	—
	Controls:	185	2	6	12 (approx.)	—	—
		186	1	3	9 (approx.)	—	—
		189	1	4	4	—	—
III	5. Exp.	220	1	Shapeless mass of epithelium			—
	6. Exp.	221	1	4	5	5	—
	7. Exp.	224	1	Shapeless mass of epithelium			—
	8. Exp.	225	2	2	2	2	—
	Controls:	219	3	3	4	4	—
		222	4	4	15 (approx.)	25 (approx.)	—
		223	2	5	7	—	—
		226	1	5	7	15 (approx.)	—
		227	1	4	11	More than 20	—
IV	9. Exp.	209	3	11	8	5	—

3rd litter. Of the six controls with complete capsules, five developed extremely well, forming fifteen to twenty-five adenomeres in 4 days, but one remained at the four-bud stage; on the other hand, none of the glands deprived of most of the capsule produced more than five adenomeres and two did not branch at all and from the 2nd day regressed to a shapeless epithelial mass.

In the single experiment made with the 4th litter no control is reported, because all the other glands of the same litter were slightly more advanced when explanted and were therefore not strictly comparable with the operated gland; they reached an advanced degree of development. The experimental explant is, nevertheless, worth describing because it showed a remarkable regression of development, a phenomenon never observed in the complete glands. At the beginning the gland grew very well and looked normal at the 2nd day, when it had formed eleven adenomeres; after this stage, however, it regressed and the adenomeres fused together until only five were present by the 4th day.

In both groups of cultures it was clear that when the gland was deprived of most of the capsule, the epithelium was confronted by a resistance to its normal expansion. This was shown by the pronounced bending of the epithelial cord which is destined to become the main duct (Text-fig. 1, lower line, and Pl. 1, fig. 6 which should be compared with fig. 7). This distortion was far greater than the small terminal hook-like bend which sometimes appeared in the duct rudiments of the intact controls, and affected a large proportion of the operated explants. The cord lengthened normally, but not finding the usual space in which to elongate, it was compelled to bend; this

is interesting in view of the fact that the capsular connective tissue is not removed from the neighbourhood of the duct but from the opposite side of the gland.

The greater variability in the development of the operated glands, as compared with that of the intact controls, may be attributed at least in part to the fact that the amount of connective tissue removed inevitably varied in different rudiments.

Histology. The question naturally arose as to whether the inferior development of the gland rudiments deprived of their capsules was due merely to injury of the epithelium during the operation. While a variable degree of trauma was probably produced, the preparations showed no more cell degeneration than was seen in the epithelium of the normal explants. There was merely an arrest of development, so that the epithelial part of the gland resembled that of a normal rudiment at a younger stage.

The histological structure of an average gland explanted after removal of most of the capsule was as follows. Even at the 3rd or 4th day of cultivation the adenomeres had a primitive structure, being solid, round and sometimes devoid of a neck. In some explants the adenomeres were composed of a superficial layer of deeply stained, cylindrical cells without either mitoses or degenerate cells, and an inner zone of more lightly stained, irregularly shaped cells with spaces between in which both degeneration and mitosis were seen. As stated above, cell degeneration was no greater than in the intact glands. Unless greatly dilated, the ducts were lined by two or three fairly regular layers of epithelial cells. As in the controls, near the opening of the duct the lumen contained a number of detached epithelial cells. When the duct was greatly dilated, as often happened in the operated rudiments, the cells of the lining epithelium were almost flat and in extreme cases were reduced to a single layer; the cavity contained detached cells.

As already mentioned, some of the glands from which the capsule was largely removed developed further than the average but later regressed. An example of such an explant is shown in Pl. 2, fig. 8. By the 2nd day this rudiment had formed eleven adenomeres but it soon began to regress, and when fixed on the 4th day the number of acini was reduced to four or five. In section they were seen to be solid, but they had a more advanced structure than that described above for a typical gland deprived of its capsule. The cells were very compact and there was no distinction between the peripheral and central cells; there was some degeneration but no more than in the controls. Each adenomere had a very distinct neck which was partly solid and partly invaded by the cavity extending from the main duct. The lumen of the main duct was not dilated, which was unusual, and was lined by three layers of cells which near the opening contained a large intracellular vacuole like the corresponding cells in the normal duct. The capsule was fairly thick, showed little differentiation and there was some concentric orientation of the cells around the acini.

Other rudiments developed less than the average; either the gland produced no adenomeres or lost them by regression and became a vesicle or a small, shapeless mass of epithelium. In such cases the histological structure was that of the main duct. If the explant was vesicular (Pl. 2, fig. 9) the epithelium was stretched and consisted sometimes of one layer and sometimes of several layers of flattened cells. Some cells projected into the cavity, some seemed on the point of detachment and others were already free and degenerating in the lumen. If the duct had originally been bent,

traces of the bends persisted as folds projecting inwards; cells in course of detachment were particularly numerous on the crests of these folds.

As described above, the epithelium sometimes formed not a vesicle, but an apparently solid mass (Pl. 2, fig. 10); this often happened if no branches developed at the beginning of cultivation. Sections showed traces of a cavity, however, and the epithelium resembled that of a main duct with an exceptionally thick wall. Most of the epithelial cells contained a large intracellular vacuole at the lower pole, as in the normal embryonic duct near its opening. The behaviour of the connective tissue in the glands from which most of the capsule had been removed differed in an important respect from that of the controls. In the latter, although there was some migration of cells and consequent spreading of the capsular tissue, this was not great and the capsule remained fairly compact. When the capsule was reduced to a thin layer around the epithelium, however, it rapidly spread into a flat, diffuse sheet of cells. It was noted that this spreading and thinning of the connective tissue was greatest in those explants in which the epithelium was reduced to a small shapeless mass, and least in those in which the epithelium showed some branching.

The results of the foregoing experiments suggest that when explanted *in vitro* the epithelial anlage of the gland retains its capacity for normal, though reduced, branching only when it can develop inside the complete capsule rudiment; when most of the capsular tissue is removed, the epithelium either fails to branch or branching stops early and often regresses.

It might be assumed, therefore, that the connective tissue has a kind of organizing action on the epithelium, which cannot develop normally in the absence of the capsular rudiment. The experiments described in the next section were made to find whether tissues other than the capsule could promote the normal development of the epithelial anlage, or whether the action of the capsule was specific.

(2) *The effect of adding other tissues to epithelial rudiments deprived of their capsules*

Method. Preliminary experiments were made to find a suitable tissue to place in contact with the epithelial rudiments after the capsule had been removed. The capsule from the opposite gland or a piece of periocular tissue were tried, but the connective tissue was too scanty. It rapidly spread into a thin layer and did not materially change the conditions of the experiment; the result was the same as in the control gland from which the capsule had been removed and to which no tissue had been added.

Fragments of limb cartilage with its surrounding tissue were then added to the rudiments. In this way a fairly solid lump of tissue was obtained which did not expand into a thin sheet. The results with this material were much more satisfactory.

It seemed interesting to study the effect on the rudiments deprived of their capsules, of adding material other than connective tissue. Experiments were therefore made in which pieces of spinal cord were added to the glands; at the stage at which it was used, the cord contains only a negligible amount of connective tissue.

In these experiments three types of explants were needed, namely (1) epithelial rudiments deprived of their capsules and placed in contact with other tissue; (2) control rudiments deprived of their capsule and not placed in contact with other

tissue; and (3) a second set of controls in which the capsular rudiment was left intact. Since each embryo has only two mandibular glands, one of the two controls had to be taken from another foetus. To minimize the effects of casual variability among embryos of the same age, individuals were selected in which the gland was at exactly the same stage of development, namely flask-shaped with a distinct neck and without the notch which indicates the beginning of branching. For each observation, both the glands of the one embryo were cultivated in the same hanging-drop culture, one being deprived of its capsule rudiment and placed in contact with other tissue, and the other being grown entire. Both the glands of the second embryo were deprived of their capsules; then other tissue was added to the one rudiment, while the other was left denuded. The third embryo of the litter was treated as the first, the fourth one as the second, and so on.

Thus the control was represented alternately by a gland with an entire capsule and one with the capsule reduced to a minimum; in each observation the two pairs were comparable, being grown in as nearly identical conditions as possible. If there were enough embryos in a litter, a culture was also made in which one gland was deprived of its capsule and the other explanted entire as described in the previous section.

Of the thirteen explants studied, cartilage was added to seven (from four litters), and spinal cord to six (from two litters). Four rudiments were fixed after 3 days in culture, eight after 4 days, and one after 5 days; seven glands were mounted whole and six were serially sectioned.

Results. Table 3 shows the number of adenomeres formed in the operated rudiments to which cartilage had been added and in the corresponding controls from which the capsule had been largely removed and not replaced by other tissue (the control 224 relates to two experiments).

Table 3. *Cultures with addition of cartilage*

Number of adenomeres at different periods of cultivation.

No. of the culture		Culture period: 1 day	2 days	3 days	4 days	5 days
(*a*) Cultures in which the addition of cartilage did not produce any effect						
Exp.	186	1	1	1	—	—
Control	187	1	2	2	—	—
Exp.	188	2	2	4	—	—
Control	188	2	3	6	—	—
Exp.	224	2	2	2	3	—
Exp.	226	1	1	1	1	—
Control	224	2	2	2	3	—
(*b*) Cultures in which the addition of cartilage produced a better branching						
Exp.	221	1	6	7	15 (approx.)	—
Control	221	1	4	5	5	–
Exp.	209	6	10 (approx.)	15 (approx.)	20–25	—
Control	209	3	11	8	5	—
Exp.	174	4	5	5	15 (approx.)	20–25 (approx.)
Control	172	3	5	6	6	6

In four cultures the operated gland showed no improvement or even a retarded development, as compared with the control glands. Two rudiments developed into a

dilated duct with vacuolated cells and three or four short-necked adenomeres; the other two formed merely an epithelial cord which in one explant was solid at the end where the adenomeres should have developed and hollow at the opposite end which corresponded with the terminal part of the main duct.

In the other three cultures, the presence of the cartilage completely transformed the situation. Not only did the glands branch more richly than their corresponding controls, but they branched much more profusely than any other explanted glands deprived of their capsules in the course of this investigation. After 4 days' cultivation one of these rudiments had formed about fifteen adenomeres grouped in four lobules, while the corresponding control from which the capsule had been removed but not replaced by cartilage, produced only five buds during the same period. The second gland differentiated even further than was usual for explants with intact capsules; it formed many adenomeres of a rather advanced histological development, in which there was no distinction between the peripheral and central cells; these were united with the main duct by rather long, hollow intermediate ducts. The third gland (Pl. 2, fig. 11) which was cultivated for 5 days, also developed vigorously, producing many adenomeres and well-formed, hollow intermediate ducts. The control gland (Pl. 2, fig. 12) of another embryo of the same litter also developed rather more than was usual for a rudiment deprived of most of its capsule, and formed six adenomeres and long, hollow ducts; this was probably due to imperfect elimination of the capsule, as indicated by the fact that the sublingual gland, which ordinarily was removed with the capsular tissue, was also present in this explant. Nevertheless, the control presented a remarkable contrast to the gland associated with cartilage (cf. Pl. 2, figs. 11, 12).

The development of the six glands deprived of their connective tissue to which spinal cord had been added, is summarized in Table 4, in which the controls represented by the glands deprived of most of the capsule without addition of other tissue, were grouped together according to the two litters used for this part of the research.

Table 4. *Cultures with addition of spinal cord*

Number of adenomeres at different periods of cultivation

Litter	No. of the culture		Culture period: 1 day	2 days	3 days	4 days
(*a*) Cultures in which the addition of spinal cord did not produce any effect						
I	Exp.	225	1	2	2	2
	Control	225	1	2	2	2
(*b*) Cultures in which the addition of spinal cord produced a better development						
I	Exp.	220	1	2	2	4
	Exp.	222	1	5	12 (approx.)	25 (approx.)
	Exp.	227	1	2	3	8
	Control	220	1	1	1	1
		221	1	4	5	5
		224	1	1	1	1
		225	1	2	2	2
II	Exp.	185	1	5	6–7	—
	Exp.	187	2	5	9–10	—
	Control	187	1	2	2	—

Only one gland to which spinal cord had been added showed no improvement as compared with its control. This specimen and its control each produced two acini and a hollow, rather bent duct, but a spherical, well-defined capsule formed round the gland associated with the cord, whereas the connective tissue surrounding the control spread into a thin sheet.

The remaining five explants to which spinal cord had been added were much better developed than the controls without the cord. One rudiment (no. 220) after 4 days' growth, had formed four adenomeres only, which is within the range of the controls, but the control gland from the other side of the same embryo grown in the same culture (no. 220) did not branch at all.

Another explant associated with spinal cord and also cultivated for 4 days formed at least two dozen well-developed adenomeres and showed very little degeneration; there were no intermediate ducts but a single hollow duct. Sections showed that the connective tissue cells had a certain concentric orientation around the adenomeres as in the normal organ and in the intact explants; there were also traces of developing blood vessels. This explant, and also the third one which reached the number of eight regularly shaped buds all enclosed in a comparatively thick, rounded mass of connective tissue were more developed than the four controls from embryos of the same litter.

The last two explants in contact with spinal cord, from the same litter, were grown for 3 days. One (Pl. 3, fig. 13) formed six or seven adenomeres and the other produced nine or ten. Both explants developed much better than the control from which most of the capsule had been removed (Pl. 3, fig. 14), although rather less than the control gland with intact capsule (Pl. 3, fig. 15).

The fact that some of the rudiments deprived of their capsules and to which cartilage or spinal cord had been added developed no better than their controls was probably due to technical reasons, e.g. imperfect union between the cartilage or cord and the gland.

An interesting difference in the behaviour of the connective tissue was observed between the rudiments to which cartilage or spinal cord had been added and the controls not associated with other explants. In the former the connective tissue often reconstituted almost as large a mass round the epithelium as the original capsule, and the normal concentric arrangement of the cells around the acini was restored. In the controls, on the other hand, the capsular remains spread into the usual thin, diffuse outgrowth described above.

II. EXPERIMENTS ON GLANDS SHOWING EARLY BRANCHING

(1) *The effect of removing part of the capsule on the development of the epithelium*

Method. In this series of experiments the glands already contained three to four adenomeres when first explanted and most of the capsule was already invaded by epithelium. Consequently, merely a small peripheral dome of unoccupied connective tissue could be removed without damaging the epithelium, so that there was only a small difference between the amount of connective tissue in the operated gland and in the untouched control.

Part of the capsule was removed from ten glands, and twelve controls were cultivated intact. Three of the operated glands were compared with controls from the same embryos; the other controls were from litter-mates at exactly the same stage of development.

Of the glands from which the capsules had been partially removed, one was fixed after 2 days, two after 3 days, four after 4 days, one after 5 days, one after 6 days and one after 7 days. One of the glands with intact capsules was fixed after 2 days, two after 3 days, six after 4 days, one after 5 days, one after 6 days and one after 7 days.

Results. Of the twelve glands with intact capsules, eleven developed almost as in the normal embryo; they branched profusely and formed adenomeres which increased in number and diminished in size as development advanced and underwent a corresponding histological differentiation. In one gland, without apparent cause, development stopped after some initial branching and the number of acini regressed.

Only three of the ten glands deprived of part of their capsules showed poor branching. In the remaining seven, the branching at first sight appeared equivalent to that of the intact explants. Four of the seven rudiments, however, differed in certain respects from the controls; the adenomeres seemed to be rather fewer than in the latter and they were also larger. Sections showed that the larger adenomeres were at an earlier stage of development than those of the intact glands, being for the most part still solid while a cavity had already appeared in the latter.

Thus the results obtained in these experiments were consistent with, though less striking than, those obtained in similar experiments on younger rudiments. Moreover, a new fact emerged, not seen in the other experiments, namely that histological differentiation was more advanced when the capsule was kept whole.

(2) *The effect of adding other tissues to glands deprived of part of their capsules*

Method. Eight rudiments, from four different litters, were deprived of part of their capsules, and each was placed in contact with pieces of cartilage. Eight other rudiments, from five different litters, were cut in the same way, but placed in contact with pieces of spinal cord. Each operated gland had two controls: a gland with intact capsule, and one from which part of the capsule had been removed and not replaced by other tissue. One control was obtained from the opposite side of the same embryo from which the operated gland was taken, and the other from a litter-mate at the same stage of development.

The cultures were observed and drawn daily, and fixed at intervals as follows. Those associated with cartilage: one after 3 days, five after 4 days, one after 6 days and one after 7 days; those with spinal cord: one after 2 days, six after 4 days and one after 6 days.

Results. There was no significant difference between the controls with intact capsules and the glands in which part of the capsules had been removed and replaced by some other tissue.

From observations on the living cultures, it appeared that six of the explants to which cartilage had been added grew and branched to about the same extent as controls from which capsular tissue had been removed and not replaced by other material; in the remaining two, it was obvious that the glands with cartilage had

grown better than the controls without this addition, and in one of the two the adenomeres were obviously smaller than in the control. Of the explants associated with spinal cord, one grew badly and was inferior to the control without cord; three grew about as well as the controls, and in four the glands with spinal cord branched much more profusely than the controls.

In these cultures the adenomeres were so numerous and superimposed, however, that in the living glands it was impossible to count them or to see their histological structure clearly. A study of the whole mounts, and especially of the histological sections, showed that the explants deprived of part of their capsules and placed in contact with other tissues, formed more numerous and smaller acini and reached a more advanced degree of histological differentiation than the controls to which no tissue had been added (cf. Pl. 3, figs. 16–19).

These results confirmed those of the previous experiments on older rudiments, and supported the view that the presence of tissue other than the normal capsule favours epithelial differentiation. This was not shown in the experiments on the early rudiments, probably owing to the fact that the explants did not reach a sufficiently advanced stage of histological development.

DISCUSSION

We must now consider what factors are responsible for inhibiting the development of the epithelial anlage of the submandibular gland when most of the capsular rudiment is removed.

It seems unlikely that the inferior development was due solely to trauma. In the first place, as described above, there is no histological evidence of greater cellular damage in the glands deprived of their capsules than in the intact controls. In the second place, whatever damage was inflicted, it did not prevent the glands from developing almost as well as the intact rudiments when cartilage or spinal cord were added after removal of the capsular tissue.

As already stated, the degree of epithelial development attained by the glands after removal of their capsules was usually correlated with the extent to which the remaining connective tissue spread out on the medium; thus development was least when the diffuse outwandering of the capsular tissue was greatest, and most advanced when the surrounding tissue was most compact. From this it seems clear that the capsule has an important influence on the development of the epithelial anlage. In this connexion the observations of Rienhoff (1922) on the development *in vitro* of the chick metanephros are interesting, This author found that the epithelial elements only differentiated normally where the surrounding stroma remained thick and compact; when there was a diffuse outgrowth into the culture medium the rudimentary tubules failed to develop further and merely formed indifferent sheets of cells.

At first sight, the fact that, when another tissue was substituted for the missing part of the capsule, the gland developed almost as well as when the capsule was intact, suggests that the influence of the capsule is not specific. A closer examination of the results, however, shows that they do not exclude the possibility of a specific action of the capsular rudiment. As described above, when cartilage or spinal cord was added to the defective gland, the epithelium became surrounded by a much larger and more compact mass of connective tissue than in similar explants not placed in

contact with other tissue. When cartilage was added, it was possible that this capsular material might have been derived from the perichondrium, but this could not be true when pieces of spinal cord were used. At most, only a trace of connective tissue was left adherent to the cord, and it was far less in amount than that which remained attached to the glandular epithelium. It can thus be assumed that, at least when spinal cord was added to the rudiment, the new capsule originated chiefly from the remains of the original capsular rudiment.

It is also uncertain whether the added tissue promoted the growth and development of the gland by a direct action on both the capsular and epithelial constituents, or whether it had a direct action on one constituent and only a secondary effect on the other. Thus it is not known whether the regeneration of the capsular tissue was the primary effect and was responsible for the improved development of the epithelium, or whether it was the epithelium that was stimulated and by its increased growth evoked a corresponding proliferation of the connective tissue. The latter hypothesis seems improbable, but it may well be that the added tissue had a beneficial effect on both elements of the gland.

Why the addition of other tissue to the defective gland rudiment should have promoted the growth of the latter is not known. Possibly the presence of another organ provided a more normal environment for the gland. It is also possible that the increase in the total mass of the explant which resulted from the addition was beneficial. This latter view receives some support from the fact that I failed to get satisfactory development *in vitro* either from rudiments earlier than the 13th day or from small fragments of even more advanced stages (15th day and older). A certain minimum size of explant may be necessary for the normal development of the tissue.

It will be seen that the results of the experiments described in this communication are not easy to interpret, and more work is required. Although there is little doubt that, at least *in vitro*, the capsular rudiment is essential for the normal development of the epithelial anlage, there is at present no evidence as to whether the capsule has a specific organizing action, whether it merely provides the mechanical conditions necessary for the expression of developmental potencies already inherent in the epithelium or, finally, whether after removal of the capsule the epithelium fails to develop because the total mass of the explant is too small.

SUMMARY

1. Experiments were made to compare the development *in vitro* of rudiments of the submandibular gland of the mouse (*a*) when the gland was explanted intact, and (*b*) when most of the capsular rudiment was removed.

2. Intact glands explanted at the single bud stage branched in the same way as in normal development, but much more slowly, and in 3–4 days' cultivation formed four to fifteen adenomeres and a long, straight duct.

3. Similar glands from which most of the capsule had been removed developed very little; some showed no branching, others began to branch and then regressed. The duct could not extend properly and became bent and sometimes greatly dilated. The more compact the surrounding connective tissue, the better the development of the defective explants.

4. When a piece of cartilage or spinal cord was added to a rudiment deprived of

most of its capsule, the epithelium often developed almost but usually not quite as well as in the intact control, and far better than in the defective glands to which no tissue had been added.

5. In rudiments from which most of the capsular tissue had been removed, a new capsule was formed when the gland was placed in contact with spinal cord or cartilage. At least in the explants associated with spinal cord, the new capsule was formed from the remains of the old one. It is not known whether this regeneration of the capsule was the cause or the effect of the better development of the epithelium.

6. Similar experiments were made with slightly older rudiments in which branching had already begun. At this stage only a very small part of the capsule could be removed, so that the difference in anatomical development between the intact and the defective rudiments was usually slight. The addition of cartilage or spinal cord, however, improved both the anatomical development and the histological differentiation of the defective explants.

7. The significance of the results is discussed.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Frontal section of the mouth of a mouse embryo at the 13th day of gestation. The epithelial anlage of the submandibular gland forms a single bud (*s.m.*) surrounded by a thickened connective tissue or capsule (*c.*) *t.*: tongue. Zenker; haematoxylin, eosin. ×60.

Figs. 2, 3. Submandibular gland anlagen taken from the same embryo at the stage of a single bud and cultivated together for 2 days. (cult. 165). Fig. 2: the gland from which most of the capsule was removed; it developed two adenomeres and the connective tissue is very scarce and thin. Fig. 3: the intact gland; it developed about four adenomeres and a regular capsule. Zenker; carmalum, whole mount. ×48.

Figs. 4, 5. Submandibular gland anlagen taken from the same embryo at the single bud stage and cultivated together for 3 days (cult. 145). Fig. 4: the gland deprived of most of its capsule; it became a small vesicle. Fig. 5: intact gland; it developed four adenomeres and a duct; on the right side the sublingual gland (*s.l.*) appeared and produced a bud. Zenker; carmalum, whole mount. ×48.

Figs. 6, 7. Submandibular anlagen of the same embryo, taken at the stage of a single bud and cultivated together for 2 days (cult. 184). Fig. 6: gland from which most of the capsule was removed; the duct (*d.*) is bent. Fig. 7: gland with whole capsule: the duct is straight; on the right side, the sublingual gland (*s.l.*) is seen. Susa; carmalum, whole mount. ×48.

PLATE 2

Fig. 8. Submandibular gland deprived of most of the capsule at the single bud stage and cultivated for 4 days (cult. 209). It has developed comparatively well and has formed a hollow duct and solid adenomeres. Susa; haematoxylin, eosin, section. ×120.

Fig. 9. Submandibular gland deprived of most of the capsule at the single bud stage and cultivated for 3 days (cult. 164). Note the vesicle with group of cells becoming detached from the wall and projecting into the cavity. Zenker, haematoxylin, eosin, section. ×100.

Fig. 10. Submandibular gland deprived of most of the capsule at the single bud stage and cultivated for 4 days (cult. 220). It became a very small epithelial mass, with a small cavity and intracellular vacuoles. Susa; haematoxylin, eosin, section. ×240.

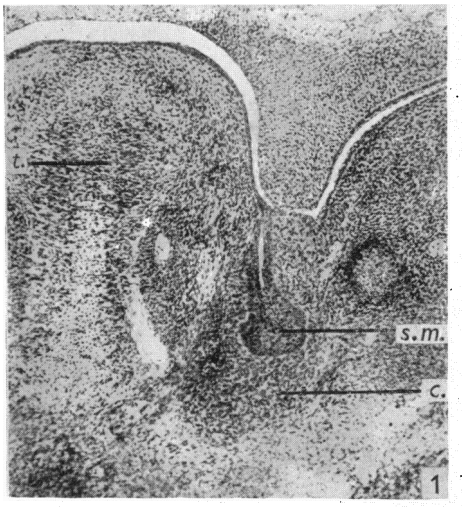
Figs. 11, 12. Gland rudiments from different embryos of the same litter both deprived of most of the capsule and cultivated for 5 days. Fig. 11: associated with cartilage (cult. 174). Fig. 12: without any addition (cult. 172). Although the development of the second explant is fairly good and a sublingual gland also (*s.l.*) has formed, the development of the first is more advanced. Susa; carmalum; whole mount. ×48.

PLATE 3

Figs. 13–15. Two gland rudiments from one embryo (Figs. 13, 15) and one from a second embryo of the same litter (Fig. 14) cultivated for 3 days. Fig. 13: gland deprived of most of its capsule and grown in association with a piece of spinal cord (cult. 185); it developed much better than the gland shown in Fig. 14 (cult. 187) deprived of most of its capsule and without any addition, which gave only two small adenomeres and a dilated duct. The intact gland of Fig. 15 developed similarly to the gland with spinal cord (the duct is missing here because it was accidentally lost during explantation). Susa; carmalum, whole mount. ×48.

Figs. 16, 17. Submandibular gland rudiments taken from the same embryo at the stage of four adenomeres, both deprived of part of the capsule and cultivated together for four days (cult. 216). Fig. 16: without any addition. Fig. 17: with a piece of spinal cord. Fewer and bigger adenomeres have been formed in the first gland, more and smaller adenomeres in the second. Susa; carmalum, whole mount. ×48.

Figs. 18, 19. Two submandibular gland rudiments taken from the same embryo at the stage of four adenomeres and cultivated together for 4 days, both deprived of part of the capsule. Fig. 18: without addition. Fig. 19: with a piece of spinal cord. Fewer and larger adenomeres have developed in the first gland and the differentiation of the duct is less advanced; more and smaller adenomeres and a more advanced development of the ducts are seen in the second gland. Susa; haematoxylin, eosin (Fig. 18); azan (Fig. 19); section. ×70.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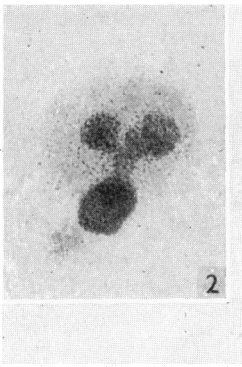
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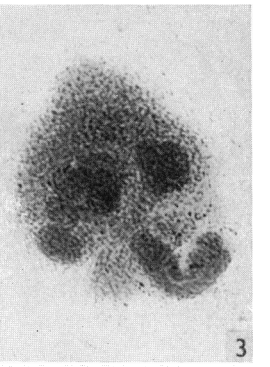
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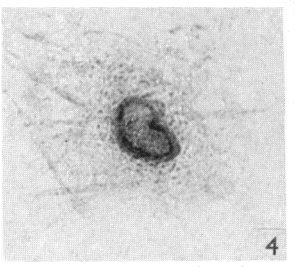
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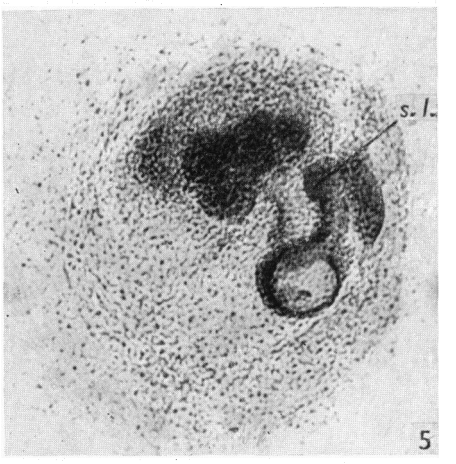
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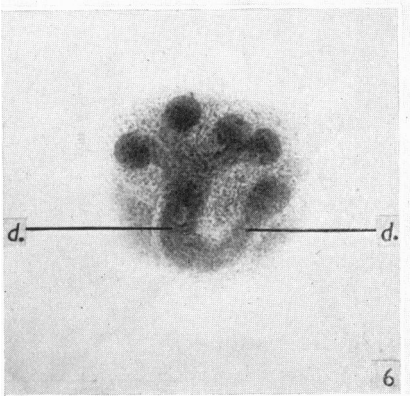
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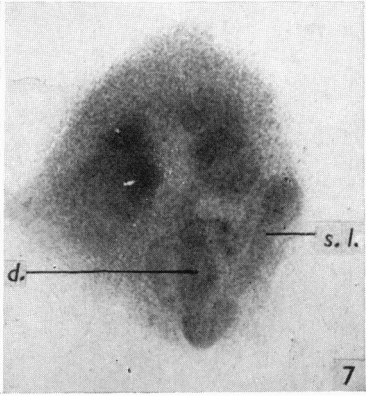
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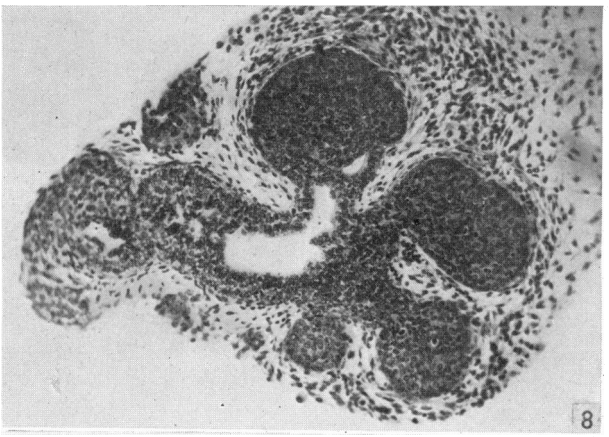
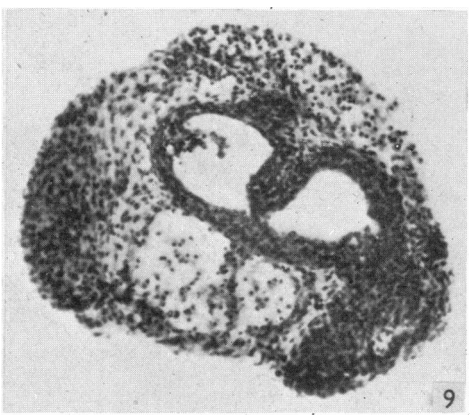
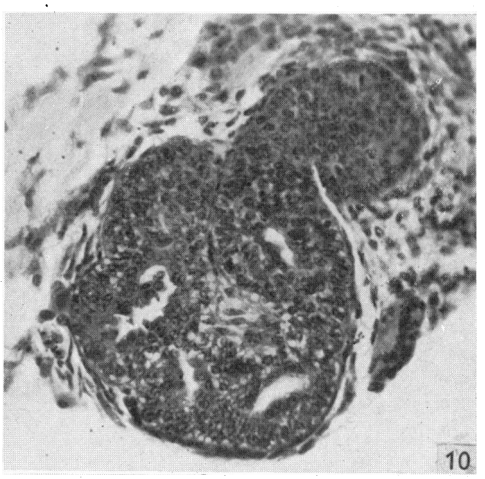
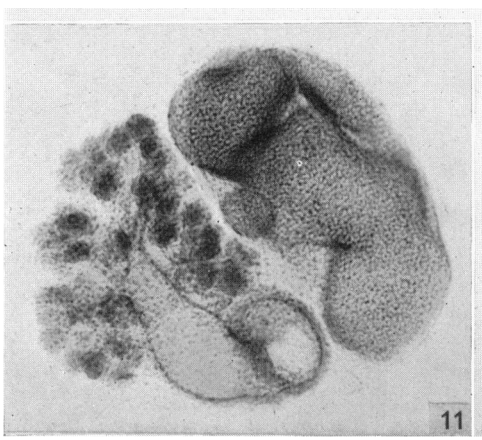
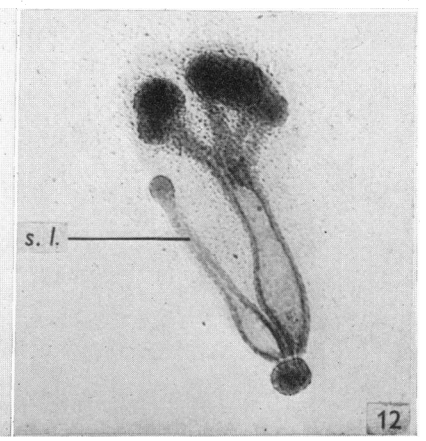


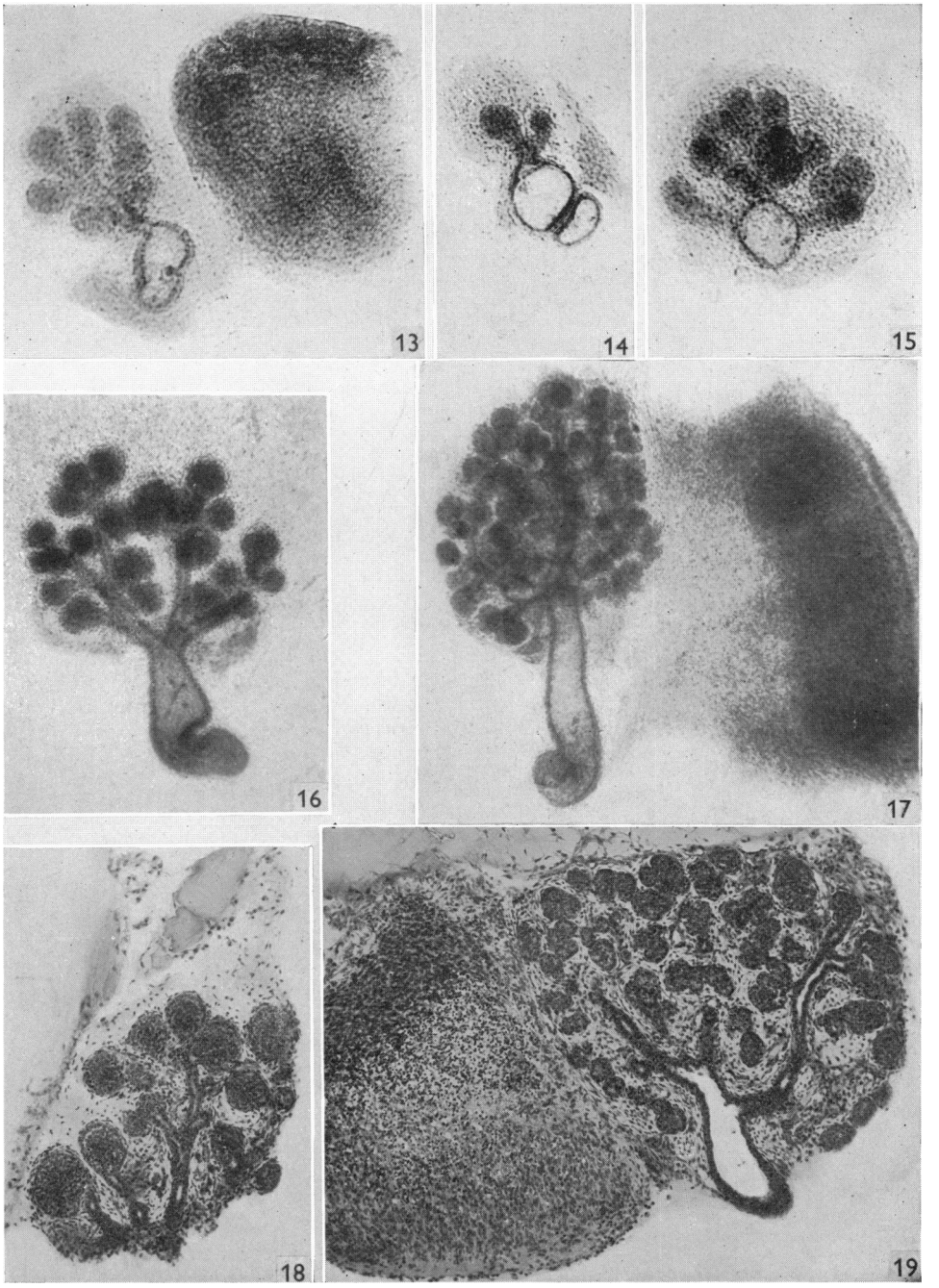








BORGHESE—EXPERIMENTS ON SUBMANDIBULAR GLAND OF *MUS MUSCULUS*



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