The Behaviour in vitro of Dissociated Embryonic Pituitary Tissue

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WITH TWO PLATES

INTRODUCTION

It was previously reported (A. Moscona & H. Moscona, 1952) that the tissues of limb-buds and mesonephroi of early chick embryos can be dissociated into suspensions of discrete viable cells which, under certain conditions of cultivation in vitro, reaggregate into clusters and re-establish a tissue-like association. Upon further cultivation in vitro these primary cellular associations became transformed into organized tissue patterns, the development of which proceeds to the level of typical histological differentiation.

Owing to the nature of the experimental material studied so far, it has mainly been the capacity of the aggregates for re-establishing typical intercellular relationship that has come prominently into view. The present observations were aimed at examining the capacity of cells, aggregated from a discrete state, to resume and complete differentiation on the cellular level, e.g. to achieve a cytologically characteristic secretory status.

The normally developed cells of the anterior lobe of the pituitary carry a distinct mark of their state of differentiation—the secretory granules. Previous work has shown (H. Moscona & A. Moscona, 1952) that this degree of development can be achieved during cultivation in vitro by the isolated rudiment of the anterior lobe from the early chick embryo. An attempt was therefore made to dissociate the anterior lobe tissue into discrete, viable cells by enzymatic digestion of the intercellular cement and to study the capacity of such cells to aggregate and to differentiate in vitro.

MATERIAL AND METHODS

The experiments were made with anterior pituitary rudiments of chick embryos. The minute size of the rudiments made it preferable to take them at the latest possible stage, and it was found that the largest amount of still undifferentiated tissue could be obtained on the 8th day of incubation. At this stage the rudiment of the anterior lobe consists of a vesicle partially filled with elongated

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epithelial cords projecting from the walls (Plate 1, fig. A), and embedded in quite dense connective tissue. The intercellular substance of the connective tissue gives a positive McManus–Hotchkiss reaction, and the mucopolysaccharide material can be clearly demonstrated by this procedure as rather dense fibrils and granules. In some cells, granules that resist digestion with saliva are also revealed, and possibly represent the precursors of the future mucopolysaccharide material of the intercellular substance (Gersh & Catchpole, 1949).

The procedure of partial and complete disintegration of the pituitary tissue was based on that devised for limb-bud and mesonephric tissue of early chick embryos (Moscona, 1952).

The rudiments were dissected aseptically and after being rinsed in Tyrode solution were transferred to a saline lacking calcium and magnesium salts, in which they were incubated for 15 minutes at 38° C. The saline was then replaced by a 3 per cent. solution of commercial trypsin. During digestion the two parts of the pituitary could easily be separated owing to the loosening of the connective tissue between them; the posterior rudiment was rejected and the anterior cut into small pieces. The pH of the trypsin medium was gradually raised to 8.4–8.6 by the addition of 1 per cent. KOH solution. After about 15 minutes of incubation in this fluid the connective tissue was greatly hydrated and swollen, the epithelial tubules thus becoming clearly discernible as separate entities. The viscosity of the medium increased, presumably owing to the dissolved breakdown products of the intercellular substances. In contrast to the much younger tissues of the limb and the mesonephric rudiments, further disintegration of the pituitary tissue could not be achieved even after prolonged treatment with trypsin.

After exposure to trypsin, the pituitary tissue was therefore submitted to the action of Clostridium welchii collagenase. The tissue was transferred to fresh saline and the enzyme, in powdered form, was added in a concentration of approximately 0.5 mg. of collagenase to 0.5 c.c. of fluid. After 10–15 minutes incubation in this solution at 38° C. the tissue broke down into a shapeless mass of loose mesenchymal cells and tubule fragments bound together by a viscous substance which was probably of a different nature from that released after trypsin treatment. At this stage the saline was replaced by several changes of Tyrode solution; care was taken not to disturb the main mass of cells but some, mainly from the connective tissue, were seen free in the fluid and were subsequently washed out. When brought in contact with Tyrode for a few minutes the partially disintegrated tissue regained a certain degree of compactness; in one series of experiments it was then explanted and cultivated by the watch-glass technique (Fell & Robison, 1929). The medium consisted of a clot composed of 2 parts of fowl plasma and 1 part of embryo extract, and the explants were transferred to fresh medium every 2 days.

1 Clostridium welchii collagenase was kindly supplied by the Wellcome Research Laboratories through the courtesy of Mr. R. D. Thomson.
In other experiments incubation in the collagenase solution was prolonged for a further 5–10 minutes, when the epithelial cells of the tubules became loosened completely and were easily dispersed into a suspension by squirting them through a pipette of a suitable bore. They were then washed by centrifugation in the Ca- and Mg-free saline and Tyrode solution, and were finally resuspended for cultivation in 0·2 c.c. of a fluid culture medium in the cavity of a hollow-ground slide; the cavity was covered by a cover-slip sealed to the glass with molten paraffin-wax. Such a preparation was suitable for continuous observation of the cells.

The culture medium used was a fluid exudate from a fowl plasma-embryo extract clot. This was prepared by mixing plasma and embryo extract in equal quantities in a tube, crushing the ensuing clot and incubating it for 1 hour at 38° C. (H. B. Fell, personal communication). The decanted exudate was centrifuged to remove any extraneous cells.

The tissues were fixed in Zenker-formol for 1–3 hours. Sections were stained with Heidenhain’s azan, Ehrlich’s or Delafield’s haematoxylin and eosin, or by the McManus–Hotchkiss periodic acid-leucofuchsin procedure.

Cell suspensions were fixed by placing a drop of the suspension on a cover-slip and exposing it for 10 minutes to acetic acid-formalin vapour and then floating the cover-slip face downwards on Zenker’s fluid for 30 minutes. The cells were stained with either haematoxylin or azan and mounted on a microscopical slide.

**THE BEHAVIOUR OF THE PARTIALLY DISINTEGRATED PITUITARY TISSUE**

The development in vitro of the isolated pituitary rudiment, as described in detail in a previous paper (H. Moscona & A. Moscona, 1952) served as a standard of comparison for the behaviour in culture of the partially disintegrated pituitary tissue.

In a series of seven experiments, anterior pituitary rudiments of 8-day embryos were partially disintegrated by treatment with alkaline trypsin and subsequently by the action of collagenase as described in the section on techniques.

Histological examination of the partially disintegrated tissue fixed immediately after treatment revealed extensive structural changes. The epithelial cords were widely separated from each other by an optically structureless substance, in which small groups of cells and individual cells were embedded (Plate 1, fig. B). Most of the epithelial cords were broken into fragments and detached from the wall of the hypophyseal vesicle; the number of mesenchymal cells was greatly reduced. The appearance of the tissue indicated that the relationship between the mesenchymal and the epithelial components was profoundly disturbed in terms of their spatial arrangement as well as their relative proportions.

During the first few days of cultivation in vitro, the partially disintegrated
tissue developed into typical cords and lobules, and became surrounded by a fibroblastic outgrowth similar to that of the untreated rudiments in vitro. Sections of explants fixed after 1 day in culture showed that the spaces between the cords and individual cells were considerably reduced. On the 2nd day the characteristic arrangement of cords and lobules was evident; they were packed closely together separated only by narrow strands of connective tissue (Plate 1, fig. C). During the following 2 days many mitoses were present. After 5 days in vitro typical acidophil and basophil cells had differentiated (Plate 1, fig. D). In the acidophil cells, most of the granules which stained orange with azan were formed on one side of the nucleus in the usual way, so that the nucleus became shifted to a terminal position. The basophils were of globular form with a slightly eccentric nucleus; minute dust-like blue granules were discernible in their protoplasm.

The formation of glandular cords and lobules and the appearance of chromophilic cells in the cultures of partially disintegrated pituitary tissue were comparable to similar processes in untreated pituitary rudiments cultivated in vitro.

THE BEHAVIOUR OF DISCRETE CELLS

Five to six rudiments were pooled for each experiment on complete dissociation of the anterior pituitary tissue. All suspensions were prepared by the method described in the section on technique. Two series of explants were made: (a) five cultures of discrete cells were grown for various lengths of time in the liquid culture medium; (b) eight cultures of cell aggregates were explanted on to the surface of a plasma-embryo extract clot and cultivated by the watch-glass technique.

Freshly isolated cells reacted by various changes in their form and behaviour to the conditions of isolation and the method of dissociation. In general these changes were similar to those described for the isolated chondrogenic and mesonephric cells (A. Moscona & H. Moscona, 1952). Two types of cells could be distinguished: relatively small cells, which protruded cytoplasmatic processes of various shapes and lengths; and larger cells, which tended to round up (Plate 1, fig. E). The formation of the protrusions might be interpreted as due to hydration of the cytoplasm resulting from the lytic action of the solutions used on the cell surface; similar reactions to lytic agents were observed in unfertilized sea-urchin eggs by Runnström & Monné (1945) and in isolated amphibian cells by Holtfreter (1948). The changes in the shape and behaviour of the cells were reversible as long as the nucleus was not affected.

When resuspended in the liquid culture medium the cells retracted their protrusions and rounded up. After about 30 minutes many of them began to move on the floor of the culture vessel, gliding by means of pseudopodia. Others accumulated in the centre of the concavity presumably by gravitation.

Series (a). After 12–24 hours in the liquid culture medium most of the discrete cells reaggregated into clusters of various sizes and shapes; the clusters were
usually elongated, but a few had a globular form. During the next 24 hours the aggregates increased in length up to 0.1–0.2 mm.; the primary clusters appeared to unite in linear arrangement, the whole colony assuming a bead-like form. On more prolonged cultivation in the liquid medium these secondary aggregates did not develop and eventually degenerated.

Histological examination revealed that the primary clusters formed after 24 hours consisted of 3–4 globular masses loosely attached to one another (Plate 1, fig. F). Aggregates fixed after 48 hours in the liquid medium were composed of numerous round masses, some of them solid and others vesicular; mitoses were present. The whole structure was surrounded by a flattened cellular capsule (Plate 1, fig. G). The impression gained was that the colony consisted mainly of one type of cells, presumably epithelial.

Series (b). After 48 hours of incubation in the liquid medium the larger aggregates were explanted on the surface of a plasma-embryo extract clot for further cultivation. These explants behaved in a surprising way. They appeared transparent and healthy for a long time in vitro, but they did not develop to any appreciable extent. There was a slight liquefaction of the clot, but no fibroblastic outgrowth nor spreading of the tissue in the form of epithelial sheets. The cultures remained in a quiescent state, in which they could be maintained for more than 2 weeks, and eventually degenerated.

Sections of explants fixed after 1 day of cultivation showed clearly that the tissue consisted exclusively of epithelial cells. Mesenchymal elements were not included in the aggregates and did not form a peripheral outgrowth. A few mitotic figures were present at this stage, but after 3–4 days in vitro the mitotic activity subsided completely. In sections of cultures fixed after 14 days in vitro the cells appeared healthy, but they retained their rounded form and undifferentiated state (Plate 2, fig. H).

THE EFFECT OF ADDING CARTILAGE TO THE AGGREGATES

The substantial difference between the untreated pituitary explants and the pituitary cell aggregates was the absence of mesenchymal elements in the latter. A series of nine cultures was therefore made in which the epithelial aggregates were allowed to grow in contact with an extrinsic source of mesenchymal tissue. This was done by placing the aggregates in the immediate vicinity of an explanted fragment of cartilage from the phalanx of a 10-day embryo.

During the first 2 days' cultivation of the combined explants, a fairly dense fibroblastic outgrowth from the phalangeal perichondrium spread towards and partially surrounded the pituitary cells. The two tissues came into intimate contact so as to form a single unit, which could be easily handled during transfer or fixation. On the following day the fibroblasts completely surrounded the aggregates with a thin capsule, which thickened during further cultivation. An abundant outgrowth formed around the whole culture.

Sections of combined cultures fixed after 3 days in vitro showed that the fibro-
blastic tissue had encapsulated the aggregates completely and after 4 days had penetrated between the pituitary cells. The homogeneous epithelial tissue of the aggregates thus became subdivided into 'lobules' of epithelium surrounded by mesenchymal tissue derived from the fibroblastic outgrowth (Plate 2, fig. 1). The pituitary cells looked healthy and mitotic activity was resumed. A few cyst-like structures filled with degenerating cells were found in the pituitary tissue. In sections of cultures fixed after 6 days in vitro chromophilic cells were seen to have differentiated (Plate 2, figs. J, K); they were predominantly acidophils and were situated mainly at the periphery of the lobules; basophils were very scanty.

Thus the addition of mesenchymal tissue to the aggregates of epithelial pituitary cells resulted in the transformation of the latter into a gland-like tissue, in which mitotic activity and cellular differentiation were resumed.

DISCUSSION

The method of dissociation of tissues into discrete cells, as devised for the limb and mesonephric tissues of early chick embryos (A. Moscona, 1952), when applied to the 8-day pituitary rudiment does not result in complete disintegration. This is probably due to the structurally more advanced and chemically more complex state of the connective tissue and of the cement substance of the pituitary rudiment at this stage.

On the assumption that at this age the intercellular fibrillar substance of the connective tissue might be of a collagenous or procollagenous nature, a procedure was evolved based on the combined action of trypsin and the collagenase of \textit{C. welchii}; by this means the anterior lobe tissue was dissociated into discrete, viable cells. \textit{C. welchii} collagenase was shown to dissolve native and degraded collagen (Oakley, Warrack, & Warren, 1948) and its action on the pituitary tissue supports the histochemical indications that collagenous material is present in the connective tissue of the gland at this stage.

The aggregates formed in the resulting cell suspensions consisted almost exclusively of epithelial cells. The reason for the disappearance of mesenchymal cells after the dissociation procedure was obscure at the time of the experiments. The recent observations of Shaffer (1956), however, showed that the \( \alpha \)-toxin of \textit{C. welchii} caused the selective destruction of mesenchymal cells \textit{in vitro}. The concentration of the \( \alpha \)-toxin present in the amount of collagenase used in our experiments was far above the effective minimum required for the differential destruction of mesenchyme. Thus the pure epithelial pituitary aggregates were obtained by the selective elimination of the connective tissue cells. It was interesting that these epithelial masses neither grew nor differentiated in the absence of connective tissue, but merely survived in a quiescent state.

The addition of connective tissue to the aggregates, however, caused them to resume their growth and development; first mitotic activity reappeared and ultimately the cells differentiated. These results imply an intimate histodynamic relationship between the two components of the glandular tissue. Flint (1903)
and afterwards Moral (1915–16) suggested that a reciprocal morphogenetic action of the connective tissue and the epithelium exists in the developing sub-maxillary gland. Heidenhain (1921) in his ‘synthetic theory of tissues’ advanced the idea that the growing glands proliferate and develop as tissue systems, based on a close morphological and physiological relationship between all the constituents. Experiments in vitro with chick metanephros (Rienhoff, 1922) and mammalian submandibulary glands (Borghese, 1950) provided substantial evidence of the reliance of the epithelial anlage upon the presence of connective tissue in morphological development.

Although it can be assumed that in developing glands there exists an intimate relationship between the epithelial and connective tissues, there is at present no evidence as to the nature of their interactions. The complexity of the problem is stressed by the observations of Grobstein (1953 a, b, c), which suggest that the morphogenesis of some glandular epithelia, e.g. mouse submandibular gland, depends upon the specific properties in the mesenchyme normally associated with them. On the other hand, the present experiments with chick pituitary in vitro showed that the typical differentiation of the glandular epithelium can be achieved in association with extrinsic homologous mesenchymal tissue.

These results are corroborated by the more recent experiments of Grobstein (1955) in which it has been shown that the capacity of inducing and promoting the differentiation in vitro of some isolated glandular epithelia, e.g. nephric tubules, is not limited to the original mesenchyme of the glandular rudiment.

**SUMMARY**

1. A method for the partial or complete disintegration of the pituitary tissue of the 8-day embryonic chick is described.

2. The anterior lobe, after being partially disintegrated by incomplete dissolution of the intercellular substance, developed in vitro into typical glandular tissue.

3. After dissociation of the anterior lobe into a suspension of discrete cells, the epithelial cells reaggregated under suitable conditions in vitro.

4. The epithelial aggregate remained in a quiescent but apparently healthy state during further cultivation.

5. The addition of perichondrial connective tissue to the aggregates caused them to resume growth and development and to differentiate into typical anterior lobe tissue.

6. The mutual relationship of the connective and epithelial tissues in developing glands is discussed.

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REFERENCES


EXPLANATION OF PLATES

PLATE 1

Fig. A. Longitudinal section through the anterior lobe of the pituitary of an 8-day chick embryo, showing the hypophyseal lumen (HL) and the developing cords. Ehrlich's haematoxylin, eosin. × 480.

Fig. B. Section through the anterior lobe after partial disintegration by treatment with solutions of trypsin and collagenase. Azan. × 240.
FIG. C. Section through a culture of partially disintegrated anterior lobe tissue after 2 days in vitro showing formation of cords and lobules. Ehrlich’s haematoxylin, eosin. ×160.

FIG. D. Section through a culture of partially disintegrated anterior lobe tissue after 5 days in vitro showing differentiated acidophil (A) and basophil (B) cells. Azan. ×1,140.

FIG. E. Smear of a suspension of pituitary cells in the treatment medium. Many cells show pseudopodial protrusions (p); others are rounded (r). Delafield’s haematoxylin, eosin. ×960.

FIG. F. Section through a primary aggregate formed during 24 hours in a suspension of anterior lobe cells in liquid culture medium. Azan. ×240.

FIG. G. Section through a group of clusters formed in a suspension after 48 hours of cultivation in liquid medium. Azan. ×240.

Plate 2

FIG. H. Section through a group of aggregates cultivated on a plasma-embryo extract clot for 14 days. Note the healthy appearance of the majority of cells. Azan. ×720.

FIG. I. Section through a culture of aggregates grown alongside a piece of cartilage for 4 days, showing penetration of fibroblastic strands between the epithelial cells and formation of small lobules. Azan. ×480.

FIG. J. Section through a culture of aggregates combined with cartilage, after 6 days in vitro, showing a glandular lobe in which mitoses (m) and chromophilic cells (ch) appear. Azan. ×960.

FIG. K. Another section through the same culture as above, showing mitoses and acidophil cells under higher magnification; A, acidophil cells; M, mitosis; C, cartilage; PT, pituitary tissue. Azan. ×1,140.

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Plate 1