Differentiation of Argyrophil and Argentaffin Cells in Organotypic Cultures of Embryonic Chick Intestine

by Valerio Monesi

From the Biological Division of the National Committee for Nuclear Research, Rome

WITH TWO PLATES

INTRODUCTION

The problem of the differentiation of enterochromaffin cells in the isolated intestine of the chick was first tackled by Simard & van Campenhout (1932) and by Ghidini (1940) by means of chorioallantoic grafts. They showed that enterochromaffin cells, demonstrated by argentaffin methods, differentiate in the grafts in the same way as they do in normal developmental conditions.

These results disproved any passage of enterochromaffin substance (enteramine) from the lumen of the gastro-intestinal tube, but still left the question open as to whether this substance is synthesized in the enterochromaffin cells themselves or whether it reaches them through the blood circulation from other sites of the organism. In the latter case the intestinal wall would work merely as an organ of deposition or excretion of circulating enteramine. Organotypic cultures of intestine, in which the organ is completely isolated from any humoral connexion with the whole organism, may be the means of solving the problem concerning the ability of intestinal cells to elaborate the substance in question.

In this connexion other problems referring to the histogenesis of enterochromaffin cells may be considered, such as: (1) whether they are especially differentiated epithelial cells of endodermic origin, as is believed by most authors (Parat, 1924 a, b; Cordier, 1926; Simard & van Campenhout, 1932; Vialli & Erspamer, 1936; Erspamer, 1954; Vialli, 1954) or whether they are formed in the connective tissue and then migrate into the epithelium, as claimed by Kull (1913, 1925) and Dias-Amado (1925 a, b); and (2) whether the same succession of stages as has been shown to occur in normal developmental conditions from argyrophil pre-enterochromaffin to argentaffin enterochromaffin stage takes place in vitro. This question is discussed in another paper by the present author (Monesi, 1960). The two stages mentioned above are also believed, on theoretical

1 Author's address: Biological Division of the National Committee for Nuclear Research, Research Group for Embryology, Sincrotrone, Frascati, Rome, Italy.


In order to know whether the enterochromaffin cells can differentiate in the isolated intestine cultivated in vitro, it is essential first of all to determine the developmental stage in which no such cells are present in the material to be used for the cultures. The experimental research in vitro was therefore preceded by a preliminary systematic study of the intestine of chick embryos at different ages, at intervals of 24 hours, from the 8th day of incubation until hatching, so as to establish the age and the sites in which enterochromaffin cells appear. The details of this research are published elsewhere (Monesi, 1960) and only the conclusions are given here. The first argentaffin cells, demonstrable with the Masson–Hamperl method, appear in the intestine of the chick embryo at the 15th–16th day of incubation in two places, the duodenum and the umbilical loop near its apex. In later stages they spread to the whole of the small intestine. Argyrophil cells, positive by the Bodian method, differentiate at an earlier stage and may be found as early as the 14th day, in the same parts of the intestine. Consequently, between the 14th and the 15th–16th day, the enterochromaffin system is represented only by argyrophil cells. This supports the theory put forward by Erspamer (1937, 1938, 1954), Vialli & Erspamer (1939), and Vialli (1954) that the argyrophil, non-argentaffin granules are the forerunners of the typical enterochromaffin ones, which combine both characteristics, being at the same time argyrophil and argentaffin. For a short period, beginning with the appearance of the argentaffin cells, i.e. at the 16th and 17th day of incubation, argyrophil cells show a remarkable prevalence over argentaffin cells, as they are four times as numerous as the latter. In more advanced stages the numerical relationship comes down to about 2:1, and remains so throughout life. Most of these cells, both argyrophil and argentaffin, are situated from their first appearance in the epithelium: only a very small number of them may be found, with the same frequency at any age, in the connective tissue of the lamina propria.

**MATERIAL AND METHODS**

Pieces of intestine of chick embryos, from the 9th to the 19th day of incubation, were cultured in vitro. From what has been said above, the stages until the 13th day doubtless precede the first appearance of argyrophil (14th day) and argentaffin cells (15th–16th day). The experiments made with embryos older than the 13th day were expected to show the behaviour of the argyrophil (14th–15th day) or of the argentaffin (16th day onwards) cells already in existence.

The parts of the intestine chosen for the cultures were the descending parts of the duodenum and the summit of the umbilical loop. These parts, besides being easy to identify, correspond to the sites where enterochromaffin cells begin to differentiate, and therefore offer a better chance of positive results.

The embryos belonged to the same breeds (New Hampshire and Rhode Island)
used for the preliminary in vivo research already quoted. However, to allow for individual variability in the age of appearance of enterochromaffin cells, control tissues were taken, at the moment of explantation, from all embryos employed for the cultures; these control tissues were obtained from the intestine immediately contiguous to the piece which was used for culturing. The sites of controls and cultures were alternatively exchanged.

The technique employed was to culture on the surface of a clot, in a watchglass contained in a Petri dish, according to Fell & Robison (1929). The clot consisted of 5 drops of a non-heparinized adult cock plasma and 5 drops of extract from 9-day chick embryos. Some cultures were made in embryonic extract only in order to avoid the possibility that some enterochromaffin substance might reach the tissue through the plasma contained in the medium.

From each embryo, and from each of the two parts of the intestine selected, 3–5 cultures were made depending upon the size of the embryos. The cultures were kept in an incubator at 37° C. for 2–6 days, and the medium was changed every 2nd day. It was not possible to continue cultivation beyond 6 days because of the constant appearance of general degeneration.

At the end of the experiments all cultures were washed in Tyrode and fixed, some in 10 per cent. neutral formol, others in a mixture of ethyl alcohol (90 parts), formol (5 parts), and acetic acid (5 parts). In addition, to provide a general idea of the pattern of the cultures, a few sections were stained in haematoxylin-eosin, and some were fixed for this purpose in Susa fluid.

The histochemical research on enterochromaffin cells was carried out by means of two methods: (1) the argentaffin reaction of Masson–Hamperl, after fixation in formol, which according to Erspamer (1937, 1938, 1954), Vialli & Erspamer (1939), and Vialli (1954) demonstrates the typical enterochromaffin cells. The immersion in ammoniacal silver nitrate always lasted 24 hours; and (2) the argyrophil reaction of Bodian after either of the two fixations mentioned above. This shows both typical enterochromaffin cells and argyrophil pre-enterochromaffin ones. The modification suggested by Dawson & Barnett (1944) was adopted, which consists in repeating twice the treatment with protargol and the subsequent reduction. Moreover, at the end of the second impregnation, and before reduction, the preparations were taken out of the incubator at 37° C. and put into another incubator at 60° C. for 4 hours. This treatment is specially useful for the cultures of intestine of young embryos, in which argyrophil granules are very difficult to impregnate.

The difference between the number of Bodian positive argyrophil cells and Masson positive argentaffin ones should indicate the number of pre-enterochromaffin cells. However, Hamperl (1952) and Hellweg (1952) claim that a small part, about 5 per cent. of the total enterochromaffin system, consists of only argentaffin, non-argyrophil cells.

It is generally admitted (Vialli & Erspamer, 1939; Erspamer, 1954; Vialli, 1954) that the two types of cells belonging to this system do not represent
AND ARGENTAFFIN CELLS

cellular categories, but successive stages of the same secretory cycle. In other words, pre-enterochromaffin, argyrophil granules represent an early step in the synthesis of the enteramine, which ends with the argentaffin stage. However, this conclusion cannot be completely generalized. In fact, in some conditions (Erspamer, 1937; Vialli & Erspamer, 1939; Dawson, 1948; Dawson & Moyer, 1948; Vialli, 1954) the enterochromaffin system is represented by argyrophil cells only, so that this stage is the final one.

RESULTS

Cultures of intestine of 9- and 10-day embryos kept their normal structure, even after 6 days of culture, and went on differentiating morphologically and cytologically. The villi, which had just begun to form when the culture was begun (Plate 1, fig. 1), increased in number and reached their definitive shape during the period of cultivation (Plate 1, fig. 2). It may be remembered that formation of villi in vitro was observed also by Chlopin (1922) in explants of intestine of rabbit embryos.

Cultures of intestine explanted in more advanced stages from the 12th day onwards with villi which had already reached a considerable development (Plate 1, fig. 3), when transversely cut as small portions of tubes, preserved their original structure fairly well at the ends of the explants, from which the villi tended to protrude; but the central zone of the cultures, especially in the part immediately touching the clot, degenerated very rapidly and became grossly necrotic as early as 4 days after explantation. To avoid this, a different technique was used for most cultures made from these stages. A piece of intestine, after being transversely cut, was divided longitudinally into two halves. These, through muscular contraction, rolled up with the epithelium turned outward. In this case also the immediate contact with the plasma had an unfavourable effect on the preservation of the villi, which disappeared, while the epithelium flattened, as Fischer (1922) had noted in explants of intestine taken just before hatching. But all the rest of the surface which remained free preserved its villi, although these changed their shape to a greater or lesser extent, the more remarkably so the older the stage at explantation. The most frequent alteration was a rarefaction and swelling of the stroma (Plate 1, fig. 4) which, however, did not affect the structure of the epithelium and, in any case, was not general, because even in these cases quite normal-shaped villi were found (Plate 1, fig. 5).

In any case, the epithelium appeared to be the most resistant structure in the cultures, and although in some parts it was flattened it preserved its normal shape in many areas of all cultures so as to allow, with appropriate methods, recognition of the enterochromaffin cells, which are the main object of the present research.

The cultivated fragments often showed slow muscular contractions similar to that observed and widely discussed in many papers concerning cultures of intes-
tine and stomach of chick embryos (Fischer, 1922; Bisceglie, 1932; Gozzi, 1940; De Jong & De Haan, 1943; Keuning, 1948).

The results concerning the time of appearance and the general behaviour of the cells giving argyrophil and argentaffin reactions are summarized in Table 1. For each embryonic stage figures are given of the number of embryos used, the number of all cultures made, and the number of those fixed in formol at different times. For the sake of simplicity, cultures fixed at 2 and 3 days, and at 4 and 5 days, were grouped together. All cultures were serially sectioned and stained in different sections for both argyrophil and argentaffin cells so as to allow cultures of any age to be listed as negative, argyrophil positive, and argentaffin positive. It will be clear from what follows that the second and third items of this classification coincide almost completely, so that the total number in each line of any group corresponds to negative cultures plus the highest number entered in one of the positive ones.

The last column includes all cultures not utilizable from this point of view, whether accidentally lost after fixation or infected or totally degenerated, or fixed in other ways than in formol.

In no culture of intestine of 9-day chick embryos did argyrophil or argentaffin cells develop, even after 6 days of culture.

Cultures of intestine of 10-day embryos, containing no argyrophil or argentaffin cells at the time of explantation, developed neither of these cells in 2-5 days; but after 6 days of cultivation, two cultures showed the presence of a small number, 4-5 in each culture, of argentaffin cells (Plate 2, fig. 6). In one of these cultures a few cells giving the argyrophil reaction were also found.

In cultures of intestine made from 12- and 13-day-old chick embryos, which therefore still belonged to a stage at which both argyrophil and argentaffin cells are absent, these cells first begin to appear after 4 days in vitro (Plate 2, figs. 7, 10). At the 5th and 6th day of culture their number has remarkably increased. Some of them are illustrated in figs. 8, 9, and 11 of Plate 2.

No regular appearance of argyrophil cells before the argentaffin cells was ever observed in these cultures. No general rule can be given about the frequency of the silver-impregnated cells, as their number varies remarkably from culture to culture and also in different sections of the same culture according to the state of health of the epithelium. In the most favourable cultures, fixed 4 days after explantation, the number of argentaffin cells can be from 0 to 6 in each 7-μ-thick transverse section. The number of argyrophil cells can be equal or slightly, but not significantly, superior. No attempt was made to do a systematic count of the cells, as it would not give reliable results because of the different state of preservation of the histological structure of the explants. It may be safely stated, however, that both argyrophil and argentaffin cells differentiated in vitro are always fewer than those which develop in vivo in the same time. Also, the number of silver-impregnated granules found in the cells of the cultivated intestine is smaller than that found in the whole embryo in normal
<table>
<thead>
<tr>
<th>Age of the embryos (days)</th>
<th>Number of embryos used</th>
<th>Total number of cultures made for each age</th>
<th>After 2–3 days</th>
<th>After 4–5 days</th>
<th>After 6 days</th>
<th>Total number of cultures lost or fixed otherwise than informol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Containing neither argyrophil nor argentaffin cells</td>
<td>Containing argyrophil cells</td>
<td>Containing argentaffin cells</td>
<td>Total number</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>43</td>
<td>2</td>
<td>...</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>52</td>
<td>4</td>
<td>...</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>36</td>
<td>7</td>
<td>...</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>13</td>
<td>4</td>
<td>44</td>
<td>8</td>
<td>...</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>54</td>
<td>12</td>
<td>7</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>60</td>
<td>7</td>
<td>8</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>20</td>
<td>...</td>
<td>10</td>
<td>10</td>
<td>...</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>15</td>
<td>...</td>
<td>8</td>
<td>8</td>
<td>...</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>12</td>
<td>...</td>
<td>10</td>
<td>10</td>
<td>...</td>
</tr>
</tbody>
</table>
conditions. In the cultures, the granules are more frequently limited to the basal pole of the cells, and the number of cells containing granules throughout their cytoplasm is much smaller.

Most argyrophil and argentaffin cells which have developed in vitro make their first appearance in the epithelium, i.e. they are found in the same situation as in vivo. Only very few are situated in the connective tissue of the lamina propria.

In cultures from 14- and 15-day embryos, belonging to a stage in which a few argyrophil cells are already present at explantation, numerous argyrophil and argentaffin cells were found after only 2 days of cultivation. The number of the former increased in culture; the latter were formed during cultivation. In 4 days' time the number of argentaffin cells in a transverse 7-μ-thick section ranged from 2 to 10 when the cultures came from 14-day embryos, and from 5 to 20 when they came from 15-day-old embryos. The number of argyrophil cells is more or less equal, or perhaps very slightly higher than that of the argentaffin cells. The number of both increased remarkably after 4 and 5 days of cultivation. The total amount of granules of both types increased when compared with the cultures of intestine of 12- and 13-day-old embryos; the number of cells containing granules throughout the cytoplasm also increased.

Embryos of 16, 17, and 19 days contain numerous argyrophil and argentaffin cells; in cultures of these stages the number of both types of cells appears to increase during cultivation.

In order to avoid the possibility that some enteramine contained in the adult cock plasma used for the medium was the source of the enterochromaffin granules differentiated during cultivation, some cultures were prepared using as a medium only extract made from 9-day-old embryos. Although the behaviour of the cultures was less favourable, the appearance of silver impregnable cells was observed.

**DISCUSSION**

The present research shows that enterochromaffin cells differentiate in the intestine of the chick embryo cultivated in vitro. They appear in organotypic cultures of intestine explanted from 10-, 12-, and 13-day-old embryos, belonging to stages before the first argyrophil and argentaffin cells are produced. The two cell types appear in vivo only at the 14th and 15½—16th day respectively. The absence of such cells at explantation time was proved not only by a systematic study of other embryos of the same breed and age, incubated with those used for the experiment, but also by a systematic study of a strictly contiguous zone of intestine from the same embryos used for the cultures. In cultures from embryos of later stages the argyrophil cells, present from the 14th day onwards, and the argentaffin cells, first appearing at the 15½—16th day, increased in number during the period of cultivation.

However, the total number of both types of cells differentiating in vitro is
evidently always smaller than that found in normal conditions at the same total age in vivo, and the number of granules is also inferior.

In culture, no stage was found in which argyrophil cells only are present; this is contrary to what occurs in vivo where, as shown elsewhere (Monesi, 1960), argyrophil cells precede the appearance of argentaffin cells by two days. It cannot, however, be concluded that the usual succession from the argyrophil, pre-enterochromaffin stage to the final argentaffin or enterochromaffin proper, does not take place in culture. It may be suggested that the passage from the first to the second stage occurs in vitro more quickly than in vivo, so that the former is unlikely to be observed. This assumption is perhaps supported by the observation that in culture, the frequency of argyrophil cells is more or less the same or very slightly higher than that of the argentaffin cells, whereas in vivo it is 3 or 4 times as much in the early stages of their differentiation, and comes down to twice in more advanced stages and at hatching.

As in vivo, most enterochromaffin cells, either argyrophil or argentaffin, which differentiate in culture are situated from their first appearance in the epithelium, occupying its whole thickness from the basement membrane to the intestinal lumen. The impression that one sometimes gathers, both in vivo and in vitro, that some are outside the epithelium, probably derives from the fact that the nuclei of enterochromaffin cells are often at a lower level than those of the remaining epithelial cells (Plate 2, figs. 10, 11). The observations by Masson (1928) and Feyrter (1953) seem also to confirm this tendency of enterochromaffin cells to bulge towards the lamina propria while remaining above the basement membrane.

However, apart from that, in the cultures as well as in natural developmental conditions a small number of cells containing argyrophil or argentaffin cells were found outside the epithelium, i.e. in the connective tissue of the lamina propria. This finding does not depend upon tangential sections of the epithelium. As in vivo, in no case is there a stage in which the enterochromaffin cells have, exclusively or prevalently, an extra-epithelial situation: the first argyrophil or argentaffin cells which differentiate in culture are mostly in the epithelium, very few being in the connective tissue; the frequency of the latter does not change when cultivation is prolonged. It may therefore be concluded that, like the results obtained in the study of development in vivo (Monesi, 1960), the results from cultures disprove the theory of Kull (1913, 1925) and Dias-Amado (1925 a, b) that enterochromaffin cells differentiate in the mesenchyme and subsequently migrate into the epithelium. On the contrary, it is more likely that extra-epithelial enterochromaffin cells are, as suggested by Simard & van Campenhout (1932), originally intra-epithelial cells which then wander into the connective tissue. Of course, all these considerations refer only to the cells when they are identifiable through specific methods: it cannot be excluded that in the differentiation of enterochromaffin cells an earlier stage may exist in which they do not contain granules, or in which the granules do not give the characteristic reactions.
The differentiation of enterochromaffin cells as shown to take place in the isolated intestine cultured \textit{in vitro} gives substantial support to the theory of the synthesis inside the intestinal cells of the enterochromaffin substance, or enteramine. Experiments with chorioallantoic grafts (Simard & van Campenhout, 1932; Ghidini, 1940) proved that this substance is not derived from the lumen of the gut. The fact (Simard & van Campenhout, 1932) that the cells in question appear in grafts made from a stage preceding that of the establishment of the normal nervous connexions (92nd hour of incubation according to van Campenhout, 1931) is against the theory put forward by Danisch (1924) that these cells are nervous or formed under the influence of the nervous system.

The present research, which separates the intestine from the influence of the blood circulation, proves that the enteramine is elaborated by the enterochromaffin cells themselves and does not derive from other parts of the organism, and that the enterochromaffin system cannot be considered as an organ of deposition or excretion of enteramine. Nor can it be believed that enteramine contained in the plasma used as culture medium is deposited in the cultivated intestine. Apart from the fact that the plasma content of enteramine is in any case extremely low, and probably zero according to the most recent work (Erspamer, 1954), it should be noted that enterochromaffin granules were observed also in cultures in which the medium consisted only of extract of chick embryos of 9 days of incubation.

**SUMMARY**

1. Argyrophil, Bodian positive cells, and argentaffin, Masson–Hamperl positive cells differentiate \textit{in vitro} in organotypic cultures of intestine of chick embryos explanted before argyrophil and argentaffin cells are known to differentiate, which is at the 14th and the 15½–16th day of incubation, respectively.

2. During culture \textit{in vitro}, argyrophil and argentaffin granules differentiate simultaneously; the number of cells demonstrable with either method does not show any significant difference throughout the duration of the cultures, which were incubated up to a maximum of 6 days. This result is at variance with what occurs \textit{in vivo}, where argyrophil cells differentiate earlier than argentaffin cells and are in earlier stages 3 or 4 times as frequent as the latter, becoming only twice as numerous at a later stage.

3. As in development \textit{in vivo}, argyrophil and argentaffin cells differentiating in the intestine of the chick embryo are mainly situated in the epithelium. Only very few are found in the connective tissue of the lamina propria.

4. Simard & van Campenhout (1932) and Ghidini (1940) showed that enterochromaffin granules differentiate in chorioallantoic grafts of intestine of chick embryos and they disproved in this way any kind of derivation of the enterochromaffin substance (enteramine) from the lumen of the gastrointestinal tract, as well as its formation under the influence of normal nervous connexions. It is now shown that the specific granules differentiate also in intestine cultivated \textit{in}}
vitro and therefore in conditions which exclude any other possible mode of correlation with the whole organism, and particularly any uptake of enteramine from the blood, thus strongly supporting the conclusion that it is synthesized in situ.

ACKNOWLEDGEMENTS

Thanks are due to Professor E. Borghese and Professor M. Vialli for their many helpful suggestions and criticisms and to Dr. E. Vivori for her very kind help with the English text.

REFERENCES


V. MONESI

Plate 2
AND ARGENTAFFIN CELLS


EXPLANATION OF PLATES

**PLATE 1**

Fig. 1. Intestine of a 9-day chick embryo, near the apex of the umbilical loop, showing a few villi just forming. Formol; haematoxylin and eosin. ×165.

Fig. 2. Intestine of a 9-day chick embryo, near the apex of the umbilical loop, cultivated for 5 days. The villi have increased in number and taken a more definite shape. Formol; carmalum. ×165.

Fig. 3. Intestine of a 12-day chick embryo, near the apex of the umbilical loop, showing numerous villi. Formol; haematoxylin and eosin. ×90.

Fig. 4. Intestine of a 12-day chick embryo, near the apex of the umbilical loop, longitudinally cut when explanted and cultivated for 6 days. In spite of great morphological deformation, almost normal villi are still present in some parts of the culture, as in the centre of the figure; most of them are short and broad: the epithelium is consistently normal. Formol; haematoxylin and eosin. ×90.

Fig. 5. Intestine near the apex of the umbilical loop, explanted at the 13th day and cultivated for 5 days. A detail showing very good preservation of the villi with their epithelium. Alcohol-formol-acetic acid; haematoxylin and eosin. ×295.

**PLATE 2**

Fig. 6. Piece of umbilical loop explanted at the 10th day of incubation and cultivated for 6 days. An argentaffin cell, which appeared during culture. Formol; Masson–Hamperl. ×1950.

Fig. 7. Piece of umbilical loop explanted at the 12th day of incubation and cultivated for 4 days. Two cells showing argentaffin granules at their bases, which appeared during culture. Formol; Masson–Hamperl; Carmalum. ×430.

Fig. 8. Piece of umbilical loop explanted at the 12th day of incubation and cultivated for 6 days. An argentaffin cell which appeared during culture. Formol; Masson–Hamperl. ×1950.

Fig. 9. Piece of umbilical loop explanted at the 12th day of incubation and cultivated for 6 days. An argyrophil cell which appeared during culture. Formol; Bodian. ×1950.

Fig. 10. Piece of duodenum explanted at the 13th day of incubation and cultivated for 4 days. An argyrophil cell which appeared during culture. Formol; Bodian. ×1950.

Fig. 11. Piece of duodenum, explanted at the 13th day of incubation and cultivated for 5 days. An argyrophil cell which appeared during culture. Formol; Bodian. ×1950.

*(Manuscript received 10:xii:59)*