A GRADUAL DECREASE IN NUCLEOLAR SIZE WITH THE MATURATION OF COLUMNAR EPITHELIAL CELLS IN THE ADULT RAT INTESTINE UNDER NORMAL AND VARIOUS EXPERIMENTAL CONDITIONS

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SUMMARY
The columnar cells, which form over 90% of the epithelium in the small intestine, undergo rapid and continuous renewal and maturation. Samples from duodenum, jejunum, upper, mid- and terminal ileum of young male rats were processed for histology. The average maximal nucleolar area was determined in 10-cell-wide bands of the basal, mid and upper levels of crypts and villi, respectively, by image analysis; in the duodenum, it was 2.8, 2.1, 1.7, 1.5, 1.3 and 0.8 (in \( \mu \text{m}^2 \)) in the respective epithelial levels from crypt base to villus top. Although villus size decreased by 68% from duodenum to terminal ileum, nucleolar area was similar at each respective epithelial level along the intestine. This indicated that nucleolar size was related to cell maturity, rather than to the size of epithelium. In other groups of rats, the duodenum was examined after administering specific inhibitors. Methotrexate (within a day) and cycloheximide (within 3 h) did not significantly affect nucleolar size, indicating that the decrease in size was not under the influence of immediate synthesis of nucleic acid or protein. On the other hand, tunicamycin (within a day) delayed the decrease and actinomycin D (within 3 h) caused a maximal decrease in all nucleoli. This implied that a glycoprotein factor and some changes in DNA were involved in the decrease in nucleolar size. The rate of protein synthesis in duodenum was then measured by grain count per cell area in autoradiographs made after 1 h of injection of \([\text{H}]\)leucine. From crypt base to villus base, the grain count doubled while the nucleoli decreased to nearly half of their size in the crypt base. When actinomycin D injection preceded the \([\text{H}]\)leucine administration, all nucleoli decreased markedly and the grain counts increased by about 30% in all epithelial levels. It thus appears that the decrease in nucleolar size stimulates protein synthesis, possibly by the release of ribosomal material or some other factor. Protein synthesis in turn has been shown to be related to cell maturation. It is concluded that the nucleolus is involved in some manner in the regulation of the maturation and renewal of the epithelial cells.

INTRODUCTION
In a recent paper (Altmann & Leblond, 1982), marked changes were shown to take place in the size and ultrastructure of the nucleoli of the columnar epithelial cells of the jejenum as these cells migrate. These cells initially arise in the crypt base from the divisions of the stem cells (Cheng & Leblond 1974). Mitotic activity continues in the lower two thirds of the crypts in the so-called progenitor cells. At least 90% of these cells are committed to becoming columnar cells, which on reaching the villi will

Key words: nucleolus, maturation, epithelial cells.
function as absorptive cells. As the cells arising from the mitoses migrate into the upper third of the crypt, the cell cycle stops and differentiation into absorptive cells begins. The cells emerging on the villus are large and show morphological signs of being differentiated (Leblond, 1981). Further maturation takes place as the cells migrate to the mid-villus, as the cells show an increase in the production of the 'brush border' glycoprotein enzymes, and also there are quantitative and qualitative changes in the RNA synthesis (Bennett, Leblond & Haddad, 1974; Uddin, Altmann & Leblond, 1984). In about the upper third of the villus, the cells start to show degenerative changes (Falconer, 1982; Leblond, 1981), which culminate at the villus tip where the cells are finally extruded.

This whole rather complex phenomenon, which is collectively referred to as 'cell renewal' (Leblond & Stevens, 1948), is associated with specific changes in the morphology of the nucleolus. These changes have been described in qualitative and quantitative terms for the rat jejunum (Altmann & Leblond, 1982). Essentially, these changes involve, from crypt base to villus top, a gradual transformation of the large reticular nucleoli into small compact ones with a 16-fold decrease in nucleolar volume. The decrease in nucleolar volume can be measured in light-microscopic sections (Snow & Altmann, 1983). This latter technique provides a way of estimating the nucleolar size for a large number of samples and cells. This method was used in the present work to examine what happens to nucleolar size under various conditions of renewal so as to obtain some clues regarding the relation of nucleolar size to cell maturation and renewal. First, nucleolar size was measured in five regions along the small intestine. Quantitative differences in epithelial renewal have been described in these regions previously (Altmann & Enesco, 1967). Secondly, nucleolar size was examined under the influence of specific inhibitors such as methotrexate, cycloheximide, actinomycin D and tunicamycin. The effects on renewal of the first three of these drugs have been described previously (Altmann, 1974, 1975; Abdel-Wahab, 1977). Thirdly, the rate of protein synthesis was estimated in the epithelial cells by \( ^{[3H]} \)leucine radioautography. The results indicate that the nucleolar changes represent an integral part of cell renewal and associated changes of protein synthesis.

MATERIALS AND METHODS

Histological technique

Young adult male Wistar rats weighing 200–300 g were used. After an overdose of chloroform, the abdominal cavity was opened. The small intestine was immediately removed and pulled out to its full length. Taking the whole length of small intestine between pylorus and caecum as 100%, 2 cm long samples were taken at the following locations: 0–1% (duodenum), 25% (jejunum), 50% (upper ileum), 75% (mid-ileum), 99–100% (terminal ileum). The samples were cut open and flattened on a piece of cardboard. They were then immersed in Carnoy's fixative for 4–6 h. The samples were cleared in chloroform and embedded in paraffin. Longitudinal sections were cut at intervals of \( 5 \mu m \) and were stained with haematoxylin plus eosin or periodic acid–Schiff reagent (PAS).

For the drug studies and radioautography, the duodenal samples only were used.
Decrease in nucleolar size of cells with maturation

Measurement of nucleolar size

Well-oriented portions of the histological sections were selected under the low-power objective. Using longitudinally sectioned crypts and villi, a band of about 10 cell positions was selected at crypt base, mid-crypt, crypt top, villus base, mid-villus and villus top. Within these bands, the largest nucleoli were selected under the oil-immersion objective. The outlines of these nucleoli were then drawn using a Wild camera lucida system. The areas of these drawings were then measured using a Zeiss Videoplan image analyser.

The variation in size between the nucleoli was very small so the necessary number of nucleoli to be measured per sample was around eight, to obtain a standard error less than 10% of the mean. About 15–20 nucleoli were measured in each band per sample and the mean was calculated.

The basic method of measuring nucleoli by light microscopy has been worked out (Snow & Altmann, 1983). Toluidine Blue staining, and a mild digestion with DNase were used in that study. It appears, however, that a staining with haematoxylin light without DNase treatment is sufficient, because haematoxylin stains the nucleoli light purple and the surrounding heterochromatin dark blue or purple. The perinucleolar chromatin was distinguished from the nucleolus in this manner and was left out of the drawings.

Administration of the specific inhibitors

Groups of 5–6 rats were used for this study. One group received a large dose of methotrexate (Lederle): an initial subcutaneous injection of 5 mg, followed by 5 mg mixed into the food daily. This dose was previously found to be lethal after a 3-day latent period (Altmann, 1974). The animals in the present experiment were killed a day after the injection.

Another group received cycloheximide (Sigma) in a dose of 5 mg per animal, subcutaneously. The animals were killed 3 h later. This dose of cycloheximide was previously found to cause rapid atrophy of the epithelium, and finally denudation started at about 6 h (Altmann, 1975).

Three groups received actinomycin D (Cosmegen, Merck) in a single intraperitoneal injection of 1 mg per kg body weight. Groups were killed at 3, 6 and 24 h, respectively, after the injection. The dose of 1 mg/kg body weight is just sufficient to inhibit transcription without causing other toxic effects (Imondi, Lipkin & Balis, 1970).

Finally, a group received tunicamycin (Sigma) intraperitoneally in 3 injections at 10 h intervals: 0–5, 0–3 and 0–1 mg, respectively, per animal. The animals were killed 24 h after the first injection.

Radioautography

The aim of the radioautography was to measure the average rate of protein synthesis per cell. Tritiated leucine (Amersham) was administered to a group of rats at a dose of 1 μCi/g body weight. The animals were killed 1 h later. A second group received actinomycin D, 1 mg/kg body weight as above, and then [3H]leucine an hour later, as above. These animals were killed 2 h after the injection of actinomycin. Histological sections were prepared as usual and stained with haematoxylin and eosin. The coating technique of Kopriwa & Leblond (1962) was then used (with Kodak NTB2 emulsion), followed by 3 weeks of exposure.

The grain counts were carried out under the high dry objective and with the camera lucida system. Using longitudinally cut crypts and villi, well-outlined cells sectioned along their long mid-axis were selected within each of the six bands described above. The outlines of the cells were then drawn and these drawings was then superimposed on the corresponding microscopic images. All the silver grains were counted within these outlines. These counts are assumed to be proportional to the average rate of protein synthesis.

The outlines of the cells were also used to obtain an approximate estimate of the average cell size by measuring their area with the Zeiss Videoplan image analyser.

The grain count per cell within a band varied little, so that measuring about 5 cells per band was sufficient to get a standard error less than 10% of the mean. About 10 cells per band were measured in each sample and the mean was then calculated.

Other histometric measurements

Crypt size was measured by the so called 'crypt size index' described elsewhere (Altmann, 1972); it is the number of epithelial nuclei per representative crypt section. A crypt was considered
representative when it was sectioned along its full length and mostly along its lumen.

Villus size was measured also by an earlier method (Altmann, 1972). The 'villus size index' is the number of epithelial nuclei per representative villus section. Such a section was cut along the longitudinal axis of the villus.

**RESULTS**

*Pattern of change in nucleolar size in the various intestinal regions*

Nucleolar size was found to be about 2-7 \( \mu m^2 \) in the crypt base in all the intestinal regions. This size was diminished to about 1-9 \( \mu m^2 \) in mid-crypt and to 1-6–1-5 \( \mu m^2 \) in the crypt top. This was the pattern in all the intestinal regions (Figs 1, 2). The decrease can be shown to be linear, the line of best fit being: 

\[
n = 2.67 - 25.4 \times 10^{-3} \times C_p
\]

where \( n \) is the nucleolar size and \( C_p \) is the number of crypt cell positions. The correlation coefficient (\( r \)) was 0.98, indicating a significant straight line. The total number of crypt cell positions would be given by the number of cells on one side of

![Graph](image)

Fig. 1. Nucleolar area is plotted against crypt and villus position. Abbreviations used in Figs 1–3: CB, crypt base; MC, mid-crypt; UC, upper crypt; VB, villus base; MV, mid-villus; VT, villus top. This curve illustrates the extent by which the nucleolus decreases in area along the duodenal epithelium. This is inferred from two-dimensional measurements. The decrease in volume would be even more marked.
Decrease in nucleolar size of cells with maturation

Villus size and the number of cells constituting the villus epithelium were shown previously to display a marked decreasing gradient from the duodenum to the terminal ileum (Table 1) (Altmann & Enesco, 1967; Altmann & Leblond, 1970). The half value of the villus size index, taken as the number of villus cell positions ($V_p$), would also show a corresponding gradient. The maximal nucleolar size was 1.38 $\mu m^2$ on average at the villus base in all intestinal regions. It decreased to about 1.18 $\mu m^2$ in the mid-villus and to about 0.40 $\mu m^2$ in the villus top (Figs 1, 2). Thus, the decrease in nucleolar size was of the same extent in all intestinal regions regardless of the

Fig. 2. Crypt nucleolar area is plotted against crypt position on the left. The values from the five intestinal regions were averaged. On the right, villus nucleolar area is plotted against the number of cell positions on the villus. Each of the five intestinal regions is represented separately, the total number of cell positions being gradually less from duodenum to terminal ileum. The lines of best fit are also shown for the pooled values in the crypts on the left, and for the regional values in the villi on the right. The slopes for the different regions are shown to increase gradually from duodenum to terminal ileum.
Table 1. Pattern of nucleolar size in intestinal regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Crypt size index</th>
<th>CB</th>
<th>MC</th>
<th>UC</th>
<th>VB</th>
<th>MV</th>
<th>VT</th>
<th>Villus size index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>107.4 ± 1.54</td>
<td>2.79</td>
<td>2.06</td>
<td>1.71</td>
<td>1.51</td>
<td>1.25</td>
<td>0.81</td>
<td>302.2 ± 10.23</td>
</tr>
<tr>
<td>Jejunum</td>
<td>100.6 ± 0.93</td>
<td>2.79</td>
<td>1.99</td>
<td>1.70</td>
<td>1.43</td>
<td>1.17</td>
<td>0.76</td>
<td>244.6 ± 6.37</td>
</tr>
<tr>
<td>Upper ileum</td>
<td>91.2 ± 0.98</td>
<td>2.66</td>
<td>1.90</td>
<td>1.43</td>
<td>1.34</td>
<td>1.12</td>
<td>0.76</td>
<td>164.2 ± 1.80</td>
</tr>
<tr>
<td>Mid-ileum</td>
<td>90.2 ± 1.24</td>
<td>2.73</td>
<td>1.85</td>
<td>1.45</td>
<td>1.30</td>
<td>1.15</td>
<td>0.80</td>
<td>111.6 ± 1.96</td>
</tr>
<tr>
<td>Term. ileum</td>
<td>85.8 ± 1.16</td>
<td>2.72</td>
<td>1.79</td>
<td>1.51</td>
<td>1.29</td>
<td>1.22</td>
<td>0.75</td>
<td>97.8 ± 0.86</td>
</tr>
<tr>
<td>Average</td>
<td>95.04 ± 1.92</td>
<td>1.92</td>
<td>1.56</td>
<td>1.37</td>
<td>1.18</td>
<td>0.78</td>
<td>184.08</td>
<td></td>
</tr>
</tbody>
</table>

differing villus size indices. This implies that nucleolar size is related not to villus size *per se*, but rather to the degree of differentiation of the villus epithelial cells. This in turn means that as the villi get shorter along the intestine, the average decrease in nucleolar size per cell position rises. This decrease would thus be \(4.64 \times 10^{-3}, 5.64 \times 10^{-3}, 7.06 \times 10^{-3}, 8.96 \times 10^{-3}\) and \(11.04 \times 10^{-3}\) \(\mu m^2\) in the five respective intestinal regions (Fig. 2). The total number of villus cell positions would be, in this case, half the value of the villus size index.

The decrease in nucleolar size along the villi was linear in each intestinal region (Fig. 2), the correlation coefficients for the lines of best fit ranged between 0.92 and 0.99. The values given above for the decrease in nucleolar size per cell position are equivalent to the slope of the curve for nucleolar size *versus* villus cell position. The \(Y\) intercept was about 1.4 in each intestinal region. The general equation expressing nucleolar size on villus \(n_v\) would thus be: \(n_v = 1.4 - s \cdot v_p\), where \(s\) is the slope and \(v_p\) is the villus cell position. The slope \(s\) increases linearly with increasing distance from the pylorus. Its value can be obtained from the following equation:

\[s = (4.24 + 0.0645d) \times 10^{-3},\]

where \(d\) is the percentage distance from the pylorus (correlation coefficient for this equation, \(r = 0.99\)).

**Nucleolar size after specific interferences with metabolism**

**Methotrexate.** After 1 day, methotrexate caused a decrease in the crypt size index of about 14% and a decrease in the villus size index of about 29% in the duodenum (Table 2). The nucleolar size increased by 22% in crypt base and by 20% in mid-crypt; at the other epithelial levels nucleolar size showed no significant difference from the control values. Thus, the nucleoli decreased in a nearly normal manner along the epithelium during treatment with methotrexate. This decrease, however, was slightly accelerated in comparison with controls because it started at a higher level at the crypt base and spanned diminished crypts and villi. The migration of the cells during methotrexate treatment has previously been shown to continue (Altmann, 1974).
Decrease in nucleolar size of cells with maturation

Cycloheximide. Within 3 h, cycloheximide caused a decrease in the crypt size index of 7% and a decrease in the villus size index of 60% in the duodenum (Table 2). Nucleolar size values were similar to those in the controls except at the middle and top of the much shortened villi. In the mid-villus, nucleolar size was the same as that of the control villus base, and in the villus top it was the same as that of the control mid-villus. It appears from these results that the upper half of the villus epithelium was lost after the cycloheximide injection, and that cell migration stopped and nucleolar size remained unchanged in the cells. Indeed, the loss of the upper villus and the cessation of cell migration has been shown previously after cycloheximide administration (Altmann, 1975).

Actinomycin D. Within 3–6 h after the administration of actinomycin D, no significant change in crypt size index was noticed. The villus size index showed only a slight decrease, of about 9% (Table 2). Nucleolar size at all epithelial levels decreased to 0.5–0.6 μm², which seems to represent a maximal decrease. At 24 h, the nucleoli were still similarly diminished, but by this time quite a number of crypt and villus epithelial cells were lost, resulting in a decrease in the crypt size index of 29% and a decrease in the villus size index of 36%.

Tunicamycin. Within a day after injection of tunicamycin there was a substantial increase in the crypt size index and a substantial decrease in the villus size index. Adding the two indices together, a value of 278 was obtained for the treated animals. The corresponding value in the controls was 410. In the treated animals, 60% of this value belonged to the crypts and 40% belonged to the villi. In the controls, 26% belonged to the crypts and 74% belonged to the villi. Thus, while some loss of epithelium took place in the treated animals, a shift in the ratio of crypt cell number to villus cell number took place. Nucleolar size decreased normally along the epithelium, but now over elongated crypts and shortened villi. The average decrease

Table 2. Effects of inhibitors on nucleolar size in the duodenum

<table>
<thead>
<tr>
<th></th>
<th>Crypt size index</th>
<th>CB</th>
<th>Maximal nucleolar size (μm²)</th>
<th>Villus size index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MC</td>
<td>UC</td>
</tr>
<tr>
<td>Control</td>
<td>107.4 ± 1.54</td>
<td>2.79</td>
<td>2.06</td>
<td>1.71</td>
</tr>
<tr>
<td>MTX (1 d)</td>
<td>92.6 ± 2.7</td>
<td>3.39</td>
<td>2.48</td>
<td>1.78</td>
</tr>
<tr>
<td>CXM (3 h)</td>
<td>99.9 ± 3.9</td>
<td>2.56</td>
<td>2.14</td>
<td>1.78</td>
</tr>
<tr>
<td>ACT (3–6 h)</td>
<td>115.5 ± 2.7</td>
<td>0.58</td>
<td>0.59</td>
<td>0.57</td>
</tr>
<tr>
<td>ACT (24 h)</td>
<td>76.09 ± 2.3</td>
<td>0.48</td>
<td>0.48</td>
<td>0.54</td>
</tr>
<tr>
<td>TM (1 d)</td>
<td>165.6 ± 1.6</td>
<td>2.49</td>
<td>1.88</td>
<td>1.23</td>
</tr>
</tbody>
</table>


in nucleolar size per cell position was $1.39 \times 10^{-2} \mu m^2$ for the crypts and $6.42 \times 10^{-3} \mu m^2$ for the villi. The corresponding control values were $2.01 \times 10^{-2}$ and $4.63 \times 10^{-3} \mu m^2$. Thus, the decrease along the crypts slowed down considerably after injection of tunicamycin. The changes on the villus cannot be fully assessed because, within a day, renewal changes only the crypt and lower villus cell population (Leblond & Messier, 1961).

$[^3H]$leucine uptake

The grain count per cell area in the normal duodenum (Table 3, Fig. 3) increased from 17.1 at the crypt base to 35.9 at the villus base. This increase may be referred to as the ascending phase of protein synthesis. It involves a 2.1-fold increase. This was followed by a descending phase along the villus from a grain count of 35.9 at the villus base to 12.2 at the villus top, that is almost a threefold decrease.

Two hours after the injection of actinomycin D, in a dose sufficient to inhibit DNA-dependent RNA synthesis, $[^3H]$leucine uptake increased 1.3-fold at all epithelial

![Fig. 3. Counts of silver grains in radioautographs are plotted against crypt and villus positions. The grain count is expressed per average area of cells sectioned along their long mid-axis.](image-url)
TABLE 3. PROTEIN SYNTHESIS AND CELL SIZE IN THE DUODENUM

<table>
<thead>
<tr>
<th>Regions</th>
<th>CB</th>
<th>MC</th>
<th>UC</th>
<th>VB</th>
<th>MV</th>
<th>VT</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]leucine grain count per cell area, controls</td>
<td>17·1</td>
<td>2·4</td>
<td>26·6</td>
<td>35·9</td>
<td>20·5</td>
<td>12·2</td>
</tr>
<tr>
<td></td>
<td>±0·8</td>
<td>±0·8</td>
<td>±0·5</td>
<td>±1·0</td>
<td>±1·3</td>
<td>±0·8</td>
</tr>
<tr>
<td>[3H]leucine grain count per cell area, ACT-treated</td>
<td>22·2</td>
<td>28·5</td>
<td>34·6</td>
<td>45·5</td>
<td>24·0</td>
<td>13·5</td>
</tr>
<tr>
<td></td>
<td>±1·0</td>
<td>±1·0</td>
<td>±1·7</td>
<td>±1·1</td>
<td>±0·9</td>
<td>±0·9</td>
</tr>
<tr>
<td>Average cell area, controls (μm²)</td>
<td>72·3</td>
<td>81·8</td>
<td>97·6</td>
<td>165·8</td>
<td>147·8</td>
<td>82·8</td>
</tr>
<tr>
<td></td>
<td>±6·0</td>
<td>±4·3</td>
<td>±5·1</td>
<td>±7·5</td>
<td>±6·9</td>
<td></td>
</tr>
<tr>
<td>Average cell area, ACT-treated (μm²)</td>
<td>86·4</td>
<td>110·1</td>
<td>132·2</td>
<td>188·7</td>
<td>147·3</td>
<td>100·0</td>
</tr>
<tr>
<td></td>
<td>±4·4</td>
<td>±3·8</td>
<td>±2·3</td>
<td>±8·5</td>
<td>±4·4</td>
<td>±4·4</td>
</tr>
</tbody>
</table>

CB, crypt base; MC, mid-crypt; UC, upper crypt; VB, villus base; MV, mid-villus; VT, villus top, ACT, actinomycin D.

The increase was only 1·2-fold in the mid-villus and 1·1-fold in the villus top (Table 3, Fig. 3).

In the normal duodenum, both the ascending and the descending phases of [3H]leucine uptake were linear (Fig. 3). Denoting leucine uptake by \( L \) and crypt cell position by \( C_p \), the equation for the ascending phase is: \( L = 16·2 + 0·27 C_p \). Denoting villus cell position by \( V_p \), the equation for the descending phase is: \( L = 34·7 - 0·16 V_p \). The correlation coefficients for the two equations were 0·91 and 0·99, respectively.

**Average cell area**

The average cell area appeared to be proportional to the [3H]leucine uptake (Table 3) by a factor of 4 between crypt base and villus base, and by a factor of 6–7 for the mid-villus and upper villus. This proportionality was also present after the actinomycin D treatment (Table 3, Fig. 3).

Similarly, the cell area like the [3H]leucine uptake, also displayed an ascending phase between crypt base and villus base and a descending phase between villus base and villus top.

The increase in cell area after actinomycin D treatment was about 1·3-fold at levels between crypt base and villus base. In the mid-villus and villus top levels the increase was smaller.

**DISCUSSION**

The probable mechanism of the decrease in nucleolar size

The decrease in the nucleolar size seems to be an orderly progression from a large reticulated nucleolus in crypt base cells to a small compact nucleolus in the villus top cells. According to previous observations (Altmann & Leblond, 1982), an essential change is the coalescence of the fibrillar centres. The reticulated nucleoli were seen...
to contain about 12 fibrillar centres in a typical section. In the successive epithelial levels this number decreased to 10, 7, 4, 3 and 1, respectively. At the same time, the size of the individual fibrillar centres increased, so that the total area occupied by the fibrillar centres remained the same, that is about 0.9 \( \mu m^2 \).

The electron-microscopic images of the fibrillar centres suggested spherical structures. If they are spherical, they probably correspond to the arrangement observed by Mirre & Stahl (1981) in the nucleoli of mouse oocytes, in serial sections. Accordingly, the fibrillar centres would be condensations of the nucleolar deoxyribonucleoprotein (DNP) network and would be interconnected by strands of extended chromatin. These strands in the intestinal cells may run in the system of interstitial (or 'vacuolar') spaces, as these canal-like spaces appeared to provide for communication between the individual fibrillar centres (unpublished observation by the author). During the decrease in nucleolar size, the most conspicuous and rapid event was the diminution and eventual disappearance of the interstitial spaces. This, in combination with the coalescence of the fibrillar centres, indicated a kind of condensation or perhaps a 'heterochromatinization' of the DNP framework.

During the formation of the nucleolus (nucleologenesis) at the end of mitosis (Chouinard, 1975) or during meiosis (Mirre & Stahl, 1981), the DNP framework develops from the extension of the nucleolar organizing regions (NOR) of the chromosomes. The framework would therefore be continuous with the extranucleolar chromatin, which has also been seen to condense with advancing cell maturation (unpublished observation by the author). The hypothesis is then that the decrease in nucleolar size is a result of some influence on the cells, which leads to the condensation of the DNP framework.

Further observations showed that each fibrillar centre was surrounded by an incomplete ring of pars fibrosa, which in turn was surrounded by spots of pars granulosa. In the reticulated nucleoli, then, several of these combined structures were aligned to form the structure that was formerly called the nucleolonema. The coalescence of the fibrillar centres would bring about a merging of the surrounding structures as well. In the extreme case there would be one remaining fibrillar centre surrounded, usually, by one band of pars fibrosa, which in turn would be in contact with one or two spots of pars granulosa. By this time, however, the total volume of the pars fibrosa and pars granulosa is greatly reduced, while that of the fibrillar centre is unchanged (Altmann & Leblond, 1982). The basic situation at this stage is that the nucleolar components, which were originally dispersed in several spots in the nucleolus, are now segregated into a few discrete bands or spots. This extreme case has been referred to in the literature as 'nucleolar segregation'.

Nucleolar segregation has been known for some time to be produced by certain drugs that interact with the DNA templates and thereby inhibit transcription (Simard, 1970). It seems that nucleolar segregation is very similar, if not identical in its end result, to the natural decrease in the nucleolus (Adamstone & Taylor, 1972). The natural phenomenon, however, may take much longer. It has been reported that the drugs used for producing nucleolar segregation interact with the extended chromatin and may condense it (Schoefl, 1964). At the same time, transcription is
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inhibited but the processing of the still available ribosomal RNA continues, so that ribosomes may still form (Daskal, 1979).

Since the natural decrease in the size of the nucleolus was not seen to be associated with degeneration or fragmentation, it is probable that the major cause of the decrease in size was the continued processing of the preribosomal material present. At the same time, the transcription of the preribosomal material became progressively less. This latter event has been demonstrated by radioautography (Uddin et al. 1984).

In conclusion, the primary event in the decrease in the size of the nucleolus along the epithelium seems to be a condensation of the DNA framework. This would result in a concomitant decrease in transcription. The formation and release of ribosomal material probably continue by the continued processing of the stored preribosomal material. This latter event could be the main cause of the decrease in the overall nucleolar size.

Relation of the decrease in nucleolar size to the renewal of the epithelium

Along the small intestine, a distinct regional variation in renewal has been shown (Altmann & Enesco, 1967). This variation was associated mainly with the gradient in villus size. In the crypts the number of cells produced and the speed by which the cells migrate to the villi do not change significantly with intestinal region. Thus, about the same number of cells move through the villi at about the same speed. But the speed of maturation into extruding cells on the villus increases toward the ileum (Altmann, 1976). So it is really the point of extrusion that changes. For example, the duodenal villus epithelial cells are capable of staying on the villus for 40 h before extrusion. On the other hand, in the terminal ileum, the time from the emergence of cells on the villus base to extrusion at the villus top is only 17 h.

Some key events in the course of the renewal of columnar cells are the birth in crypt base, inhibition of cycling in upper crypt, emergence on villus at the villus base, and extrusion at the villus top. The present results indicate that all these events are characterized by a specific nucleolar size regardless of the difference in size of the epithelial structures. Thus, when the speed of maturation is slow, as in the duodenal epithelium, the speed by which the nucleoli decrease is also slow. In the ileum, the speed of maturation is increased and there is a corresponding increase in the rate of diminution of the nucleoli.

These findings become more significant when it is considered that factors in the intestinal lumen establish the villus size gradient and the associated changes in the process of renewal (Altmann & Leblond, 1970; Altmann, 1971). Therefore, the factors that elevate villus size in the duodenum and the jejunum, and the factors that reduce villus size in the ileum, should also have an effect on the nucleoli. However, this remains for further study. It is perhaps sufficient to conclude, now, that nucleolar size and structure are closely associated with the differentiation and maturation of the columnar epithelial cells.

Relation between renewal, protein synthesis and nucleolar size

Previous studies in our laboratory using cycloheximide (Altmann, 1975) and
$[^3]H$]leucine radioautography (Altmann, 1976) have shown that a major factor controlling villus size is the rate of protein synthesis in the villus epithelial cells. This is visualized in the following manner; the ability to stay on the villus would be determined by the ability to replenish certain surface proteins responsible for cell adhesion; when this ability is lost, there is a minimal uptake of tritiated leucine; this minimum is reached faster in the ileum than in the duodenum or the jejunum. Alternatively, when cytoplasmic protein synthesis is blocked by cycloheximide, an apparently normal but accelerated extrusion leads to the loss of the villus epithelium within hours.

As the present results and also previous results (Altmann, 1976) on $[^3]H$leucine uptake have indicated, the movement of cells along the crypts up to the villus base is accompanied by a marked upsurge of protein synthesis. At the same time, there is a marked reduction in nucleolar size. Using a comparable scale, the two processes can be compared. Therefore, taking maximal protein synthesis and maximal nucleolar size as 100, the following values are obtained at the successive levels from crypt base to villus base: 47·6, 62·4, 74·1 and 100·0 for protein synthesis; and 100·0, 73·8, 61·3 and 54·1 for nucleolar size. Both progressions are linear when plotted against crypt cell position and yield a slope of +0·74 and −0·74, respectively (y intercepts were 45·2 and 97·7; correlation coefficients were 0·91 and 0·99). The two processes thus seem to be related. A plausible explanation would be that the nucleoli that are decreasing in size provide new ribosomes, which are added to the existing stock. Stored mRNA could then become involved in additional protein synthesis.

From villus base to villus top, protein synthesis declined while the nucleoli were still decreasing in size. Apparently, a rapid degradation of some part of the protein synthetic machinery took place. Most probably, ribosomes were degraded, as a decline in cytoplasmic basophilia along the villi has been observed (Padykula, 1962). Possibly, this ribosomal degradation could not be offset by the ribosomes provided by the continuing decrease in nucleolar size.

If the natural decrease in nucleolar size can increase protein synthesis, experimental induction of such a decrease should also be able to do so provided the basic mechanisms of the two phenomena are similar. As described before, this acceleration of protein synthesis did take place after treatment with actinomycin D and, also this protein synthesis added new cytoplasm to the cells.

The decrease in nucleolar size caused by single injections of actinomycin D persisted for 24 h in our experiment, indicating either that the change in the DNA molecules was persistent or that the decreased state was most stable regardless of the fate of the DNA molecules. The important difference between the 3 to 6-hour and the 24-h samples was the considerable decrease in the crypt and villus size indices in the latter. The remaining cells had to function from RNA templates that were already transcribed and stored. The existence of such templates in the intestinal epithelial cells has been inferred (Altmann, 1974, and unpublished data).

Actinomycin D in appropriate doses leads to a complete suppression of DNA-dependent RNA synthesis by forming complexes with the active parts of the DNA molecule (Harber & Müller, 1962; Reich, 1963, 1964). It has been shown that the
alteration of the DNA molecule by actinomycin D is primarily responsible for 'nucleolar segregation', and that inhibition of RNA synthesis without specific alterations in the DNA would not alter the nucleolus (Reich, 1964; Goldblatt, Sullivan & Farber, 1969). In experiments using high doses of methotrexate, which inhibited RNA synthesis, no effect was seen on the nucleoli of the intestinal columnar cells (Abdel-Wahab, 1977). Methotrexate inhibits RNA synthesis but has no effect on the existing DNA molecules. In the present experiments also, methotrexate did not affect the nucleoli. The nucleoli decreased in the usual manner along the shortened crypts and villi. This implies that no immediate RNA synthesis was needed for the decrease in nucleolar size.

*Indications from the effects of cycloheximide and tunicamycin*

Several ultrastructural signs of cell maturation along the villus have been described (Falconer, 1982). In cells of the upper villus, for example, the Golgi lamellae are gradually replaced by vacuoles. In cells that are about to be extruded, additional vacuoles arise from the endoplasmic reticulum and from the nuclear envelope. When cycloheximide, a translational inhibitor of protein synthesis, is given to rats, an accelerated extrusion takes place so that within hours the extrusion zone appears at the level of the former mid-villus or villus base (Altmann, 1975). Cycloheximide administration also causes a rapid cytoplasmic maturation in villus levels near the extrusion zone (Falconer, 1982). This cytoplasmic maturation is not accompanied by a decrease in the nucleolar size. The present experiment with cycloheximide has also shown that when the extrusion zone reached the level of the former mid-villus, nucleolar size remained unchanged.

The signs of cytoplasmic maturation and the subsequent extrusion of cells have been attributed to an increasing inability of the cells to synthesize proteins and thereby to renew their organelles and their glycoprotein coat (Altmann, 1976). Under normal circumstances, this inability probably stems from the decrease in the nucleolar size, as the diminished nucleoli can no longer replenish the decreasing ribosomal content. When this inability is superimposed on the cells by an extraneous agent such as cycloheximide, rapid maturation and extrusion take place, resembling morphologically the natural cytoplasmic events.

A remaining question is: what may cause the natural decrease in the size of the nucleoli? Is it caused by a specific cell product or is it a spontaneous progression of the nucleolus into a more stable state? No definite answer can be given but the experiment with tunicamycin may provide the initial clue.

Tunicamycin inhibits the glycosylation of nascent protein (Takatsuki, Arima & Tamura, 1971), so that the cells become deficient in certain biologically important glycoproteins. This results in inhibition of certain aspects of differentiation and cell cohesion in many cell culture systems (e.g. see Cates, Kaur & Sanwal 1984; Thesleff & Pratt, 1980; Lam & Siu, 1982). Also, surface properties of cells change, especially the binding capacity of receptors. (e.g. see Prives & Bar-Sagi, 1983; Keefer & DeMeyts, 1981; Duksin & Bornstein, 1977; Solanki, Logani & Slaga, 1982; Rosen, Chia, Fung & Rubin, 1979). Only a few effects *in vivo* of the injection of tunicamycin have been studied, especially on the intestine (Michaels, 1980).
Our present initial experiment with this drug showed a marked influence on renewal, namely the crypts elongated at the expense of the villi. Since about a day is required for the renewal of crypts and lower villi (Leblond & Messier, 1961), we allowed a day for the effects of tunicamycin. Although not all aspects of the changes have been clarified as yet, it appears that the crypt to villus transformation was delayed and the crypts became elongated accordingly. Decrease in nucleolar size was correspondingly delayed. It therefore appears that the production of some glycoproteins is involved in bringing about the decrease in nucleolar size as well as certain aspects of differentiation.

CONCLUSIONS

Just over a decade ago, Harris (1972) concluded from his cell fusion experiments that the nucleolus had a regulatory role in protein synthesis. The cytoplasm can only start to produce proteins in the fused cells when the nucleolus appears in the reactivated nucleus. Conversely, protein synthesis ceases when the nucleolus is inactivated. Harris proposed that the nucleolar region exercises a kind of control over gene expression.

Our present study has shown a connection between nucleolar morphology and protein synthesis; that is, when the nucleolus decreases in size some factor is released into the cytoplasm and elicits a marked increase in protein synthesis. This factor may be ribosomal material but could be an unknown substance.

Additional evidence for nucleolar regulation of protein synthesis was provided by the increase in this synthesis in actinomycin-treated animals, in which all the nucleoli rapidly decreased in size until they reached a minimal size.

Whatever the exact mechanism of nucleolar regulation may be, it is now certain that the nucleolar changes represent an integral part of the renewal process and by regulating protein synthesis they probably guide the cells through the various phases of differentiation and maturation. In the duodenum and the jejunum, the decrease in nucleolar size is slow and extrusion occurs relatively late. In the ileum this decrease is accelerated so that the extrusion of cells occurs relatively early. We have pointed out above that the slowing as well as the accelerating of renewal are accomplished by intraluminal 'villus enlarging' and 'villus reducing' factors, which may therefore offer a future tool for manipulating the nucleoli.

The life cycle of the columnar cell may therefore be rewritten, including the nucleolar events and the changes in protein synthesis. At the crypt base, an active reticulated nucleolus and a high but average rate of protein synthesis would characterize the cells. As the cells migrate from crypt base to villus base, the nucleoli decrease in size and the rate of protein synthesis per cell increases, both at about the same rate. From villus base to villus top a gradual decline in protein synthesis is associated with increasing maturation and eventual extrusion. These events may be determined by the continuing decrease in nucleolar size, which would result in the nucleolus being no longer able to provide for protein synthesis. The time needed to reach this stage from the early progenitor stage is probably determined by a delicate balance between various factors.
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such as ribosomal degradation and replenishment, and villus enlarging and reducing factors.

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