Observations on the Origin and Significance of the Nuclear Envelope-Limited Monolayers of Chromatin Unit Threads Associated with the Cell Nucleus

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Summary

Monolayers of chromatin structural units about 330 nm in width enclosed on both sides by extensions of the nuclear envelope, called sheets, and located either in the cytoplasm (c••n••c type), or within the nucleus (c••n••n type), are common in cultured cells of Burkitt's lymphoma. The sheets are absent from mitotic cells except at telophase where, unlike interphase, type c••n••n is more numerous than c••n••c. The degree of nuclear asymmetry is defined in terms of the increase in enclosing membranes over that required to enclose the same area in a circular configuration. The percentage number (Pt) of cells with nucleus-associated sheets averaged over all stages in the cell cycle, increases with cell viability and with nuclear asymmetry. However, during the cycle there is a marked diminution in Pt during the S-phase of DNA synthesis when nuclear asymmetry itself does not change. Hence, it is suggested, and data on other cell types support the hypothesis, that nuclear asymmetry is a necessary but not sufficient factor in causing sheets to form. Microtubules are present within the cytoplasm and their morphological arrangement suggests a role in determining nuclear asymmetry. Treatment with a microtubule depolymerizing agent, colcemid, does not alter either the existing nuclear asymmetry or Pt, but when cells are treated early in S-phase the reappearance of sheets in the G2 phase of the cell cycle is considerably delayed. The reappearance takes place when the microtubules are still depolymerized. It is suggested that synthesis of membrane in excess of what is needed to enclose a sphere results in nuclear asymmetry and associated membrane-enclosed monolayers, the resulting nuclear conformation, including the distribution of membrane between types c••n••n and c••n••c, depending on what is energetically favoured. No biochemical function has yet been assigned to sheets.

Introduction

Recent electron-microscope studies (Davies, 1967, 1968a, b; Davies & Small, 1968; Davies & Haynes, manuscript in preparation) have shown that the condensed chromatin in a variety of vertebrate cells contains a structural unit about 170 nm in diameter, well definable primarily because of its tendency to form an ordered layer, or layers, of definite geometry on the surface of the nucleus in contact with the enveloping membranes: in addition, the outer layer gives rise, by delamination and membrane flow, to monolayers confined between paired membranes, shown to be

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extensions of the nuclear envelope. These monolayers constitute a dramatic manifestation of chromatin structure. They occur only in certain cell types and may be termed (Davies & Tooze, 1966) the envelope-limited sheets of chromatin, or simply the sheets. The nearly constant width of the sheets, wherever they are found, average about 37.0 nm, provided the strongest evidence for the general occurrence of a similar structural unit, hence called the unit thread; the unit thread consists of folded DNA and protein. This larger dimension 37.0 nm is due, it was assumed, to certain projections which radiate from the central 17.0-nm unit.

As well as helping to elucidate chromatin structure the sheets may have a cell physiological role, and its elucidation may help in understanding nuclear function. Oocytes are known to be engaged in activities vital to the future embryos: in certain maturing fern oocytes, of Dryopteris felix-mas, sheets are very common (P. Bell, personal communication). Nuclei with associated sheets have been reported in normal human foetal retina, as well as in retinoblastomas both in vivo and in vitro (Popoff & Ellsworth, 1969). Indeed one of the very first reports of envelope-limited sheets was in these tumours originating in nervous tissue (Allen, Latta & Straatsma, 1962). However, Burkitt’s lymphoma originating in the connective tissue of man, is most suitable for the study of sheets since they are also very numerous in these cells, and furthermore unlike the retinoblastomas, the cells can be easily maintained in continuous culture (Epstein & Barr, 1965).

In this paper we describe the way in which the numbers of sheets associated with the cell nucleus vary with cell viability and during the cell cycle. The occurrence of sheets is shown to depend, at least in part, on the asymmetric shape of the cell nucleus and the factors which may influence nuclear shape are therefore examined and discussed.

**METHODS**

**Maintenance of the cells of Burkitt’s lymphoma in vitro**

Cells of 3 cell lines of Burkitt’s lymphoma (EB 1, EB 2 and EB 3) were provided by Prof. M. A. Epstein and were maintained in suspension culture in this laboratory. The cultures, at an initial concentration of 1.0 x 10⁶ cells/ml, were incubated at 37 °C in Eagle’s Minimal Essential Medium (MEM) (Wellcome Research Laboratories, Beckenham, Kent) supplemented with 10% foetal bovine serum, 100 units/ml of both benzyl penicillin and streptomycin, and non-essential amino acids (all supplied by Flow Laboratories, Ayrshire, Scotland). Cell suspensions, in 20-ml aliquots, were kept stationary in hard glass, rubber-stoppered, conical flasks of 100 ml capacity; the cells sank to the bottom of the flasks but did not attach to the glass surface. Feeding, by re suspension at the above concentration in fresh medium, was carried out every 3 or 4 days.

**Cell viability and growth**

Cell viability was determined by suspending duplicate samples of cells in 0.05 % trypan blue in Eagle’s MEM. The percentage number of viable cells, namely those excluding the dye, was calculated from several fields containing a total of 500 cells. The viability varied from about 70 to 95 %.

Cell numbers were determined using a haemocytometer with Improved Neubauer ruling.
Fixation was in 3% glutaraldehyde in Eagle’s MEM, osmolality 590 mosmol, for 1 h at 20 °C. A modification of the technique of Achong & Epstein (1965) was used for the preparation of the cells as concentrated pellets. A 5-ml sample of the cell suspension was centrifuged very gently at 1000g for 5 min in a wide-bore tube to produce a loose pellet of cells; these cells were resuspended in 5 ml of fixative and were reconcentrated into 0.5 ml. This cell suspension was drawn up into 0.8-mm bore capillary tubes which were then heat sealed at one end; the tubes were spun in a Hawksley Micro-Haematocrit centrifuge at 1200g for 5 min to produce a consolidated cylinder of cells. The sealed end of the tube was broken off and the cylinder of cells extruded into fresh fixative. After washing in Eagle’s MEM containing 10% sucrose, the pellets were postfixed in 1% OsO4, also made up in Eagle’s MEM with 10% sucrose; these operations were carried out at 4 °C. The pellets were dehydrated in graded ethanols and embedded in Araldite. Sectioning was carried out using a diamond knife (Dehmer, Germany) on an A. F. Huxley-Cambridge Ultramicrotome (Mark 1). Sections were stained with 2% aqueous magnesium uranyl acetate for 30 min at room temperature, followed by Reynold’s lead citrate (1963) for 2 min. They were examined in a Siemens Elmiskop I at 80 kV with a 200-μm condenser aperture and a 50-μm objective aperture; viewing and photography was carried out at either 5000 or 40000 times.

**Autoradiography**

S-phase cells were identified by the fact that they utilize tritiated thymidine (³H-TdR). Cell cultures were incubated in Eagle’s MEM containing ³H-TdR at a final concentration of 0.5 μCi/ml for 15 min. Excess ³H-TdR was removed by washing in fresh medium before preparation of the cells for electron microscopy. 100-nm sections of the labelled preparations were prepared for EM autoradiography by the method of Pelc, Coombes & Budd (1961) using a 14% solution of Ilford L4 emulsion. Exposure was for 4 weeks at -20 °C; the autoradiographs were developed in Kodak D19b at 20 °C for 2 min.

RNA synthesis in the cells was detected by autoradiography following labelling of the cells with tritiated uridine (³H-UdR). Cell cultures were incubated in Eagle’s MEM containing ³H-UdR at a concentration of 0.5 μCi/ml for 15 min. After suitable washing autoradiographs were prepared as above.

**Hypertonic media**

The osmolality of normal Eagle’s Medium (250 mosmol) was increased to 550 mosmol by addition of sodium chloride and cells were incubated in this hypertonic medium for times up to 30 min and then reimmersed in normal medium; samples were removed for electron microscopy at 10-min intervals.

**Cell synchronization**

A chemical synchronization technique, the double block and release method of Galvazi & Bootsma (1966) was employed on the EB2 cell line to obtain a culture in which all the cells were, in theory at least, at the beginning of the S-phase. Without going into details, 5-fluoro-deoxyuridine (FUdR) was added to the growth medium at a final concentration of 5 x 10⁻⁶ M to inhibit DNA synthesis, and thymidine (TdR), at a concentration of 2 x 10⁻⁴ M, added after appropriate time intervals to release this inhibition. In order to choose the appropriate times it was necessary to calculate the times spent in G₁, S and G₂ from a labelled mitosis curve (Quastler & Sherman, 1959). The total cell cycle time was 30.5 h, with G₁ about 2 h, S about 14 and G₂ about 5 h. With all the cells theoretically at the beginning of S-phase the reinitiation of DNA synthesis led to an unexpected slow increase in cell number by 20%, followed by a wave of cell divisions occurring between about 20 and 24 h later. The cell number increased finally by about 70%, rather than the expected 100%. These data indicate that by no means had all the cells proceeded synchronously towards division.
Fig. 1. Schematic diagram showing the 3 different types of monolayer limited on both sides by nuclear envelope.  
Fig. 1. Schematic diagram showing the 3 different types of monolayer limited on both sides by nuclear envelope. $c$, cytoplasm; $n$, nucleus. Imperfections or interruptions occur at $i$. The arrangement of unit threads adjacent to the nuclear envelope gives rise to less-electron-dense bands $b_1$, $b_3$, and electron-dense bands $b_2$, $b_4$. The monolayer of unit threads enclosed between envelopes gives rise to 3 bands. $S'$ is the width of the monolayer measured between centres of the inner components of the envelopes.

The centrosome classification method

This method for determining the position in the cycle of a cell in an unsynchronized population is based on the fact that the centrosome, or centrosphere, with its contained centrioles and pericentriolar structures has a fine structure which varies with, and is characteristic of, the stage in the cycle. This was established in a study on HeLa cells (Robbins, Jentzsch & Micali, 1968) synchronized by the method of Terasima & Tolmach (1961), a method which unfortunately is not applicable to Burkitt's cells since they do not attach themselves to the glass surface of the culture vessel. Robbins et al. (1968) showed that, in HeLa cells, duplication of each member of the centriolar pair begins at or near initiation of DNA synthesis and ends at the end of S-phase. Tokuyasu (1972) identified early S-phase nuclei in cultured human lymphocytes by observing the fine structure of the centrioles. A method for placing cells in the cycle which does not rely on obtaining favourable views or serial sections of the centrioles themselves has the advantage of allowing the position of larger numbers of cells to be determined. Haynes (1973) has shown, by DNA labelling, and serial sectioning of a few cells, that the overall changes in the centrosomal complex, including the pericentriolar structures, are similar in Burkitt's cells to those already reported in HeLa cells and can be used to ascertain the stage in the cycle. Briefly the centrosomal structure as a function of stage in the cycle is as follows. In $G_1$ cells the centrosome, the region excluding ribosomes, is small and contains an orthogonally arranged pair of centrioles, and a few spindle tubules, rather less than the number in the telophase centrosome. These virtually disappear as the cell enters the S-phase. During S the procentrioles arising from each of the centrioles in the $G_1$ pair lengthen, the centrosome is enlarged and it contains large numbers of membrane-limited vesicles. In late S and early $G_2$ cells, a stage referred to as $S-G_2$, large numbers of amorphous electron-dense bodies (Fig. 23) appear throughout the centrosome, membrane-limited vesicles are still present, and there are numerous microtubules. By late $G_2$ the now fully replicated centriole pairs have further separated and have migrated to opposite poles of the cell, each centrosome is smaller, the numbers of vesicles and electron-dense bodies reduced and characteristically there is a high number and concentration of microtubules. From a study of cells sectioned through the centrosome it was usually possible from the appearance of its size and the contained pericentriolar structures to place cells in 4 classes, namely $G_1$, $S$ (early and mid-$S$), $S-G_2$ (late $S$ and early $G_2$) and $G_2$ (late $G_2$).
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Classification of sheets

The sheets may be divided into 3 basic types designated c- n- c, c- n- n and n- n- n (Fig. 1), depending on the material encountered, either cytoplasm (c) or nucleoplasm (n), when traversing a line drawn perpendicular to the plane of the sheets (Davies, 1968a). Variants such as c- n- c and c- n- n (Fig. 1) may occur but are much less common in the Burkitt cells, as were the type n- n- n; over 95% of sheets were types c- n- c and c- n- n. Types c- c either form pockets (Fig. 1 at 1) which often contain cytoplasmic organelles or may join two lobes (Fig. 1 at 2).

The percentage of cells with sheets, P,

In any one section all the cells were examined for sheets on the fluorescent screen at a magnification of ×5000 electron-optical and ×10 optical. Two or three sections from different blocks of the same culture yielded N cells, N being about 500. If n is the number of cells with sheets then P, = 100n/N. Clearly P, bears a close but not easily determined relationship to the actual percentage of cells with sheets attached to their nuclei. P, is obviously a lower limit since the section thickness of 100 nm was only about one-hundredth of the diameter of the entire nucleus, about 10 μm.

L, l, P, and P,.

Cells were randomly selected for photography by choosing the one in the upper left-hand corner of each grid square. L, is the average length of nuclear envelope obtained by measuring the total length of nuclear envelope, including that enveloping monolayers, in a number N of nuclei and dividing by N. l is the average length of nuclear envelope enclosing monolayers and is obtained by adding up the total length in the n nuclei containing sheets among the N cells photographed and dividing by N. N was about 30 for each culture. In a c- n- c sheet only half the length of envelope is included in l since the other half would normally be present in the absence of a monolayer: in a c- n- n sheet both enclosing membrane pairs are included. By definition L, = L, the average length of nuclear envelope not involved in sheet formation. The ratio 100/L, or P, is the percentage of nuclear envelope involved in formation of sheets. P, would seem to be a better measure of sheet formation than P, since any cell nucleus would be scored as bearing a sheet whether it is long or short. However P, is easier to measure on much larger numbers of cells and is likely to be the more accurate for that reason.

Asymmetry of nucleus

The circle is the 2-dimensional geometric figure which has the minimum perimeter for a given enclosed area. Deforming a circle, keeping the enclosed area constant leads to an increase in perimeter. A measure of the asymmetry, D, of a given 2-dimensional figure of perimeter C, can be obtained from the expression (C, - C)/C, where C, is the circumference of a circle enclosing the same area as the given figure. For any circle D = 0. A value of D = 1 corresponds to C, = 2C, that is a 100% increase in perimeter over the circle of equal area. Fig. 2 is included to demonstrate the range of asymmetry values which arise when a circle is transformed into various shapes maintaining the enclosed area constant.

The average degree of asymmetry, D, in nuclei of about 30 cells per culture randomly selected (see section L, l, P, ) was calculated from the above formula using the measured average perimeter L, (= C, and C, calculated from the measured average area enclosed by the nucleus. D is the average asymmetry calculated from L rather than L,; the area enclosed within the sheet could be neglected in calculating both D, and D. D, gives a measure of the asymmetry of the 3-dimensional nucleus from which the 2-dimensional sections are derived. No attempt was made to determine, by serial sectioning and nuclear reconstruction, the precise relationship between the 2- and 3-dimensional asymmetries.
Fig. 2. The calculated asymmetry $D$ when a circle, area $A$, is changed into: $\lambda$, an ellipse enclosing the same area with axial ratios $b/a$ (abscissa) varying from 1 to 10; $\beta$, a number $n_0$ (abscissa) of circles the sum of whose areas is equal to that of the original circle; $\gamma$, 2 circles of unequal area, individually $A_1$ and $A_2$ where $A_1 + A_2 = A$ and $A_1/A$ is the abscissa; and $\delta$, a circular indentation of area $A_1$ enlarges within a circle of area $A_1$ ($A = A_1$ when $A_1 = \infty$) such that the segmented area (cross-hatched) remains constant, or $A_2 - A_1 = A$.

RESULTS

Cells with unknown position in the cycle

Values of $S'$. The value of $S'$ (Fig. 1) for $\cdots n \cdots c$ sheets was $32 \pm 2$ nm average for 154 cells fixed only in 3% glutaraldehyde in MEM (590 mosmol) and $32 \pm 2$ nm for 141 cells in above fixative followed by 1% OsO$_4$. The values of $S'$ were found to vary somewhat with osmolality of fixative over the narrow range studied, but at each osmolality there were never large differences in $S'$ between the fixation in glutaraldehyde alone, or followed by OsO$_4$, as was reported by Davies & Tooze (1966) in newt erythroblasts. Thus at 520 mosmol the average value of $S'$ was slightly larger, $34.0 \pm 3$ nm for glutaraldehyde followed by OsO$_4$ and $32.0 \pm 3$ nm for glutaraldehyde alone.

In electron micrographs the unit threads appear as dots, or 'granules', and dashes (cf. Fig. 1) depending on whether or not they are oriented normally to the plane of the section. In this material the quality of fixation was such that they were not as well defined as, for example, in chicken reticulocytes (Everid, Small & Davies, 1970).

Cells labelled with RNA precursors. In cells with tritiated uridine incorporated into the RNA, the electron-microscope autoradiographs did not reveal any preferential labelling, or indeed any labelling at all, over the envelope-limited sheets. However, the resolution of the method on these cells is probably not sufficient to exclude entirely
the possibility that there is labelled RNA in the sheets, although this appears to us to be unlikely.

Variation of $P_s$ with viability. In the cell line EB1 the cells have few sheets at any viability: $P_s$ varied from about 3% at 57% viability to 13% at 92% viability (Fig. 3). In EB2 there was a very rapid increase of $P_s$ with viability, from about 10% at 64% viability to 63% at 93% viability (Fig. 3). In line EB3 cell cultures had a quite high percentage of cells with sheets, varying from 29% at 53% viability to 43% at 96% viability (Fig. 3).

Variation of $D_t$ with viability. In all 3 lines, EB1, EB2, EB3, the value of the asymmetry $D_t$, averaged over all the cells in the culture increased with the viability of the cells in the culture (Fig. 4). The low values of $D_t$ for EB1 cell nuclei, from 0.03 to 0.23 with changing viability, is an indication of the fact that these nuclei are usually almost circular in cross-section.

The nuclei from EB2 cells were, on average, nearly circular at low viabilities, $D_t$ being 0.36 at 64% viability, but irregularity in nuclear form increased very rapidly with viability, the average asymmetry, $D_t$, reaching values of 1.09 (Fig. 4). Histograms showing the spread in values of individual nuclear asymmetries for 3 different values of viability are shown in Fig. 5 A–C. The disappearance of the cells from the low values of individual $D_t$ (Fig. 5A) and the shapes of Fig. 5B, C indicate that all the nuclei are
Fig. 4. Relates the average nuclear asymmetry $D_t$ (for definition see text) with percentage cell viability for 3 cell lines EBr1 (■), EB2 (●), and EB3 (▲).

becoming increasingly asymmetric; evidently the change in the average value of $D_t$ is not due to the appearance of a few very highly irregular nuclei. Similar results were obtained for the other two cell lines.

Nuclei from EB3 cells were always very irregular at all viabilities, $D_t$ ranging from 0.8 to about 1.0 (Fig. 4).

**Variation of $P_s$ and $P_t$ with $D_t$.** The values of $P_s$ and $D_t$ both increase in a similar manner with viability for all 3 cell lines. When the values for $P_s$ and $D_t$ for all 3 cell lines are plotted they are found to lie approximately on the same curve (Fig. 6), with $P_s$ generally increasing with $D_t$, but most rapidly at the highest values of $D_t$. This means that as the average asymmetry of nuclei in any population goes up there is an increasing probability that any one cell will have a sheet associated with its nucleus. Of course, approximately circular nuclei did occasionally bear sheets, and highly irregular ones often did not.

When the cells with sheets are analysed into the 2 major types, $c\cdot n\cdot c$ and $c\cdot n\cdot n$, the former are found to be more common (Fig. 7) and the percentage number $P_s$ ($c\cdot n\cdot c$) increases more rapidly with average nuclear asymmetry, $D_t$, than does the number $P_s$ ($c\cdot n\cdot n$), except at the highest asymmetries.

The other measure of the extent of sheet formation is $100\, l/L_t$ or $P_t$, the percentage of nuclear envelope enclosing monolayers. $P_t$ was measured on each of three or four cultures (36 cells per culture) from each of the 3 cell lines and when plotted against
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Fig. 5. Histograms showing numbers of cells with particular individual nuclear asymmetries $D_t$ for 3 values of cell viability: A, 64%; B, 85%; and C, 93%. All EB2 line.

Fig. 6. Curve relating the percentage of cells with sheets, $P_s$, and average asymmetry $D_t$ derived from Figs. 3, 4. EB1 (■), EB2 (●), and EB3 (▲).

$D_t$ gave a curve (Fig. 8) very similar to that already found to relate $P_s$ and $D_t$. Additionally, the numerical data in Fig. 8 show that even at the highest average nuclear asymmetry correlated with the maximum number of cells with sheets, less than about 10% of the total length of nuclear envelope is involved, on average, in enclosing the monolayers of unit threads.

It might be thought that since the nuclear asymmetry $D_t$ is calculated from $L_t$, which includes the length $l$ involved in sheet formation, that the increase in $D_t$ is merely due to an increase in $l$, and that the variation in $P_s$ or $P_l$ with $D_t$ is self evident.
Fig. 7. Shows how percentages of cells with c-n-c sheets, $P_c(c-n-c)$, and with c-n-n sheets, $P_n(c-n-n)$, vary with average nuclear asymmetry, $D_t$. These data are from cultures used in Figs. 3, 4; points shaded on left (e.g., ○) refer to c-n-c sheets, points shaded on right (e.g., □) refer to c-n-n sheets. Squares, EB1; circles, EB2; triangles, EB3.

However this is not so. When $D$ derived from $L$ is used, rather than $D_t$, the relationship with $P_n$ is very similar to that already found between $D_t$ and $P_n$. At maximum $P_n$ (Fig. 6) $D_t$ is 1.09, whereas $D$ is 0.9. Evidently then, the main correlation with sheet formation is the general asymmetry of the nucleus irrespective of the additional envelope employed in forming sheets which is small as already shown in Fig. 8.

Cells with a known position in the cycle: EB2 only

Cells in mitosis. Whereas in interphase the chromatin is located, from the ultrastructural observations, over the entire interior surface of the nuclear envelope, apart from the area actually occupied by the nuclear pores, prophase is recognized by the condensation of chromatin, leaving extensive areas of nuclear envelope free from chromatin (Fig. 11): in such cells there are occasional nuclear structures identical in morphology with the c-n-n and c-n-c sheets except that the monolayer of unit threads is absent and the inner space reduced in width (Fig. 12). Presumably during chromosome condensation or coiling, the monolayers, and other parts of the chromo-
Table 1. The percentages, $P_n$, of telophase cells with sheets (col. 5), with $c \cdot n \cdot n$ sheets (col. 2), with $c \cdot n \cdot c$ sheets (col. 3), and the percentage containing both types on 1 nucleus (col. 4)

The percentage of cells with sheets averaged over the entire population of the culture is shown in col. 6.

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>$P_n (c \cdot n \cdot n)$</th>
<th>$P_n (c \cdot n \cdot c)$</th>
<th>$P_n (c \cdot n \cdot n + c \cdot n \cdot c)$</th>
<th>Total population $P_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>69</td>
<td>30</td>
<td>9</td>
<td>6</td>
<td>45</td>
</tr>
<tr>
<td>52</td>
<td>32</td>
<td>13</td>
<td>9</td>
<td>54</td>
</tr>
<tr>
<td>107</td>
<td>38</td>
<td>23</td>
<td>11</td>
<td>72</td>
</tr>
</tbody>
</table>

Some, are withdrawn into the main body with the chromosomes remaining attached to sites on the envelope. After prometaphase, defined (Fell & Hughes, 1949) by the breakdown of the nuclear envelope, the chromosomes never have attached nuclear envelope-limited sheets, as were found throughout mitosis in the erythroblasts of newt (Davies & Tooze, 1966). However, paired or stacked remnants of what we assume to be nuclear envelope are found in the cytoplasm. Presumably these arise by reorientation of nuclear envelope during its breakdown, since they are too numerous to be accounted for by empty sheets. These long lengths contain breaks (Fig. 14) but no clearly defined nuclear pores which might, if present, be better recognizable in surface views. These paired nuclear envelope fragments persist throughout division and are found in early $G_1$ cells, as shown by the centrosome classification method. How this tendency to form pairs may be related to apposition of the pair of nuclear envelopes enclosing monolayers of unit threads is not known.

Paired nuclear envelope fragments have already been reported in dividing cells (refs. in Hanaoka & Friedman, 1970; Jones, 1962), as well as in the interphase cells of Burkitt’s lymphoma (Epstein, 1961) and other cell types (refs. in Hanaoka & Friedman, 1970). In interphase they may properly be referred to as paired cisternae. Hanaoka & Friedman (1970) suggested that paired cisternae in interphase cells may be the residua of the structures formed during mitosis. We have found that in Burkitt’s cells paired cisternae are only found during $G_1$ and this supports the hypothesis that they originate during mitosis.

In several thousand cells examined at metaphase and anaphase, envelope-limited sheets were never found, which confirms the observation of Epstein (personal communication). Metaphase chromosomes did not have membranes attached to them but during anaphase paired membranes with the morphology of nuclear envelope were observed scattered between and presumably applied to chromosomes (Fig. 15). These are probably the source of the paired membranes found later within the telophase nucleus. The beginning of telophase is defined (Fell & Hughes, 1949; Boss, 1959) as the stage at which the daughter chromosomes are maximally clumped and, beginning at this time, nuclear envelope is applied to the surfaces of the extending
chromosomes within the enlarging daughter cell. Sheets are very common through-
out telophase, even when $P_s$, the percentage of cells with sheets averaged over the
entire population, interphase cells plus mitotic cells, is very low. For example (Table 1)
in culture no. 69, $P_s$ is about 5%, whereas the percentage of telophase nuclei with
sheets is very high, namely 45%. Moreover, type $c \cdot n \cdot n$ predominates over type
$c \cdot n \cdot c$, 30% compared with 9% in culture no. 69 (Table 1). Further, there is an
appreciable number of cells in which nuclei contain both types of sheet, 6% in culture
no. 69.

Cells treated with hypertonic saline. Robbins, Pederson & Klein (1970) showed that
when HeLa cells are treated with hypertonic solutions they undergo morphological
transformations, several of which grossly resemble those which occur when untreated
cells enter prophase. These include chromosome condensation with preferential
localization at the nuclear envelope and nucleoli, ruffling of the nuclear envelope,
alteration in nucleolar structure and polyribosome breakdown. So as to increase the
number of prophase-like cells available for study, cultures of Burkitt's lymphomas
were treated with standard culture medium made hypertonic by addition of sodium
chloride. It is convenient to consider the cells in this section, although such a prophase-
like condition may be induced in cells from any stage of the cycle, since most cells in
the treated culture assumed this configuration.

When treated with 550 mosmol medium, within 2 min the nucleolonemal thread
had disappeared, the interphase chromatin had begun to clump and within 20 min
the chromatin morphology closely resembled that in the typical prophase cell. During
treatment, monolayers of chromatin were retracted out of the enveloping membranes,
either partially or fully depending on time, but when the cell was returned to normal
medium after 30 min treatment in hypertonic medium, the unit threads re-entered the
emptied sheets to reform monolayers. In a detailed kinetic study cells were immersed
in 550 mosmol medium and samples withdrawn at intervals. We will refer to the
envelope-limited monolayers as sheets whether or not the monolayer of units is
complete. Let $x, y, z$ be the fractional numbers of full, partially full, and empty
sheets respectively, where $x + y + z = 1.0$. The data in Fig. 9 show that after 10 min
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Table 2. The percentage, $P_s(S)$ of labelled S-phase cells with sheets and the percentage, $P_s(G_1, G_2)$ in unlabelled $G_1, G_2$ cells

$P_s$ is the percentage of cells with sheets averaged over the total population.

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>$P_s$</th>
<th>$P_s(S)$</th>
<th>$P_s(G_1, G_2)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>22</td>
<td>6</td>
<td>36</td>
</tr>
<tr>
<td>64</td>
<td>10</td>
<td>2</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 3. Time (col. 1) after release of second FUdR block; col. 2, expected position in cell cycle of majority of cells; col. 3, the percentage of cells with sheets, $P_s$

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Expected position</th>
<th>$P_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$S$, early</td>
<td>15</td>
</tr>
<tr>
<td>2-4</td>
<td>$S$</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>$S$</td>
<td>16</td>
</tr>
<tr>
<td>12</td>
<td>$S$</td>
<td>21</td>
</tr>
<tr>
<td>16-18</td>
<td>$S$ and $G_2$</td>
<td>31</td>
</tr>
<tr>
<td>20-22</td>
<td>$G_2$</td>
<td>28</td>
</tr>
<tr>
<td>25</td>
<td>$G_1$</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 4. As a function of stage in the cycle (col. 1) identified by the centrosome classification method, the percentage number of cells with sheets, $P_s$ (col. 3)

Col. 2 is number of cells. Data from 6 cultures, numbers 45, 52, 54, 58, 64, 69. The value of $P_s$ averaged over all the cells in all the cultures (2 sections per culture) was 22.

<table>
<thead>
<tr>
<th>Stage in cycle</th>
<th>No. of cells</th>
<th>$P_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_1$</td>
<td>111</td>
<td>42</td>
</tr>
<tr>
<td>$S$</td>
<td>165</td>
<td>5</td>
</tr>
<tr>
<td>$S-G_2$</td>
<td>164</td>
<td>19</td>
</tr>
<tr>
<td>$G_2$</td>
<td>62</td>
<td>40</td>
</tr>
</tbody>
</table>

there are very few full sheets left ($x = 0.15$), only $8\%$ ($x = 0.08$) after 30 min, and at that time $84\%$ ($z = 0.84$) are completely empty. Thirty minutes after reimmersion in normal medium $80\%$ ($x = 0.80$) of the sheets have refilled completely, $15\%$ ($z = 0.15$) are still empty and the remaining $5\%$ ($y = 0.05$) are partially full. The numbers $x, y, z$ depend partly on the rate of movement and partly on the lengths, or in 3 dimensions the area of sheet, shorter sheets presumably emptying and refilling first. The times taken to empty are somewhat longer than the period of 2 min during which the initial clumping of chromatin occurs but commensurate with the longer time of 20 min taken to reach the prophase-like condition.

In untreated cultures empty sheets (Fig. 13) were very rarely seen in the interphase cells.

Cells labelled in S-phase. After labelling with $^3$H-TdR, cells synthesizing DNA, in the so-called S-phase, were recognizable in the autoradiographs by the developed silver grains lying over the nucleus. Analysis of all the cells on each section, in all
Table 5. As a function of the stage in the cycle (col. 1), the variation in the percentages of c • n • c type sheets, $P_s$ (c • n • c) (col. 4); c • n • n type, $P_s$ (c • n • n) (col. 3); $P_s$ (c • n • c + c • n • n) is the percentage (col. 5) with both types attached.

$P_s$ (col. 2) is the percentage averaged over the entire selected cell population.

<table>
<thead>
<tr>
<th>Stage in cycle</th>
<th>$P_s$</th>
<th>$P_s$ (c • n • n)</th>
<th>$P_s$ (c • n • c)</th>
<th>$P_s$ (c • n • c + c • n • n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_1$</td>
<td>48</td>
<td>9</td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td>$S$</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>$S$-$G_2$</td>
<td>20</td>
<td>3</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>$G_2$</td>
<td>57</td>
<td>6</td>
<td>45</td>
<td>6</td>
</tr>
</tbody>
</table>

about 1000 cells from 2 cultures, showed (Table 2) that there were far fewer sheets in the S-phase cells than in the unlabelled $G_1$ and $G_2$ cells.

*Cells synchronized chemically.* Samples of cells were prepared for electron microscopy at time intervals corresponding to predetermined stages in the cycle after the release of the second FUdR inhibition of DNA synthesis. Values of $P_s$ were obtained (Table 3) from 6 experiments involving 6 cultures. We attempted in each experiment to estimate $P_s$ for about 2000 cells for each time. By this method, also, more of the cells with sheets lie outside the DNA synthesis phase but the data are not as convincing as those in Tables 2 and 4; presumably this is due to poor synchrony.

*Cells identified by the centrosome classification method.* When cells were identified by the fine-structural appearance of the centrosome there were relatively few cells in the S-phase with sheets associated with their nuclei (Table 4).

When, in culture numbers 45, 52 and 58, the cells containing sheets were analysed into types (Table 5), then at each individual stage in the cycle types c • n • c were found to occur in larger numbers than types c • n • n. It was earlier shown (Fig. 7) that for cells of unknown position in the cycle $P_s$ (c • n • c) was numerically larger than $P_s$ (c • n • n). These findings are in contrast to the results on telophase nuclei (Table 1) where $P_s$ (c • n • n) was always larger than $P_s$ (c • n • c). However, in common with the telophase nuclei, there were interphase nuclei with both types of sheet attached (Table 5 and Figs. 17, 20).

In one culture, number 52, estimates were made of the percentage of nuclear envelope, $P_e$, involved in the formation of sheets. Based on fewer cells, 95, and hence probably less accurate than the data in Table 4, these data (Table 6) also show that the proportion of envelope involved in formation of sheets is reduced during S-phase.

In the same cells the average asymmetry $D_t$ was also calculated and shown not to vary much during the cell cycle (Table 7). It may appear paradoxical that whereas the percentage number of cells with sheets increases with nuclear asymmetry, $D_t$ (Fig. 6), there is an appreciable drop in $P_s$ during S-phase (Table 5), without any corresponding drop in asymmetry (Table 7). This is merely due to the fact that $P_s$ in Fig. 6 is estimated from cells at all stages in the cycle and hence does not reveal the low value of $P_s$ for the cells in S-phase.

*Nuclear asymmetry, centrioles, microtubules and colcemid treatment.* In electron
Table 6. The proportion, \( P_t \), of nuclear envelope involved in formation of sheets, as a function of stage in the cycle

<table>
<thead>
<tr>
<th>Culture no. 52.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage in cycle</td>
</tr>
<tr>
<td>( G_1 )</td>
</tr>
<tr>
<td>( S )</td>
</tr>
<tr>
<td>( S-G_2 )</td>
</tr>
<tr>
<td>( G_2 )</td>
</tr>
</tbody>
</table>

Table 7. The small variation in average asymmetry, \( D_t \), during the cell cycle

<table>
<thead>
<tr>
<th>Stage in cycle</th>
<th>No. of cells</th>
<th>( D_t )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( G_1 )</td>
<td>20</td>
<td>0.46</td>
</tr>
<tr>
<td>( S )</td>
<td>30</td>
<td>0.40</td>
</tr>
<tr>
<td>( S-G_2 )</td>
<td>30</td>
<td>0.42</td>
</tr>
<tr>
<td>( G_2 )</td>
<td>15</td>
<td>0.39</td>
</tr>
</tbody>
</table>

micrographs, due to the small fraction of the cell seen in any one section it is not in general possible to relate the type of nuclear asymmetry to the position of the centrosome and the disposition of the microtubules. Not all the observations described in this section are on cells at a known stage in the cycle, the sections not having passed through the centrosome. Some nuclei are very lobed (Figs. 18, 21, 22), the lobes sometimes being connected by \( c\cdots n\cdots c \) type sheets which may contain either small or large discontinuities, interruptions or imperfections, that is regions where the monolayer of unit threads is interrupted. Sometimes these interruptions (arrowed in Figs. 16, 18, 25, for example) may be approximately spherical or elliptical in shape, that is microlobes, the outer surfaces bearing a layer of unit threads, the inner regions having the same appearance as the larger nuclear lobes. Such imperfections also occur in the pocket-forming \( c\cdots n\cdots c \) sheets, as well as \( c\cdots n\cdots n \) sheets. In some cells, 2 or more nuclear lobes radiate out from a centre, where lie the centrioles (Fig. 21), and in these cells the overall appearance is similar to the radially segmented nuclei described by Norberg (1970). In such radially segmented nuclei we suppose that the lobes may be joined by \( c\cdots n\cdots c \) sheets; however, \( c\cdots n\cdots c \) sheets may occur on those parts of the nucleus farthest from the centre (Fig. 21). The centrosome itself often lies in an indentation in the nucleus and it, together with the associated microtubules, give the appearance of influencing the asymmetry (Figs. 22, 23). In some cells (Fig. 24) centrioles appear, from the symmetry, to be concerned with organizing the numerous Golgi bodies which lie around them, and no connexion with nuclear asymmetry is apparent, at least in the plane of sectioning. Occasionally the centrioles are found actually within a \( c\cdots n\cdots c \) pocket (Figs. 16, 17).

Cytoplasmic microtubules are found radiating out from the centrioles. Few in number in \( G_1 \), there are few if any in \( S \), but their number increases in \( S-G_2 \) and they are even more numerous later in \( G_2 \). Microtubules are frequently found within the
Table 8. Values of nuclear asymmetry, $D_t$ (36 cells each value), and percentage of cells with sheets, $P_s$ (500 cells each value), at various times after addition of colcemid

<table>
<thead>
<tr>
<th>Time after colcemid, h</th>
<th>$P_s$</th>
<th>$D_t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21</td>
<td>1.53</td>
</tr>
<tr>
<td>0.5</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>1.46</td>
</tr>
</tbody>
</table>

Fig. 10. $P_s$, the percentage of all cells with sheets, as a function of time. At zero time cells are allowed to begin S-phase by release of the chemical block; S-phase lasts about 14 h. Colcemid is added at a time shown by vertical arrow: A, after 16 h, early G$_2$; B, after 12 h, towards the end of S; and C, after 4 h, early in S. Control cells, O; colcemid-treated cells, •. Data from EB2 line.

nuclear indentations (Figs. 19, 22, 23), and often within the pockets of c--n--c sheets (Figs. 13, 25) where they lie with their long axes parallel to the surface of the nucleus.

When cultures were treated with colcemid at a concentration of 1.0 µg/ml, sufficient to cause the breakdown of the microtubules, as judged by electron microscopy, neither the percentage of cells with sheets nor the asymmetry, $D_t$, of the cell nucleus, averaged over the cells at all stages of the cycle, altered appreciably (Table 8).

In the chemically synchronized cells it was possible to treat with colcemid at various times after the release of the second FUdR block, which allows the S-phase to commence. Treatment 16 h after release, in early G$_2$, has little effect on the formation of sheets in G$_2$ (Fig. 10A). Treatment 12 h after release towards the end of S reduced the rate at which sheets were formed, but did not much change their final number (Fig. 10B). However, when treatment was even earlier, 4 h after release from the chemical block, hardly any sheets were formed until much later than usual in G$_2$, but the final value of $P_s$ approached that found in the untreated controls (Fig. 10C). Similar results were obtained in 5 similar experiments. In treated cells, microtubules did not reform and the cells were arrested at metaphase.
DISCUSSION

General considerations

Many cell nuclei are approximately spherical. Suspended droplets, when not acted on by external forces, also assume a spherical shape. The sphere is the geometric body which has the least surface area for a given volume. We assume that at the interface between the droplet and its surround, the molecules become ordered. This is an energy-requiring process and hence the most stable shape, namely the one for which the potential energy is at a minimum, requires that the surface area is a minimum. This is why droplets are spherical. The structural units, the unit threads, in cell nuclei also become ordered at the surface in contact with the enclosing membranes, a configuration which it was also assumed was the energetically favoured one (Davies, 1968a). Consider now the hypothetical spherical droplet, bounded by a membrane or envelope. A departure from spherical symmetry could arise in 2 ways. First, suppose that a change in shape arises without a change in volume; this will result in an increase in the surface area and hence will require an additional amount of envelope. For example, when a spherical rubber balloon containing an incompressible fluid is distorted, the change in shape requires a stretching of the envelope. In the case of the cell nucleus distortion at constant volume, that is at constant mass and concentration, or ratio of mass to concentration, would require either acquisition of new nuclear envelope, or, probably less likely, stretching of the existing one. Secondly, a change from a sphere to an asymmetric form without a change in surface area requires a decrease in enclosed volume. This could arise in the case of an initially spherical nucleus either by an increase in concentration brought about by loss of water or by loss of total substance. In the analogous transformation of plum to prune we may assume that the wrinkled appearance is largely due to loss of water following drying, the epidermis remaining constant in area. For any given envelope-limited, asymmetric body it is only possible to state that the amount of envelope is in excess of what is required to enclose the same volume in a spherical configuration.

Ideas about morphology or structure and function may perhaps be seen most clearly in relationship to simple viruses. Like protein molecules (Liquori, 1966), virus subunits assemble into structures which are the energetically favoured ones (Caspar, 1966). To ensure reproduction the particular structure must also be biologically effective, that is it must function. For example, a mutation which changed the nature of the subunits and resulted in the assembly of a tail unit in a bacteriophage which did not allow passage of the nucleic acid into the host bacterium would not yield a biologically effective virus, although this structure might be the energetically favoured one. Similar considerations can be applied to the cell nucleus. It has to be stated at the outset that our studies have not yet clearly revealed how biologically effective sheets are, that is no particular biochemical function has been found, although certain suggestions will be made.
Geometric factors in sheet formation

Certain geometrical considerations regarding the confining of a monolayer of unit threads by the membranes comprising the nuclear envelope were put forward earlier (Davies, 1968b). By model experiments on deformable plastic bodies it could be shown that \( c \cdot n \cdot c \) sheets and their associated pockets form by processes of nuclear invagination and evagination accompanied by movement of cytoplasm, the 2 membranes of the envelope flowing or deforming as a single entity. When a nucleus is deformed so as to produce 2 lobes separated by a sheet of the \( c \cdot n \cdot c \) type no pocket is formed. Neither \( c \cdot n \cdot n \) nor \( n \cdot n \cdot n \) sheets can form in this way, it was suggested, but these types may arise either if the inner membrane of the nuclear envelope invaginates between the \( b_2 \) and \( b_4 \) layers of units (Fig. 1), or if membrane fragments trapped within the nucleus at telophase migrate and come to lie parallel to the envelope-associated layer of units, the \( b_2 \) layer. These membrane fragments, perhaps arising from the nuclear envelope at the previous prophase, may fuse with the newly formed envelope, but apart from this fusion the above hypothesis involves membrane flow rather than membrane breakdown and fusion.

In this paper we describe the fragments of nuclear envelope-like material lying within the telophase nuclei, which fragments are rarely seen in the \( G_1 \) cell. Conceivably they may have migrated towards the surface of the cell nucleus giving rise to the \( c \cdot n \cdot n \) sheets as was suggested. However, this cannot be the only mechanism of formation, since \( c \cdot n \cdot n \) sheets largely disappear during \( S \)-phase and reappear during \( G_2 \). This suggests that the second mechanism, namely invagination of inner membrane, may also be operative.

One of our most striking findings is the correlation of \( P_s \), the percentage number of cells with sheets measured on cells at all stages in cycle, with the average degree of nuclear asymmetry, \( D_t \). Evidently with the increasing deformation of the nucleus there is an increasing probability of slip in the outer lamina, the layer of units which gives rise to the appearance of the \( b_1 \), \( b_2 \) and \( b_3 \) bands in the electron microscope. If a smooth-surfaced solid object had a laminated construction then it would be expected that during deformation the individual laminae might become separated and easily observable and hence the results on the cell nucleus are not surprising. However, the degree of asymmetry cannot be the only factor influencing the formation of sheets since the value of \( P_s \) when determined for each separate stage in the cycle decreases during the \( S \)-phase with hardly any change in \( D_t \). The nature of this second factor is not known. Withdrawal of DNA from its environment between the membranes may be necessary for its duplication.

It is known that when sheets are commonly found in association with the nucleus in other cell types, these nuclei are frequently polymorphic, for example in the neutrophilic or heterophilic granulocytes of lamprey (Davies & Small, 1968), certain fern oocytes (Bell, private communication) and in human medulloblastomas (Tani et al. 1971). Furthermore, there are polymorphic nuclei in which sheets have not been found. Nuclei in smooth muscle cells have a helical or spiralized morphology (Franke & Schinko, 1969; Lane, 1965); nuclei in epithelial cells are frequently flattened or
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ellipsoidal and thrombocytes of fish for example (Fawcett & Witebsky, 1964) are bilobed, but no envelope-limited sheets have yet been reported in them. These data on nuclei from other cells support the conclusion that nuclear asymmetry is a requisite but not the sole factor in determining the formation of envelope-limited sheets.

Role of microtubules

The way in which entire cells change shape as well as maintain their 3-dimensional forms are clearly of interest in regard to their individual functioning and to the formation of tissues and organs. The possible role of the cytoplasmic microtubule has received much attention (e.g. Byers & Porter, 1964; Porter, 1966). In the protozoan Actinosphaerium nucleofilum both the formation and the maintenance of the external form of the pseudopodial extensions, or axopods, were found to be correlated with an internal geometric arrangement of microtubules, the axoneme (Tilney, 1965; Tilney & Porter, 1967). When the microtubules were depolymerized the axopod changed form and disappeared. In a study (Gibbins, Tilney & Porter, 1969; Tilney & Gibbins, 1969) on developing embryos, changes in cell shape were also found to be associated with the presence of microtubules and did not take place in the presence of agents like colchicine, or with treatment at low temperature, which caused their depolymerization. Additionally, and unlike the case of Actinosphaerium which appears to be special, once the changes in shape had been established the new shape was not abolished when microtubules were broken down. This study led Tilney & Gibbins (1969) to emphasize the role of microtubules in the development of new cell shapes, rather than in their maintenance. Similar considerations apply to the development of shape in erythrocytes (Small, 1969; Small & Davies, 1972). The preservation of shape, it was suggested, might be taken over by extracellular factors as well as, possibly, by intracellular structures such as microfibrils. The question arises whether microtubules play a similar role in determining nuclear asymmetry and, or, the formation and maintenance of envelope-limited sheets.

Electron-microscope observations on human polymorphonuclear leucocytes by Bessis & Breton-Gorius (1967) showed that triangular chromatin-containing extensions of the nucleus were directed with their apex towards the centrioles, with microtubules parallel to the long sides of the extensions. In some extensions 2 opposing nuclear envelopes lay parallel to one another and either contained chromatin of width about 33.0 nm (fig. 6 in Bessis & Breton-Gorius, 1967) or were devoid of material staining like chromatin. Although these extensions were referred to as triangular or tubular, we suggest that the appearances in the thin section arise from wedges or sheets similar to those reported here, lying in a plane perpendicular to that of the section. In a study of changes in thrombocyte ultrastructure during clot retraction Shepro, Belamarich, Merk & Chao (1969) concluded that microtubules probably account for the actual lobation of the nucleus, but they did not describe any specific orientation of the microtubules with respect to the lobes.

When normal human leucocytes, lymphocytes and monocytes are treated with the anticoagulant sodium oxalate at an appropriate concentration for a few hours the normally spherical nuclei become lobed (Soderström, 1966, quoted in Norberg, 1969)
In some leukemias these mononucleate cells also acquire lobed nuclei. In the radially segmented nuclei the lobes or segments appear to radiate from a common centre. From light-microscope observations on whole cells and from electron micrographs, Norberg (1970) suggested that microtubules radiating from centrioles at this centre encircled the intersegmented nuclear clefts forming a sort of collar which he presumed gave rise to this constriction. In common with these workers we have noted in certain favourably cut sections, an orientation of the nuclear lobes towards the cell centre with outward radiating microtubules as well as microtubules lying in nuclear clefts and indentations. We conclude that microtubules probably do play a part in determining the nature of the nuclear asymmetry found in Burkitt’s cells but the morphological evidence relating microtubules to a specific nuclear morphology is not as convincing as it appears to be in the case of oxalate-treated leucocytes. Neither, apparently, do the pocket-forming $c \cdot n \cdot c$ sheets appear in any specific geometric orientation with respect to the lobes and the cell centre.

When mononucleate cells were treated (Norberg, 1969) with oxalate in the presence of vinblastine, an agent known to break down microtubules, the formation of polymorphic or radially segmented nuclei was considerably reduced. In a control group of 500 cells without vinblastine 302 became segmented, whereas in the presence of vinblastine only 124 out of the 500 cells counted were segmented. Evidently segmentation can occur in the absence of microtubules, broken down by vinblastine, but the fractional number is reduced. In our experiments, colcemid, another microtubule-depolymerizing agent was found not to affect either the existing average asymmetry $D_t$ or the value of $P_s$, the percentage number of cells with sheets obtained from cells at all stages of the cycle. However, when added early in $S$-phase, at a stage when $P_s$ was small, the value of $P_s$ did not increase in $G_2$ as it did in the control. These results suggest that microtubules are not required to maintain either nuclear asymmetry, or $c \cdot n \cdot c$ sheets once acquired, but that they play a role in the initial formation of $c \cdot n \cdot c$ sheets. This conclusion is parallel to that on cells where, as was already mentioned, it has been suggested (Tilney & Gibbins, 1969) that, in general, microtubules have a role in determining changes in cell shape rather than in maintaining the changed shape. Another fact which apparently supports this hypothesis is the increased number of sheets found in $S$–$G_2$ and $G_2$ compared with $S$ and the concomitant increase in number of microtubules. However, one contrary fact is the increase in $P_s$, admittedly much later in the colcemid-treated cells than in the controls, but in the absence of microtubules. Bessis & Breton-Gorius (1967) reported that the nuclear elongations were abolished when the cells were treated with colchicine at a concentration which destroyed microtubules. However, this finding does not necessarily contradict the above suggestion if it is assumed that the factors which would take over to stabilize the nuclear extensions are themselves short-lived in these cells: then in the absence of microtubules, depolymerized by the colchicine, the nuclear extensions disappear.
Role of nuclear envelope

Regarding the asymmetric shape of the Burkitt cell nucleus, it is clear that it is enveloped by membranes in excess of what are required to enclose the same volume in a spherical configuration. Whether excess nuclear envelope is utilized in enclosing the overall aspheric shape, or in enclosing monolayers of unit threads so as to form sheets, no doubt depends on what is energetically favourable. Similar considerations must apply to the way in which the nuclear envelope enclosing the monolayer, itself a small proportion never exceeding 10% of the whole envelope, is partitioned between the 2 major types, namely c·n·c and c·n·n. What is energetically favoured in turn depends upon little-understood factors, for example molecular forces existing between the layers of structural units. The nuclear chromatin is dispersed or extended during DNA synthesis in the S-phase (Tokuyasu, 1972) and this may be accompanied by a volume increase. If so, and the amount of nuclear envelope does not alter, it is conceivable that the envelope which disappears from the sheets may be reutilized in enclosing the increased nuclear volume. Disappearance of sheets during S-phase might be associated with a simultaneous flow of chromatin and envelope, since empty sheets were very rare, but this cannot be regarded as firmly established: if the process were rapid very few instances of it would be seen. In contrast, observations on both normal and hypertonic medium-induced prophases suggest a 2-step process with initial chromatin withdrawal leaving empty sheets, followed by envelope fragmentation.

It is not clear whether the nuclear envelope plays an active or passive role during formation of asymmetric nuclei with associated sheets. One possibility, which we favour, is that as a result of excess synthesis, the nucleus assumes an asymmetric conformation, the nature of which is partly determined by the localized configuration of microtubules. Alternatively the enclosing membranes could be synthesized in response to other factors which actually determine asymmetry. Fawcett, Anderson & Phillips (1971) concluded that the shape of the sperm head may be largely determined from within, by a specific genetically controlled pattern of aggregation of DNA and protein during condensation of chromatin. Before ideas can be developed further it will be necessary to learn more about how the synthesis of the membranes enveloping the nucleus is controlled. This may be related to the possible role of the nuclear envelope in maintaining an orderly arrangement of the chromosomes in the interphase nucleus (see review by Comings, 1968).

The fact that Pn, the percentage number of cells with sheets, increases with the viability of the cells in the culture shows that sheets are not nuclear transformations in dying cells. The formation of c·n·c sheets results in an increase in the area of chromatin in contact with the cytoplasm and hence the availability of the DNA molecules to cytoplasmic factors. In general, an increase in nuclear asymmetry at constant volume increases the surface area available for diffusion and it may be significant that malignant cells, with high growth rates, frequently have asymmetric nuclei (Bernhard & Granboulan, 1963; Koller, 1963). However, the resolving power of the autoradiographic technique is not sufficient to demonstrate clearly whether or not the DNA in sheets is more active in transcription than DNA at other locations in the...
nucleus. The unit threads in sheets have a similar appearance to those found in condensed chromatin where they are close packed. Also apart from the interruptions or imperfections, in sheets the monolayer is continuous with no nuclear pores. Hence, since condensed chromatin is known to be inactive in transcription it could be argued that the sheets which contain similar units might also be expected to be inactive. However activation of chromatin might consist merely in decondensation and separation of unit threads which might be active when only in a monolayer. There is a further possibility. The envelope-limited sheets frequently contain the so-called imperfections which may contain transcribable DNA and the sheets may be dynamically flowing structures which function to bring out these DNA regions into contact with the cytoplasm.

No doubt, sheets arise because they are energetically favoured configurations. However, it would be surprising if, considering their frequent presence in certain oocytes, a biochemical role were not eventually to be found for them.

We thank Prof. M. H. F. Wilkins for his encouragement and support, our colleagues in the Biophysics Department for discussion, Prof. M. A. Epstein for the provision of and help in establishing the Burkitt cells in this laboratory, and finally, members of the photographic department under the direction of Mr Z. Gabor, particularly Mrs F. Collier for help with the plates.

REFERENCES


Envelope-limited monolayers of chromatin


(Received 8 December 1972)

All electron micrographs in Figs. 11-25 are of EB2 cells

Fig. 11. Electron micrograph showing the prophase chromosomes attached to the envelope, with empty sheets both c·n·c and c·n·n. Arrow within nucleus to dispersing nucleolus. ×15000.

Fig. 12. Electron micrograph of the empty c·n·c sheet in Fig. 11. ×46500.

Fig. 13. Electron micrograph of a partially empty c·n·c sheet associated with an interphase cell; microtubules at arrows. ×35000.

Fig. 14. Electron micrograph of paired remnants of nuclear envelope in cell at metaphase. ×120000.
Envelope-limited monolayers of chromatin
Fig. 15. Electron micrograph of anaphase chromosomes with nuclear envelope-like fragments at arrows. × 13000.

Fig. 16. Electron micrograph of arrowed region, p, in Fig. 17 showing 4 centrioles and microtubules lying within a c·n·c pocket; a discontinuity in the monolayer occurs at i; 'granules' at arrow are end-on views of the unit threads. × 62000.

Fig. 17. Electron micrograph of a cell showing c·n·n and c·c·c sheets, one of which is enlarged in Fig. 16 to show centrioles in the cytoplasmic pocket, p. × 10000.
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Fig. 18. Electron micrograph of a cell showing nuclear lobes joined by $c\cdot n\cdot c$ sheets, arrowed; interruptions at $i$. $\times 15000$.

Fig. 19. Electron micrograph showing cytoplasmic microtubules (arrowed) lying within an indentation in the nucleus, adjacent to a $c\cdot n\cdot n\cdot n$ sheet. The nuclear envelope-like fragment within the nucleus has a pore (double-headed arrow). $c$, cytoplasm; $n$, nucleoplasm. $\times 49000$.

Fig. 20. Electron micrograph showing a $c\cdot n\cdot c$ sheet and adjacent $c\cdot n\cdot n$ sheet. $c$, cytoplasm; $n$, nucleoplasm. $\times 36750$. 
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Fig. 21. Electron micrograph showing 2 nuclear lobes and centrosome (arrowed) containing centriole. Note c-n-c sheet. × 10000.

Fig. 22. Electron micrograph showing 2 nuclear lobes connected by a c-n-c sheet, on the left side of which is a centriole (arrowed) lying in the indentation. × 10500.

Fig. 23. Electron micrograph of a 2-lobed nucleus like that in Fig. 22, except that a connecting sheet is not apparent, showing a centriole with attached satellites, lying in a nuclear indentation; microtubules radiate out and lie parallel to the nuclear surface. Similar microtubules occur but are not seen at lower magnification in Fig. 22. Amorphous electron-dense bodies at arrows. × 43750.
Envelope-limited monolayers of chromatin
Fig. 24. Electron micrograph showing 2 extensive c-n-c sheets associated with a nucleus. Numerous Golgi bodies surround a centriole (arrowed). x 15000.

Fig. 25. Electron micrograph showing a c-n-c sheet; microtubules (arrows) oriented parallel to the nuclear surface lie within the cytoplasmic pocket; interruption in sheet at i. x 26250.