STUDIES ON THE NUCLEOLUS

I. The Nucleolar Membrane of Rat Liver Cells

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INTRODUCTION

The structure of the nucleolus has remained an enigma ever since its discovery by Fontana in 1781 (Vincent, 1955). The electron microscope has not advanced our frontiers since the nucleolus and chromatin have not been as amenable to investigation as the mitochondria and the endoplasmic reticulum. The recent reviews on the ultra-structure of the nucleus (Whaley, Mollenhauer and Leech, 1960; Wischnitzer, 1960; Mirsky and Osawa, 1961) refer specifically to the absence of a limiting membrane for the nucleolus.

The idea that the nucleolus has a well-defined border has persisted for the past several decades despite Montgomery's (1898) suggestion that the dense nucleolar periphery is liable to be identified erroneously as a membrane. It has been recorded in the nucleoli of such diverse tissues as normal myeloblasts of man (Schridde, 1907, see Thorell, 1944), amphibian oocytes (Duryee, 1950; Callan, 1952), meristematic cells of plants (Chayen, 1952; Subramanyam, 1960) and rat liver cells (Wolman, 1957). In some oocytes it is surmised to be a lipoidal layer (Callan, 1952). Often, the membrane was seen only in unhydrolysed cells under a high resolution u.v. microscope (Chayen, 1952) or on over-differentiation of material stained with Giemsa (Subramanyam, 1960), or after special techniques intended to reveal membranes essentially similar to those seen under the electron microscope (Wolman, 1957). There are also reports that when the nucleoli extrude their contents into the cytoplasm, their membranes get incorporated into that of the nucleus (Duryee, 1950).

While elucidating the suitability of the haematoxylin squash technique for a study of the rat liver cells, it was observed that the nucleoli had a well-defined boundary (Royan, 1961). This discovery rests on the difference in the behaviour of plant and liver nucleoli to hydrolysis in N HCl at 60° C. for a few minutes. Unlike in plants, the interior of the liver nucleoli
lost their affinity for haematoxylin on hydrolysis revealing the well-stained envelope. This border may be chromatin alone, or the nucleolar membrane with its associated chromatin. The procedures described below would enable a clear separation of the nucleolar membrane from the chromatin associated with it.

**Observations**

Thin slices of the liver of rat fixed in acetic alcohol (1:3) were stored in 70% alcohol. They were downgraded to water and washed for 30 min. or more before processing them in different ways.

1. **Staining with Aceto-carmine**

Material mordanted in 4% ferric ammonium sulphate solution for periods ranging from 2-90 min. was washed in repeated changes of water for 30 min., teased into finer bits and kept in a drop of 1% aceto-carmine till they became dark in colour. The slide with the material was gently warmed over a flame, squashed under a coverslip and then sealed with paraffin wax. The colour of the cell organelles depended on the duration of exposure of the tissue to the mordant. When this was only 2 min. the colour remained reddish even after a lapse of 24 hours. A longer stay in the mordant was necessary to obtain a darker shade.

The nucleolus occupies no fixed position in the nucleus (Photos 1-4). It is sometimes seen in contact with the nuclear membrane (Photo 3). The nuclear membrane and the uneven border of the nucleolus were well defined only during the earlier stages of staining (Photos 1-3). When the nuclear and nucleolar matrices became dense with passage of time the irregularly stained patches at the nucleolar periphery were alone visible (Photo 4).

To improve the clarity of the structural details, the tissue was hydrolysed in N HCl at 60°C. (Royan, 1961) before staining with aceto-carmine. The exposure to the mordant was kept constant at 10 minutes. While in material hydrolysed for 2 min. the nucleolar matrix appeared lightly tinted, hydrolysis for more than 5 min. removed the affinity of the matrix for the dye. The nucleolar periphery in such preparations was as well defined as the nuclear membrane, but its irregularly thickened appearance rendered difficult a judgment whether the stained area is chromatin alone, or the membrane with its associated chromatin.

2. **Staining with Hematoxylin**

Unhydrolysed tissue kept for 10 min. in the mordant and 20 min. in the stain gave optimal pictures. The medium used for softening as well
as destaining the tissue was 45% acetic acid. While a fair separation of the cells could be obtained by teasing the tissue into fine bits prior to squashing in a drop of cold acetic acid, exposure of the stained bits to the same medium kept at 60° C. for 2 min. facilitated better destaining and easier separation of the cells. The tissue slices swell to two or three times their original size when kept for a few minutes in the cold or hot 45% acetic acid.

There is a general similarity in the structural details of the nucleoli in aceto-carmine (Photos 1-4) and hæmatoxylin preparations (Photos 5-7). The clarity is better in hæmatoxylin. A selection among the cells of a squash is necessary to study the membranes of the nuclei and nucleoli. The nuclear membrane is well defined in those cells which show a deeper staining. In such instances (Photos 5 and 6), however, the nucleolar details are not very clear. In those tinted relatively lightly (Photo 7) the nucleolus was seen to have a stained border. The irregular thickenings on this rind may be due to chromatin lying apposed to it. That the nucleolus has a distinct thin membrane (M) resembling that of the nucleus of the same cell (Photo 7) could be surmised from the thinness of the nucleolar ‘skin’ between the thickenings.

Since earlier experience (Royan, 1961) indicated that a mild hydrolysis in N HCl at 60° C. enabled a selective staining of the nucleolar border with hæmatoxylin, the effect of hydrolysis was explored further. No marked change was noticed when the time of hydrolysis was varied from 5-15 min. The nucleolar matrix remained unstained, or at best very lightly tinted, in contrast to the nucleolar border with its irregularly thickened configuration (Photos 8-15). There appears to be a slight swelling of the nucleolus on hydrolysis in N HCl (compare Photos 1-7 with Photos 8-14). The stained patches which gave an irregularly thickened appearance to the nucleolar border in Photos 1-3 and 7 appear stretched out along the periphery of the nucleolus in Photos 8-15. This may in all probability be due to the primary hydrolysis in N HCl and the secondary softening in 45% acetic acid. The presence of a nucleolar membrane could be surmised by the thinness of the stained rind between the irregularly thickened regions (arrows in Photos 8, 9 and 11-15). In rare instances, the nucleolar border appeared uniformly thick (Photo 10).

The cells were either uni- or bi-nucleate and each nucleus had one or more nucleoli. When there are more than one in a nucleus, they showed differences in size as well as shape (Photos 11-13). More often they were spherical. In Photo 11, the small nucleolus with a well-defined rind is lying close to the nuclear membrane. They assume a hemispherical contour
when in close contact with the nuclear border (Photos 12 and 13) and the thinness of the nuclear membrane at the region of contact with the nucleolus is suggestive of the dissolution of the nucleolar rind, and of a possible transfer of the nucleolar matter to the cytoplasm through the nuclear membrane (Montgomery, 1898; Subramaniam and Gopala Aiyar, 1935; Gates, 1942; Duryee, 1950; Brachet, 1957; Sirlin, 1961).

A small percentage of cells in any squash would be macerated by the pressure applied. Slightly ruptured nuclei offer better evidence for the presence of nuclear and nucleolar membranes. The rupture of the nucleus in Photo 14 is near the region where the nucleolus is in contact with it and the nuclear membrane is seen trailing into the cytoplasm. The stained border of the nucleolus is thin near the place of rupture (indicated by an arrow in Photo 14) and an arc-like area opposite to it gives the impression that the granular masses are attached to the thin membrane. These pictures lead one to presume that the nucleolus is bounded by a distinct thin membrane which is difficult to locate when the chromatin lying in contact with it exhibits an almost uniform distribution (Photo 10).

It is not easy to expel the nucleolus from the liver nuclei by pressure as is possible in the meristematic cells of plants. An isolated nucleolus with bits of nuclear matrix still adhering to it is illustrated in Photo 15. The configuration of its periphery resembles that illustrated in intact nuclei. Further pressure macerates the nucleolus but does not enable a separation of the nucleolar membrane from the stained bodies in contact with it. It became necessary, therefore, to elucidate whether the chromatin and the nucleolar membrane could be stained differentially by the Feulgen technique.

3. Staining by the Feulgen Technique

The tissue bits hydrolysed in N HCl at 60° C. for periods ranging from 5-15 min. were washed for 5 min. in distilled water. Unhydrolysed and hydrolysed material stained with leuco basic fuchsin for 90 to 150 min. were exposed to two changes of SO₂ water of 10 min. each, rinsed in distilled water and squashed immediately in 45% acetic acid.

When on examination, the cells appeared well scattered, the coverslips were released in 40% alcohol, downgraded to water and tinted lightly with a dilute solution of light green. They were gradually upgraded to absolute alcohol, cleared in mixtures of alcohol and xylene (3 : 1; 1 : 1; 1 : 3) followed by three changes in pure xylene and mounted in Canada balsam. Permanent mounts of Feulgen squashes processed through tertiary butyl alcohol (Royan, 1961) lacked clarity.
In unhydrolysed controls, the cell organelles were stained by the counter-stain alone. Under phase contrast, the nucleolus was dense and homogeneous with no indication of a well-defined border (Photos 16 and 17). One of the nucleoli is seen in contact with the nuclear membrane in Photo 17. The nuclear membrane appeared thick with irregular dark masses in contact with it reminiscent of the condition of the nucleolar membrane illustrated in Photos 1-15.

Hydrolysis for 5–10 min. produced a bright staining. It was less intense when exposure to hot HCl was extended to 15 minutes. Some of the Feulgen-positive structures were seen in contact with the Feulgen-negative nucleolus (Photos 18–20) while others were lying free in the nuclear matrix. The nuclear and nucleolar membranes were not easily discernible under an ordinary microscope (Photos 18–20). Neither were they distinct under phase contrast (Photos 21–23). The chromatin occurring in association with the nucleolus and the nuclear membrane were bluish-green under phase contrast and had a discontinuous distribution. The spaces between such bodies appeared as if bridged by a membrane having a different shade of colour (Photos 21–23). While Feulgen enabled the identification of the chromatin associated with the nucleolus (Caspersson, 1947; De Robertis, Nowinski and Saez, 1948; Vincent, 1955; Sirlin, 1960) the evidence for the presence of a membrane was inconclusive.

4. Secondary Fluorescence of the Nuclear Organelles on Staining with Acridine Orange

In living as well as fixed preparations, the chromatin fluoresces green and the nucleolus orange when stained with acridine orange and examined under the u.v. illumination. The nucleus of yeast is visible in living cells only under certain physiological conditions. Its general invisibility led to the disagreements witnessed in literature (Royan, 1958; Subramaniam, 1960). In such a material when the nuclear membrane is not well defined under phase contrast, it stands out under u.v. illumination on staining living cells with acridine orange. When the vitally stained cells are again examined under the phase contrast microscope, only a very small percentage exhibited a distinct nuclear membrane (Royan and Subramaniam, 1960). Further, the intra-nuclear structures were capable of differentiation into chromatin and nucleolar equivalents by their differential fluorescence. This experience gave the hope that the nucleolar membrane of rat liver cells may be capable of a clear distinction from the chromatin associated with it.

Unhydrolysed Material.—A long wash in water for 6–24 hours of the material stored in 70% alcohol was necessary to separate even a few cells by
squashing. Well-washed bits of tissue, therefore, were stained in acridine orange (1:40,000) for 5 min. and then squashed in a fresh drop of the dye. In scattered cells, the cytoplasm fluoresced green while some of its inclusions were orange. The nuclear matrix was green but the nucleolus appeared quite dense and orange in colour. In regions where the cells were closely packed together, all the cell organelles were green. A nucleolar membrane could be recognized but its clarity was masked by the density of the nucleolar matrix. The lack of contrast between the membrane and the matrix precluded presentation of a photograph.

Hydrolysed Material.—It was shown recently (Subramaniam and Royan, 1960) that acid hydrolysis of the tissue induced a reversal of the colours of the secondary fluorescence of the cell organelles. The chromatin and the nucleolus which were green and orange respectively in unhydrolysed cells were orange and green on hydrolysis. This colour reversal was found to improve the clarity of the nuclear organelles.

Hydrolysis in N HCl at 60°C for Five Minutes.—Strict control of the variables like the time of washing after hydrolysis and the duration of the storage of the tissue in bulk in 1 in 40,000 acridine orange was necessary to obtain reproducible results. Hydrolysed slices of liver, 1 mm. in thickness, washed for 2 min. in water were teased into still finer bits. One of these was stained for 15 sec. in acridine orange, squashed in a fresh drop of the stain and sealed with paraffin wax.

The secondary fluorescence emitted by the cell organelles was dull when examined immediately. Fifteen minutes later, the nuclear and nucleolar membranes, essentially similar in appearance, were clearer while the cytoplasm was dull green. After a lapse of two hours, these membranes were brighter. The chromatin, whether lying apposed to the nucleolar border or free in the matrix assumed a greenish tinge while the cytoplasm was greenish orange.

In scattered cells with dull orange nuclei, the nuclear and nucleolar membranes were green in the beginning and changed gradually to orange. This change in colour could be hastened by a wash in water for five to seven minutes before staining. The actual sequence of colours observed was dull green, greenish white, greenish yellow, dull orange and deep orange culminating in flame red. The intensity of the colours of the secondary fluorescence seems to depend on the proper scattering of the cells and the quantity of the fluorochrome available to each of these cells in a preparation (Subramaniam and Royan, 1960). If the cells are too many, or if the small number of cells under the coverslip are packed together, all the structures fluoresce only green.
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Even in these cells, the nuclear and nucleolar membranes could be located (Photos 24–26).

It is difficult to get a uniform distribution of the cells under a coverslip. To observe the complete transition in the colours of the fluorescence of the nucleus from green to orange it is only necessary to move from a region of crowding to areas where the cells are well separated. In scattered cells, the transition from green to orange progresses with time. In select instances the nucleolar membrane was olive green and the chromatin masses orange (Photos 27–28). Sometimes, portions of the nucleolar border itself fluoresced orange. In cells with deep orange nuclear matrix, the membranes were also of the same colour (Photo 29). The orange has a tendency to fade on exposure to ultra-violet unless it is very deep in colour. Representation of the nuclear and nucleolar membranes in a single photograph is difficult when both are not in the same plane (compare Photos 24–26 with Photos 27–29).

Attempts made so far to obtain an uniform staining of all cells in a squash have been a failure. In tissues hydrolysed for 5 min. neither a long wash in water extending for an hour nor a longer storage in the stain before squashing yielded better pictures. In fact, the flattening of the tissue mass into a layer one cell thick was rendered more difficult by these procedures.

Since hydrolysis for 5 min. produced only a partial reversal of the colours in the liver cells of the rat, the effect of hydrolysis for periods ranging from 5 min. to 15 min. was explored. A complete reversal of colours could be effected by hydrolysis for 15 minutes. The cytoplasm then was green and the nuclei flame red. The nuclear and nucleolar membranes visible in the initial stages were masked when the nuclear matrix began to fluoresce in shades varying from deep orange to flame red. The shorter period of hydrolysis, therefore, appeared to be better suited for a demonstration of the membranes.

Unlike haematoxylin and Feulgen, examination under an ultra-violet lamp of cells stained with acridine orange enabled a demonstration of the similarity between the nuclear and nucleolar membranes under certain specified conditions.

5. Fixed but Unstained Cells under Ordinary and Phase Contrast Types of Illumination

The nuclear and nucleolar membranes appeared relatively thick as compared to pictures of membranes in electron micrographs. This engendered the suspicion that an artificial increase in thickness may have been produced by adsorption of the stains. There was also the possibility that the thickness of the membranes in fluorescence micrographs 24–29 may be due to reflections from the membranes appearing as halos. Experience indicated that
the thickness of the membranes was more pronounced in the photographs than experienced visually. This necessitated a study under ordinary as well as phase contrast of material hydrolysed for 5 min. in N HCl at 60° C. and squashed in water.

**Hydrolysis in N HCl at 60° C. for Five Minutes.**—The nuclear and nucleolar membranes could be located even under an ordinary microscope (Photo 30). Their contrast, however, was not very satisfactory. Under phase contrast, the cytoplasm and the nuclear matrix were granular. The nuclear and nucleolar membranes, with their thickenings, were very clear (Photos 31–33). In Photos 31 and 32 the nucleolar border has an uneven contour. The thinness of the membrane could be made out in the region indicated by arrows in Photo 33. If material hydrolysed in N HCl is squashed in 45% acetic acid, the cytoplasm was almost transparent and the nuclear and nucleolar membranes were visible under the phase contrast microscope.

**Exposure to 45% Acetic Acid without Hydrolysis in N HCl.**—If slices of fixed liver tissue are squashed in a drop of water, the cells presented under phase contrast a golden yellow appearance without any structural details. The use of a softening agent like acetic acid is a necessity for a proper separation of the cells. When pieces of rat liver were kept in 45% acetic acid at 60° C. for 2–5 min. there was an enormous swelling of the tissue. If squashed immediately in a drop of cold 45% acetic acid, the structural details were not clear.

It was found that a wash in water for periods ranging from 5–20 min. after exposure to hot acetic acid produced a clarity of the structural details. If the wash is of short duration, the cells were almost transparent. This is gradually lost on keeping the material in water when the nucleolar matrix also becomes dense. The contours of the nuclear and nucleolar membranes could be seen in material washed for a short time in water. The appearances indicated that the thickenings of these membranes were due to material lying apposed to them.

To accentuate the contrast among these structures, material exposed to hot 45% acetic acid and kept in distilled water for just 2 min. was squashed in a drop of 1 in 40,000 acridine orange solution (Photos 34–39). A longer wash before staining was unsatisfactory. When examined under phase contrast, the nucleolar membrane was not as thick as in stained preparations. This is illustrated in Photos 36–39 of the same cell in different foci. Photo 39 illustrates clearly the continuous nature of the nucleolar membrane and how it could be distinguished from the chromatin lying apposed to it.
DISCUSSION

Various types of membrane systems have been described from electron micrographs (Sjöstrand, 1956; Whaley, Mollenhauer and Leech, 1960; Wischnitzer, 1960; Porter, 1961). Nuclei have been shown to be bounded by fenestrated double lamellae. It has generally been presumed that the nucleolus has no such limiting membrane. Mirsky and Osawa (1961) conclude: “Interactions between the nucleolus and other nuclear components are not limited by the properties of a membrane, a factor of much significance for interactions between the nucleus and cytoplasm or between the nucleus and the medium surrounding the cell” (p. 722).

Experience on Yeast indicated that what is seen in living nuclei (Royan, 1958; Subramaniam, 1960; Royan and Subramaniam, 1960) are not often detected under the electron microscope (Agar and Douglas, 1957; Mundkur, 1960; Hashimoto, Conti and Naylor, 1958). Sjöstrand (1957) cautions that the technique for electron microscopy has still to be perfected. Fell (1960) emphasizes that there is little hope in the near future of a strict comparison of living and fixed material under the electron microscope.

The chromatin associated with the nucleoli of animal cells often form a complete border (Vincent, 1955; Brachet, 1957). It is generally conceded that plant nucleoli originate in association with the organizer regions of the satellited chromosomes (McClintock, 1934; Gates, 1942). These satellites may be eu- or hetero-chromatic (Fernandes, 1936). Only when they are heterochromatic could the nucleoli be seen in association with them at interphase. A similar situation has been reported in different tissues of animals also (Ohno and Kinosita, 1955). The dense nucleolar periphery considered by Davis (1960) to be rare (his Fig. 6) appears to be common in the cells illustrated in this paper. Even in unhydrolysed material a denser periphery is usually observed (Photos 1-17). The chromatin associated with the nucleolus exhibits a variable disposition in the different cells (Photos 18-23).

To prove the existence of a nucleolar membrane, it should be capable of distinction from the chromocenters associated with it. Each technique has its limitations. While aceto-carmine, hæmatoxylin and the fluorochrome, acridine orange, gave indications of the presence of a membrane similar to that of the nucleus, Feulgen squashes confirmed that the chromatin associated with it has generally an irregular distribution. Convincing evidence for the presence of a distinct nucleolar membrane could be adduced from two directions. It is clear in cells hydrolysed in N HCl and examined without staining under the phase contrast microscope (Photo 34). It is better defined in cells exposed to the swelling action of 45% acetic acid at 60° C. for 2-5 min.,
stained with acridine orange (1: 40,000) and then examined under phase contrast.

**SUMMARY**

Evidence for the presence of a nucleolar membrane in rat liver cells is marshalled from a variety of techniques. The basophilia of the nucleolar matrix has to be removed in order to locate this membrane. Unlike acetic-carmine and haematoxylin, Feulgen enabled the specific identification of chromatin but not the nucleolar membrane. Under specified conditions, the nuclear and nucleolar membranes could be seen in squashes of hydrolysed cells stained with acridine orange (1: 40,000) and examined under u.v. illumination. Convincing proof for the presence of a nucleolar membrane was obtained by examination under phase contrast, cells, either hydrolysed in N HCl at 60°C for 5 min.; or lightly tinted with acridine orange, after exposure to 45% acetic acid at 60°C for 2-5 min.

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DESCRIPTION OF PHOTO-MICROGRAPHS

Photos 1–39 are of the Nuclei of Rat Liver Cells. Excepting Photos 16–23 the others are from temporary mounts

PLATE XVIII

PHOTO 4. The Nucleolus becomes denser with progress of time.
PHOTO 7. The nucleolar “skin” (M) between the irregular thickenings is thin and resembles the nuclear membrane (N).
PHOTOS 8–15. Hydrolysed in NHCl at 60° C. and stained with haematoxylin. The nucleolar matrix is unstained and the arrows indicate regions where the nucleolar membrane could be recognized, × ca. 2,700.
PHOTO 10. The nucleolar border is uniformly thick.
Photos 12 & 13. The nucleolus assumes a hemispherical contour when it is in close contact with the nuclear membrane.

Photo 14. The nuclear membrane (N) is indicated by an arrow at the place of its rupture.

**Plate XIX**

Photo 15. A rare instance of an isolated nucleolus.


Photos 21-23. Feulgen—light green. Phase Contrast, × ca. 5,200. The thickenings on the membrane are Feulgen-positive. Arrows indicate the region of the nucleolar membrane.

Photos 24-29. Hydrolysed in N HCl at 60°C. for 5 min. and stained with acridine orange (1:40,000). Ultra-violet illumination, × ca. 4,100.

Photos 24-26. The nuclear membrane is in focus.

Photos 27-29. The nucleolar membrane is in focus.

Photos 27 & 28. The nucleolar membrane was olive green while the chromatin masses were orange.

Photo 29. The nuclear matrix, and the nuclear and nucleolar membranes were deep orange.

**Plate XX**

Photos 30-33. Unstained. Hydrolysed in N HCl at 60°C. for 5 minutes.

Photo 30. Ordinary illumination. The nuclear and nucleolar membranes are visible, × ca. 2,500.

Photos 31-33. The nucleolar border has an uneven contour. The thin nature of the membrane is shown by arrows in Photos 32 and 33. Photos 31 and 33, × ca. 2,500. Photo 32, × ca. 4,100.

Photos 34-39. Softened with 45% acetic acid at 60°C. for 2 minutes and squashed in a drop of acridine orange (1:40,000). Phase Contrast. The nucleolar membrane is as thin as the nuclear membrane. Photos 34-38, × ca. 2,400. Photo 39, × ca. 5,200.

Photos 37-39. The same cell in different foci.

Photo 39. The nucleolar membrane (M) could be distinguished from the chromatin (CHR) lying apposed to it.

CHR: Chromatin; N: Nuclear Membrane; M: Nucleolar Membrane.