

## CHARACTERIZATION OF NUCLEAR STRUCTURES CONTAINING SUPERHELICAL DNA

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### SUMMARY

Structures resembling nuclei but depleted of protein may be released by gently lysing cells in solutions containing non-ionic detergents and high concentrations of salt. These nucleoids sediment in gradients containing intercalating agents in a manner characteristic of DNA that is intact, supercoiled and circular. The concentration of salt present during isolation of human nucleoids affects their protein content. When made in 1.95 M NaCl they lack histones and most of the proteins characteristic of chromatin; in 1.0 M NaCl they contain variable amounts of histones. The effects of various treatments on nucleoid integrity were investigated.

### INTRODUCTION

Structures resembling nuclei but depleted of protein may be released by gently lysing cells in 1.0 M NaCl (Cook & Brazell, 1975, 1976). These nucleoids sediment through gradients containing intercalating agents in a manner characteristic of DNA that is intact, supercoiled and circular. We concluded that nucleoid DNA was subject to the same kind of topological constraint restricting rotation of one strand of the duplex about the other as that found in circular DNA molecules. In this paper, human nucleoids are characterized further. Techniques for manipulating nucleoids have been studied in some detail since the DNA within them proves to be resistant to shearing forces and is useful for studies on its conformation and template activity (Colman & Cook, 1976).

### MATERIALS AND METHODS

#### *Cells*

Cells were prepared as described in the accompanying paper (Cook & Brazell, 1976) and elsewhere (Jost, Lennox & Harris, 1975).

#### *Mixtures used for lysing cells*

A number of mixtures, all adjusted to pH 8.0 but containing different salt concentrations, were used to lyse cells. They are designated by parentheses containing the final salt concentration

of the mixture after the addition of the cells. Lysis mixture (0.4 M NaCl) contains sodium chloride, ethylenediamine-tetra-acetic acid, tris-(hydroxymethyl)-aminomethane, Brij and glycerol such that on the addition of 1 volume of phosphate-buffered saline containing cells to 3 volumes of the mixture, the final concentrations of the constituents become 0.4 M, 0.1 M, 2 mM, 1 % and 10 %, respectively. (For the purpose of calculating the final concentration of sodium chloride, the contribution of the phosphate-buffered saline is neglected.) Lysis mixture (1.0 M NaCl) contains sodium chloride, ethylenediamine-tetra-acetic acid, tris-(hydroxymethyl)-aminomethane and Triton X-100 such that on addition of 1 volume of phosphate-buffered saline containing cells to 3 volumes of the mixture the final concentrations of the constituents become 1.0 M, 0.1 M, 2 mM and 0.5 %, respectively. Lysis mixture (1.95 M NaCl) is similar to lysis mixture (1.0 M NaCl) except that the final concentration of sodium chloride is 1.95 M.

### *Sucrose and glycerol gradients*

Nucleoid conformation was analysed in 'isokinetic' sucrose gradients (Cook & Brazell, 1975). Sucrose gradients (15–30 % sucrose; 4.6 ml; pH 8.0) contained sodium chloride (1.0 or 1.95 M), tris-(hydroxymethyl)-aminomethane (0.01 M), and ethylenediamine-tetra-acetic acid (0.001 M) in addition to variable amounts of ethidium bromide. Glycerol gradients (30–50 % glycerol; 4.6 ml; pH 8.0) contained sodium chloride (0.4 M), tris-(hydroxymethyl)-aminomethane (0.01 M), and ethylenediamine-tetra-acetic acid (0.001 M) in addition to variable amounts of ethidium bromide. Generally, 200- $\mu$ l samples were applied to the gradients which were spun at 20 °C in an SW 50.1 rotor on a Beckman L2-65b centrifuge at speeds and times indicated in the legends to the figures. Gradients were analysed by passing them through an absorbance monitor operating at a wavelength of 254 nm. The position of nucleoids in the gradient was indicated by a peak in the optical density trace, and the distance the nucleoids travelled down the gradient was determined by measuring the distance on the trace from the meniscus to the peak. Fractions from the gradients were collected in tubes carried on an Ultrarac 7000 fraction collector (LKB-Produktur AB, Sweden). Nucleoids were then pelleted from the fractions and stored at -70 °C until required for protein analysis. Six gradients were generally spun together in one rotor, and at least one gradient of the six served as a reference. The distances travelled by nucleoids in other tubes were expressed as ratios relative to the distances sedimented by nucleoids in the reference tube. The contents of the reference tube are indicated in the legends to the figures.

Isokinetic sucrose gradients (15–30 %; 4.6 ml; pH 8.0) containing 1.0 M NaCl and underlaid with 0.5 ml of 60 % sucrose saturated with CsCl were prepared and used as described by Cook & Brazell (1975). (The underlay catches any rapidly sedimenting material and prevents it pelleting on the bottom of the tube.) 150  $\mu$ l of lysis mixture (1.0 M NaCl) and 50  $\mu$ l of phosphate-buffered saline containing radioactive cells were applied to these gradients, the gradients were then spun at 5000 rev/min for 25 min at 20 °C, fractionated, and the radioactive content insoluble in trichloroacetic acid determined as described below.

HeLa nucleoids were prepared in bulk on 'step' gradients. These were made by layering 10 ml of a 15 % sucrose solution over 2.5 ml of a 30 % sucrose solution. Both sucrose solutions (pH 8.0) contained 1.95 M NaCl, 0.01 M tris-(hydroxymethyl)-aminomethane and 0.001 M ethylenediamine-tetra-acetic acid. 0.5 ml of phosphate-buffered saline containing about  $5 \times 10^6$  cells was added to 1.5 ml of lysis mixture (1.95 M NaCl), mixed and after 15 min at room temperature the mixture was layered on top of the step gradient. Gradients were spun at 2500 g for 25 min at 4 °C and the white aggregate containing the majority of the nucleoids was removed from the interface between the 15 and 30 % sucrose. (Sometimes some nuclear material remains floating above the 15 % sucrose or pellets on the bottom.)

### *Caesium chloride density gradients*

Three millilitres of a 2:3 (w/v) solution of caesium chloride in distilled water in a centrifuge tube were overlaid with 150  $\mu$ l of lysis mixture (1.0 M NaCl) followed by 50  $\mu$ l of phosphate-buffered saline containing cells. After 15 min the tube was spun at 38000 rev/min for 48 h in the SW 50.1 rotor on a Beckman L2-65b ultracentrifuge to establish the density gradient.

An aggregate of nucleoids banded in the middle of the gradient and was collected with a needle inserted through the side of the tube.

### Enzymes

Ribonuclease A (Worthington Biochemical Corporation, Freehold, N.J., 13.8 mg/ml; 5139 units/ml), diluted 10-fold in 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0), was immersed in boiling water for 10 min to inactivate any deoxyribonuclease present. Dilutions of this heat-treated enzyme were made in 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0) and 0.02 % bovine serum albumin. Pronase (Calbiochem Ltd., Hereford; B grade, free of nucleases; activity 90 000 PUK/g) was dissolved in 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0) and allowed to self-digest at 20 °C for 1 h before use. Dilutions were made in 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0).

### Fluorescence microscopy

Nucleoids in solutions containing 100 µg/ml ethidium were counted in a haemocytometer by fluorescence microscopy using a Leitz Orthoplan microscope with the following filters: incident light, heat filter KG1 (2 mm), filter BG38, excitation filter in position 1; fluorescent light, suppression filter in position 1, barrier filter K530. Unless otherwise stated, samples were prepared for photography as follows. Cells were lysed in lysis mixture (1.95 M), an equal volume of 100 mM dithiothreitol in 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0) added, and ethidium (5 mg/ml) added to make a final concentration of 100 µg/ml. Colour photographs were taken immediately on daylight high-speed Ektachrome film using a 30-s exposure. When acridine orange (100 µg/ml) was used as a fluorescent stain, the excitation and suppression filters were placed in positions 4 and 3 respectively.

### Radiochemicals

Radioactive materials were obtained from the Radiochemical Centre, Amersham. Cells were labelled at the concentrations described in the legends with [5-<sup>3</sup>H]uridine (sp. act. 28.4 Ci/mmol), [*Me*-<sup>3</sup>H]thymidine (56 Ci/mmol), and L-[4,5-<sup>3</sup>H]leucine (58 Ci/mmol). The amount of radioactivity insoluble in trichloroacetic acid was determined by applying aliquots containing nucleoids to a 2.5-cm glass-fibre disk (Whatman GF/C). The disks were washed successively with 15 ml of 5 % trichloroacetic acid and ethanol, dried, placed in a glass vial containing 3.0 ml Unisolve 1 (Koch-Light, Colnbrook, Bucks) and the amount of radioactivity present measured in a Packard Tri-Carb liquid scintillation spectrometer (Model 3390).

### Analysis of proteins

DNA-binding proteins from mouse ascites tumour cells were prepared as described previously (Jost *et al.* 1975). DNA-cellulose columns containing double-stranded DNA were prepared as described by Litman (1968) with minor variations (Scherzinger, Litfin & Jost, 1973).

Chromatin was isolated by Bhoree & Pederson's (1973) method, with slight modifications. Nuclei from HeLa cells were prepared by swelling the cells in 0.01 M NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM tris-(hydroxymethyl)-aminomethane (pH 7.5) for 15 min, and then breaking them in a Dounce homogenizer. When 90–95 % of the cells had released intact nuclei, the nuclei were pelleted at 1000 g for 5 min. They were resuspended in 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0), 0.1 mM phenylmethylsulphonylfluoride, 0.2 mM β-mercaptoethanol, 0.2 % NP40 (Shell Chemical Co.) and pelleted at 2000 g for 5 min. The process was repeated 2 or 3 times without NP40 in the buffer until no perinuclear halo of adherent cytoplasm was observed in the microscope. The nuclear pellet was resuspended in 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0), 0.1 mM phenylmethylsulphonylfluoride, 0.2 mM β-mercaptoethanol, and the nuclei allowed to swell; the nucleoli were then liberated by four or five 30-s sonications (MSE ultrasonicator large tip, maximum power). Nucleoli were removed by pelleting through 1.1 M sucrose in 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0), 0.1 mM phenylmethylsulphonylfluoride, 0.2 mM β-mercaptoethanol in an SW 27 rotor at 5000 rev/min for 15 min in a Beckman ultracentrifuge. Chromatin in

the supernatant was spun through 1.7 M sucrose in the above buffer (SW27, 25000 rev/min, 2 h). Proteins were removed from chromatin by extracting the pellet with 2.0 M salt.

Proteins eluted from columns containing bound DNA or from chromatin and nucleoids were subjected to electrophoresis on polyacrylamide slab gels containing sodium dodecyl sulphate (Laemmli, 1970; Studier, 1973) which were stained and analysed by densitometry as described by Jost *et al.* (1975). Radioactivity was extracted from the gels by treating dried slices (1 mm) with 0.15 ml  $\text{H}_2\text{O}_2$  at 50 °C for 10 h, solubilized by the addition of 0.5 ml Nuclear Chicago tissue solubilizer (NCS) and counted in a toluene-based scintillant in a Packard scintillation counter.

Proteins were iodinated by the chloramine T method of Sonada & Schlamowitz (1970) with the modifications given. Chloramine T was dissolved in a solution containing 5 vol. of water and 2 vol. of 0.5 N  $\text{H}_2\text{SO}_4$ . Between 1 and 10  $\mu\text{g}$  of protein in 50  $\mu\text{l}$  0.3 M sodium phosphate buffer (pH 7.3) were added to 10  $\mu\text{l}$  chloramine T solution (1.2  $\mu\text{g}/\text{ml}$ ) and 10  $\mu\text{l}$   $^{125}\text{I}$  (10  $\mu\text{Ci}/\mu\text{l}$ ). After 2 min the reaction was stopped by the addition of 10  $\mu\text{l}$  5%  $\beta$ -mercaptoethanol. Then 10  $\mu\text{l}$  of solutions containing tyrosine 50  $\mu\text{g}/\text{ml}$  and bovine serum albumin (50  $\mu\text{g}/\text{ml}$ ) were added. Of the 100  $\mu\text{l}$  final volume, 10  $\mu\text{l}$  were mixed with 10  $\mu\text{l}$  sample buffer (Laemmli, 1970) and subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Autoradiographs were prepared with Kodak X-ray film from the dried gels. Protein pellets containing  $10^5$ – $10^6$  nucleoids obtained from sucrose gradients (15–30%) were suspended in 0.3 M sodium phosphate buffer (pH 7.3). In some experiments the suspension was sonicated exhaustively to break the nucleoids, but this treatment had no effect on the protein pattern obtained after electrophoresis in polyacrylamide gels.

### *$\gamma$ -irradiation*

Sucrose gradients containing nucleoids were irradiated as described by Cook & Brazell (1975, 1976).

## RESULTS

### *Lysis procedure*

Nucleoids are prepared in a lysis mixture that contains a non-ionic detergent to disrupt membranes and 1.0 or 1.95 M NaCl (Cook & Brazell, 1975, 1976). Release of nucleoids is readily monitored using phase-contrast microscopy. Nuclei in intact

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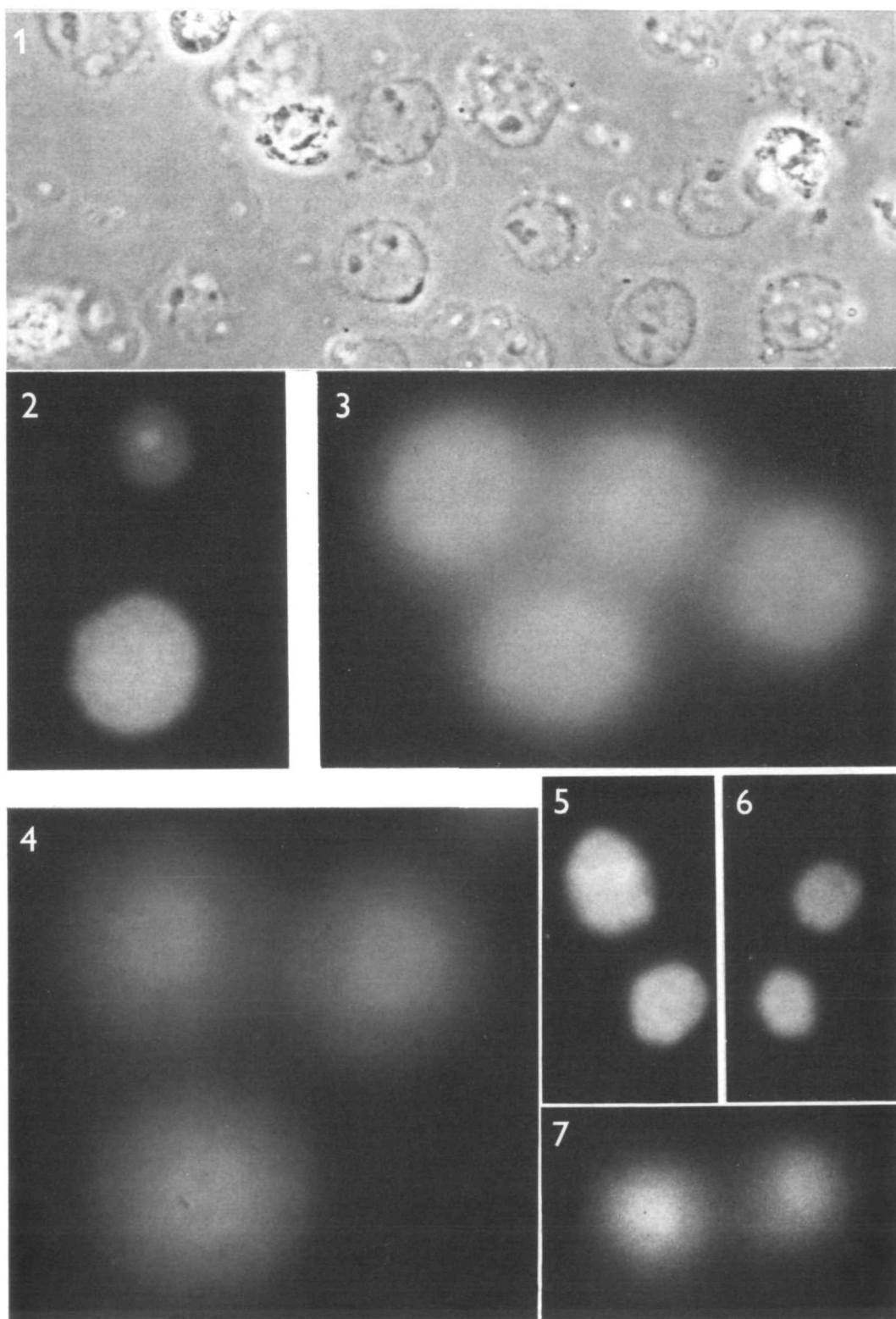
Fig. 1. HeLa nucleoids isolated in 1.95 M NaCl on step gradients and photographed in the phase-contrast microscope (Ilford Pan F film).  $\times 252$ .

Fig. 2. HeLa nucleoids prepared in 1.95 M salt, stained with ethidium and photographed in the presence of dithiothreitol in the fluorescence microscope. One nucleoid with a nucleolus is derived from an interphase cell, the other from a mitotic cell.  $\times 252$ .

Fig. 3. HeLa nucleoids prepared in 1.95 M salt, stained with ethidium and photographed in the absence of dithiothreitol in the fluorescence microscope. A brightly fluorescing halo surrounds the nucleoids.  $\times 252$ .

Fig. 4. HeLa nucleoids prepared in 1.95 M salt on a step gradient, irradiated ( $54.4 \text{ J kg}^{-1}$ ), stained with ethidium and photographed in the presence of dithiothreitol in the fluorescence microscope. A brightly fluorescing halo surrounds the nucleoids.  $\times 252$ .

Figs. 5–7. Chick nucleoids from the red blood cells of 5-day-old embryos, 12-day-old embryos and hen erythrocytes (Figs. 5–7, respectively). Nucleoids were prepared in 1.0 M NaCl, stained with ethidium and immediately photographed in the presence of dithiothreitol in the fluorescence microscope.  $\times 252$ .



cells appear dark and on lysis they become light as nuclear proteins are lost. Nucleoli are clearly visible in the nucleoids and blebs of cytoplasmic material and membrane ghosts adhere to them (Fig. 1).

### *Fluorescence microscopy*

When ethidium intercalates into DNA there is an enhancement of its fluorescence (LePecq & Paoletti, 1967) so that nucleoids stained with ethidium may be conveniently counted in a haemocytometer by fluorescence microscopy. Stained nucleoids fluoresce strongly, the brightness of fluorescence reflecting the nucleic acid content of the nucleoid. (For example, HeLa nucleoids fluoresce more brightly than those of the *Drosophila* cell-line, K85.) A photomicrograph of HeLa nucleoids stained with ethidium is presented in Fig. 2. Fluorescent nucleoli are clearly visible in nucleoids derived from interphase nuclei. (Ethidium-RNA complexes also fluoresce strongly.) The chromosomes derived from mitotic cells remain identifiable in the lysis mixture and fluoresce brightly.

Nucleoids stained with ethidium are extremely sensitive to the illumination used in the fluorescence microscope. Initially, the nucleoid has a well defined limit, but on continued illumination a rapidly expanding halo develops around the original limits of the nucleoid, which remain as a bright rim. (This effect may be caused by photoactivation of the ethidium resulting in the cutting of the DNA duplex so that DNA is able to escape from the confines of the nucleoid.) The rate of dispersion of the fluorescent material is slowed in the presence of 50 mM dithiothreitol. (Dithiothreitol perhaps competes with the DNA for any photoactivated compound and thus lessens DNA fragmentation.) In Figs. 2 and 3 photomicrographs taken in the presence and absence of dithiothreitol are presented. Since the photography of fluorescing nucleoids requires exposures of about 30 s, dithiothreitol is routinely added to stabilize the nucleoids. However, it should be noted that within about 5 min in the dark, and in the absence of ethidium, dithiothreitol will destroy nucleoid integrity.

Nucleoids which have been irradiated with  $\gamma$ -rays are surrounded by a fluorescing halo, the size of this halo being roughly proportional to the dose of irradiation (Fig. 4). High doses of radiation ( $54.4 \text{ J kg}^{-1}$ ) disperse fluorescing material in nucleoid preparations; low doses ( $9.6 \text{ J kg}^{-1}$ ) which abolish the characteristic sedimentation properties of nucleoids, have a minimal effect.

Nucleoids from the *Drosophila* K85 cell-line fluoresce weakly, but those made from the highly polytenized nuclei of the salivary glands of *Drosophila* larvae fluoresce more brightly than HeLa nucleoids (J. M. Levin & P. R. Cook, unpublished observations). The nucleoids from polytenized nuclei contain structures which, though larger and more diffuse, clearly resemble the polytene chromosomes of intact nuclei stained with ethidium. They possess the same striking banding pattern of bright and weakly fluorescing regions even in  $1.95 \text{ M NaCl}$  in the lysis mixture.

Acridine orange emits red and green fluorescence when bound to RNA and DNA, respectively. HeLa nucleoids stained with acridine orange fluoresce in the green, but are bounded by a red rim and contain brightly fluorescing red nucleoli. Even in

the presence of dithiothreitol, illumination disperses fluorescent material in nucleoids stained with acridine orange within a few seconds.

The appearance of various chick nucleoids obtained from different red blood cells is of some interest because their DNA is constrained to different degrees (Cook & Brazell, 1976). Photomicrographs of various chick nucleoids prepared in 1.0 M NaCl and stained with ethidium are presented in Figs. 5–7. The limits of chick nucleoids are not as well defined as those of HeLa, whose integrity (as estimated by fluorescence microscopy) remains intact for a number of hours. Chick nucleoids are fragile and easily broken by pipetting. This is especially so for the nucleoids derived from the red blood cells of 5-day-old embryos. Hen erythrocyte nucleoids differ from the other types studied. When hen erythrocytes are added to lysis mixture (1.0 M NaCl) fluorescent material is released which rapidly and progressively becomes more diffuse, so that within 5 min the intensity of fluorescence has diminished but extends over a greater area. The lysis mixture also becomes viscous. The nucleoids released from adult hen erythrocytes appear very similar to heavily irradiated ( $54.4 \text{ J kg}^{-1}$ ) nucleoids from the red cells of 5-day-old embryos.

#### *RNA content of nucleoids*

Nucleoids made from HeLa and XTC-2 cells contain all nuclear DNA, most of the nuclear RNA and variable amounts of protein (Colman & Cook, 1976). Little RNA escapes from nuclei during the preparation of nucleoids. HeLa cells were grown in a medium containing [ $^3\text{H}$ ]uridine ( $20 \mu\text{Ci/ml}$ ) for 15 min to label only nuclear RNA. The labelled cells were added to lysis mixture (1.0 M NaCl) floating on a sucrose gradient that contained 1 M NaCl and was underlaid with 0.5 ml of 60% sucrose saturated with CsCl (see Materials and methods). After the gradients were spun and their optical density and radioactive profiles analysed, more than 95% of the radioactivity insoluble in trichloroacetic acid and initially present in cells and applied to the gradient was found co-sedimenting with the optical density peaks produced by the nucleoids. Nucleoids therefore contain nearly all the RNA that becomes rapidly labelled in nuclei.

#### *Nucleoid conformation in different salt concentrations*

The conformation of nucleoids is analysed by sedimenting them through sucrose gradients containing ethidium. The distance sedimented by the nucleoids is expressed as a ratio relative to that of nucleoids sedimenting under standard conditions. The ratio reflects the DNA conformation within the nucleoid (Cook & Brazell, 1975). Nucleoid conformation in different salt solutions was studied for various reasons. High salt concentrations reduce the affinity of many proteins for DNA, so nucleoids might be prepared free of proteins by isolating them in 1.95 M salt. If protein–DNA binding is responsible for packaging and constraining nucleoid DNA, increasing the salt concentration might destroy nucleoid integrity or increase the length of DNA over which the topological constraint acts (see Cook & Brazell, 1975, for a discussion). Changing the salt concentration surrounding a constrained DNA also alters the number of double helical turns, and therefore changes the number of

any superhelical turns (Wang, 1969; Upholt, Gray & Vinograd, 1971; Hinton & Bode, 1975*a, b*).

In pilot studies, HeLa nucleoids prepared in 1.0 M NaCl contained small quantities of histone which varied from preparation to preparation. This variation depends upon the length of time that the nucleoids are maintained in the lysis mixture (1.0 M NaCl) before being spun through sucrose gradients: the longer the lysis time, the less histones are subsequently found in the nucleoids. Increasing the salt concentration in the lysis mixture results in more rapid dissociation of protein to give nucleoids essentially free of histones. When nucleoids prepared in lysis mixture (1.95 M NaCl) are spun in sucrose gradients containing 1.95 M NaCl, their sedimentation rate is dependent on the ethidium concentration in the gradient in the biphasic manner characteristic of DNA that is constrained (Fig. 8c; see the accompanying paper for a discussion). The rate of sedimentation of HeLa nucleoids in the presence of 1.95 M salt and high concentrations of ethidium ( $> 14 \mu\text{g/ml}$ ) is about the same as the rate in the absence of ethidium. This is not so for HeLa nucleoids sedimenting in 1.0 M salt; these sediment in high concentrations of ethidium more slowly than in the absence of ethidium (Fig. 8B). As roughly similar concentrations of ethidium are required to reduce the sedimentation rate to the minimum value in both 1.0 and 1.95 M salt, direct effects of NaCl on the winding of the duplex must be quite small at these high concentrations (cf. Upholt *et al.* 1971). The differences in shape of the 2 curves probably result from differences in the protein content of the nucleoids (cf. Figs. 16 and 17, pp. 320, 321).

Nucleoids prepared in lysis mixture (0.4 M NaCl) and spun in glycerol gradients containing 0.4 M NaCl are included in Fig. 8 for comparison. (Nucleoids tend to aggregate in sucrose gradients containing 0.4 M NaCl, so glycerol gradients were used for these experiments.) Nucleoids prepared in this way have many of the properties of nuclei and contain many nuclear proteins including the histones (E. Jost, unpublished observations). Unlike their counterparts prepared in 1.0 or 1.95 M NaCl, they appear darker in the phase-contrast microscope and they do not fluoresce as strongly when stained with ethidium. Their sedimentation rate is reduced progressively by increasing concentrations of ethidium. This sedimentation behaviour does not necessarily imply that in 0.4 M NaCl nucleoid DNA is not constrained or supercoiled, since the proteins in these nucleoids might prevent intercalation of the ethidium.

Irradiation affects the rate of sedimentation of HeLa nucleoids in 1.95 M NaCl in much the same way as it affects nucleoids prepared in 1.0 M NaCl (Fig. 9; cf. Figs. 8 and 9 in Cook & Brazell, 1975). This indicates that the topological constraint restricting rotation of one strand of the duplex about the other acts over similar lengths of DNA in the nucleoids prepared in the two different salt concentrations.

Salt might affect nucleoid conformation both directly by altering the winding of the duplex and indirectly by affecting the binding of proteins that alter the conformation of DNA. Direct and indirect effects may be distinguished by comparing the sedimentation properties of nucleoids with differing protein constitutions in



gradients containing the same salt concentration. Fig. 10 illustrates the sedimentation properties of nucleoids prepared in step gradients in 1.95 M NaCl when respun in gradients containing 1.0 M NaCl and various concentrations of ethidium. The rate of sedimentation of these nucleoids depends upon the ethidium concentration in the gradient: they behave as if their DNA were still constrained, since the curve is

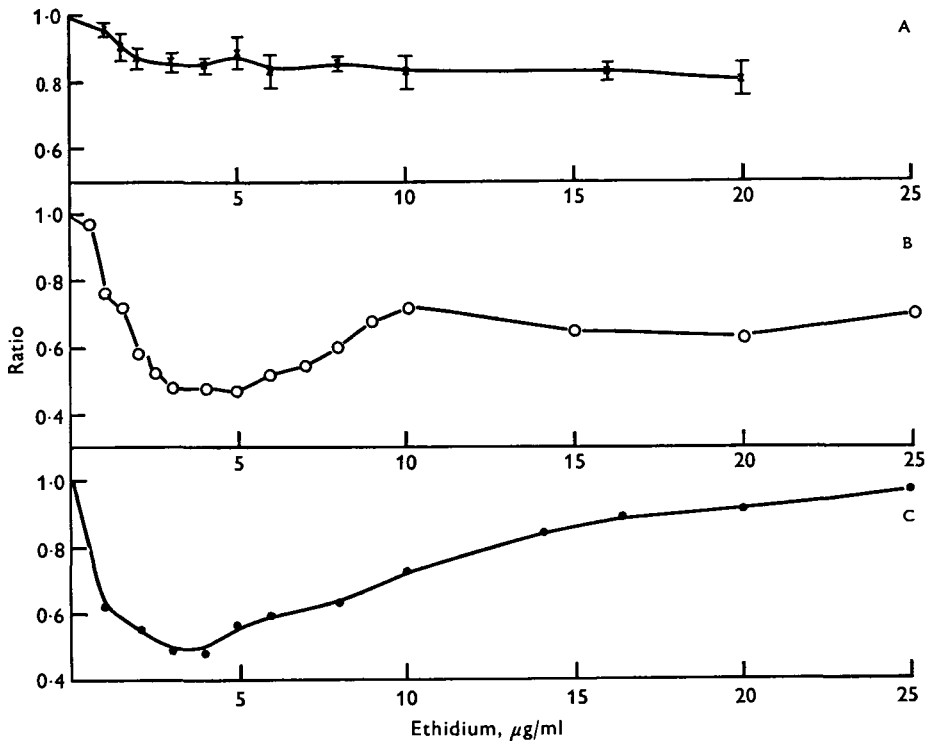


Fig. 8. Sedimentation of HeLa nucleoids through gradients containing different salt and ethidium concentrations.

The distance sedimented by HeLa nucleoids through gradients containing different salt and ethidium concentrations is expressed as a ratio relative to that of nucleoids sedimenting in a reference tube. The nucleoids were spun in glycerol or sucrose gradients containing 0.4 M NaCl (A), 1.0 M NaCl (B) or 1.95 M NaCl (C).

A, 400 µl of phosphate-buffered saline containing  $0.5-2 \times 10^5$  HeLa cells/ml were added to 1.2 ml lysis mix (0.4 M NaCl), mixed by gentle agitation and 200-µl aliquots added to 4.6 ml glycerol gradients (30-50 % glycerol; pH 8.0; 0.4 M NaCl) containing different concentrations of ethidium. 15 min after the addition of cells to the lysis mix, the gradients were spun at 5000 rev/min for 15 min before analysis as described in Materials and methods. The reference tube contained no ethidium. Error bars give the standard error of the mean.

B, 4.6 ml sucrose gradients (15-30 % sucrose; pH 8.0; 1.0 M NaCl) containing different concentrations of ethidium were overlaid with 150 µl of lysis mix (1.0 M NaCl). 50 µl of phosphate-buffered saline containing  $0.5-2 \times 10^5$  HeLa cells/ml were then added and 15 min later the gradients spun at 5000 rev/min for 25 min before analysis as described in Materials and methods. The reference tube contained no ethidium.

C, as for B, except that gradients were spun for 60 min and contained 1.95 M NaCl and were overlaid with lysis mix (1.95 M NaCl).

biphasic. Prior treatment of nucleoids with 1.95 M salt halves the rate of sedimentation in the absence of ethidium and shifts the minimum of the curve to lower ethidium concentrations; at higher ethidium concentrations treated nucleoids sediment more rapidly than their untreated counterparts. These differences presumably result from the different protein content of the nucleoids prepared in the different ways.

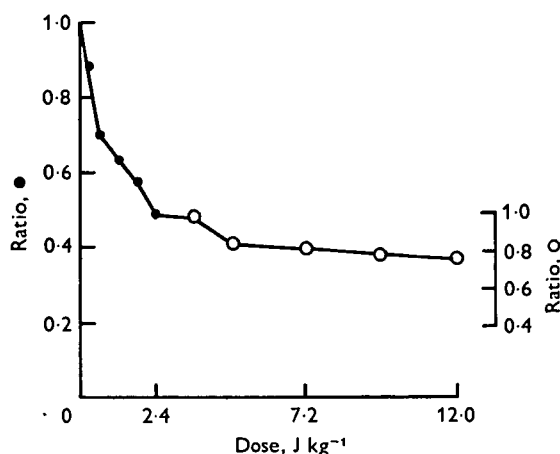


Fig. 9. The effect of  $\gamma$ -rays on the sedimentation of HeLa nucleoids in 1.95 M NaCl.

The distance sedimented by irradiated nucleoids in gradients lacking ethidium is expressed as a ratio relative to reference nucleoids sedimenting under the same conditions. Cells in phosphate-buffered saline were added to lysis mix (1.95 M NaCl) floating on top of a sucrose gradient (15–30 %) containing 1.95 M NaCl and irradiated (dose rate 1.2 J kg<sup>-1</sup> min<sup>-1</sup>) before spinning at 5000 or 10000 rev/min for 1 h. A ratio of 1 refers to unirradiated (left-hand ordinate) and irradiated reference nucleoids (right-hand ordinate) sedimenting in gradients lacking ethidium. A ratio of 1 on the right-hand ordinate is equivalent to a ratio of 0.49 on the left-hand ordinate. The alignment and scales of the ordinates permit a rough comparison of the sedimentation rates of nucleoids spun at the different speeds. ●, ratio given on left-hand ordinate, reference nucleoids unirradiated, gradients spun at 5000 rev/min. ○, ratio given on right-hand ordinate, reference nucleoids irradiated (2.4 J kg<sup>-1</sup>), gradients spun at 10000 rev/min.

Nucleoids sediment in gradients lacking ethidium as a broad band, but when ethidium is present the band becomes sharper. This effect occurs with various intercalating agents (Cook & Brazell, 1975) and with the different nucleoids studied in the accompanying paper (Cook & Brazell, 1976). It is particularly noticeable when nucleoids isolated in 1.95 M NaCl are respun in gradients containing 1.0 M NaCl. The broad distribution of radioactive nucleoids in such gradients, and the band-sharpening effect of ethidium, are illustrated in Fig. 11. When nucleoids isolated in 1.95 M NaCl are respun in 0.4 M NaCl the effect is even more marked, making the analysis of such gradients very difficult. However, the sedimentation behaviour of the nucleoids isolated in 1.95 M NaCl suggests that their DNA remains supercoiled in 0.4 M NaCl.

*The resistance of nucleoid DNA to shear*

DNA of high molecular weight is easily sheared by pipetting (Burgi & Hershey, 1961; Levinthal & Davison, 1961). If nucleoid DNA is sheared by pipetting, the DNA should lose supercoils. Nucleoids were therefore isolated on step gradients,

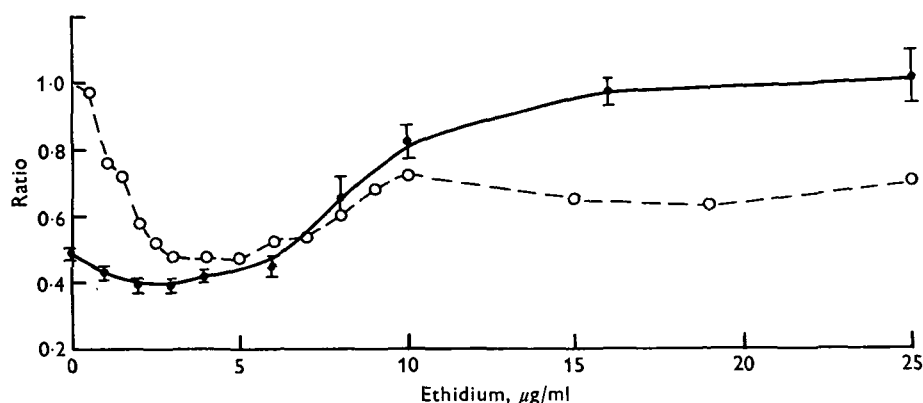


Fig. 10. Sedimentation of HeLa nucleoids isolated in 1.95 M salt through gradients containing 1 M salt and different concentrations of ethidium.

The distance sedimented by HeLa nucleoids isolated in 1.95 M salt is expressed as a ratio relative to nucleoids sedimenting in a reference tube.

HeLa nucleoids were prepared in 1.95 M salt on step gradients, counted, and diluted to 1.0 M salt with 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0). 200- $\mu$ l aliquots containing between 1 and  $3 \times 10^5$  nucleoids/ml were applied to 5 sucrose gradients (4.6 ml; 15–30 % sucrose) containing 1 M NaCl and different concentrations of ethidium. The sixth sucrose gradient (4.6 ml; 15–30 % sucrose; 1.0 M NaCl; no ethidium) served as a reference; 150  $\mu$ l of lysis mix (1.0 M NaCl) was layered on top of the gradient followed by 50  $\mu$ l of phosphate-buffered saline containing  $1-3 \times 10^5$  cells. Fifteen minutes later, the reference gradient with the 5 others was spun at 5000 rev/min for 25 min and analysed as usual.

●, the distance sedimented by nucleoids prepared in 1.95 M NaCl is expressed as a ratio relative to the distance sedimented by the nucleoids in the reference tube.  
○, the distance sedimented by nucleoids prepared by lysing cells in lysis mix (1.0 M NaCl) on top of gradients containing 1.0 M salt and different concentrations of ethidium is expressed as a ratio relative to the distance sedimented by the nucleoids in the reference tube. (Data from Cook & Brazell, 1975.)

mixed with diluent and passed 10 times through a pipette tip only 1.0 mm wide. If nucleoid DNA remained intact and supercoiled after these manipulations, the nucleoids should sediment more than one and a half times as far in gradients containing 1.0 M NaCl and 16  $\mu$ g/ml ethidium as they do in the absence of ethidium (cf. Fig. 10). The ratio of the relative distance sedimented in 16 and 0  $\mu$ g/ml ethidium ( $R^{16/0}$ ) reflects nucleoid integrity. The  $R^{16/0}$  of intact nucleoids with supercoiled DNA is generally greater than 1.5. On the other hand, nucleoids irradiated with 9.6 J kg<sup>-1</sup> to remove supercoiling remain at the top of the gradient and are characterized by  $R^{16/0} < 1$ . The  $R^{16/0}$  of the nucleoids passed through the pipette is no different from controls (Table 1), indicating that their DNA remains supercoiled and so must be resistant to shear.

### *The effect of temperature on nucleoids*

The effects of different temperatures on nucleoid integrity are illustrated in Fig. 12. HeLa nucleoids, isolated on step gradients, were diluted to 0.2 M NaCl and incubated at various temperatures for 10 min before being spun in gradients con-

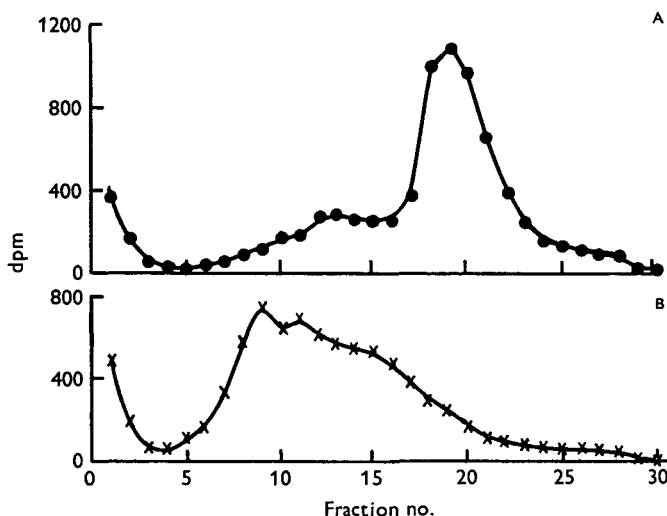


Fig. 11. Sedimentation of radioactive HeLa nucleoids, isolated in 1.95 M salt, through gradients containing 1 M salt.

HeLa cells were grown for 24 h in [ $^3\text{H}$ ]thymidine (0.1  $\mu\text{Ci/ml}$ ) and nucleoids isolated in 1.95 M salt on step gradients. The nucleoids were then spun in gradients containing 16 or 0  $\mu\text{g/ml}$  ethidium (A and B, respectively) and 1.0 M NaCl as described for Fig. 10. After spinning, 3-drop fractions were collected from the gradients, and the radioactive content of the fractions determined as described in Materials and methods. The first fraction was from the top of the gradient. More than 80% of the radioactive label applied to the gradients was recovered.

taining 16  $\mu\text{g/ml}$  ethidium. The rate of sedimentation of nucleoids is decreased by incubation at temperatures above 20 °C. After incubation at 50 °C the nucleoid suspension becomes viscous, although individual nucleoids remain visible in the fluorescence microscope when they are stained with ethidium. Temperatures above 60 °C are required to destroy nucleoid integrity completely.

### *The effect of pronase and ribonuclease on nucleoid integrity*

HeLa nucleoids, isolated on step gradients, were diluted to 0.2 M NaCl and incubated with pronase or ribonuclease at 20 °C for 10 min before being spun in gradients containing 16  $\mu\text{g/ml}$  ethidium (Figs. 13, 14). Low concentrations of both enzymes reduce the rate of sedimentation of nucleoids and, at higher concentrations, they destroy the nucleoids (as determined by fluorescence microscopy). The amount of RNA converted to acid-soluble material by ribonuclease was determined by pre-labelling the RNA in the nucleoids with [ $^3\text{H}$ ]uridine. When half the label is

Table 1. *The resistance of nucleoid DNA to shear*

Gradient no.	Variation in salt concentration	Ethidium concentration in gradient, $\mu\text{g/ml}$	Ratio
1	1.95–1.0 M	0	1.0
2	1.95–1.0 M	16	1.57
3	1.95–0.2–1.0 M	0	0.9
4	1.95–0.2–1.0 M	16	1.56

HeLa nucleoids were prepared in 1.95 M salt on step gradients and counted. One aliquot was diluted to 1 M salt and another to 0.2 M. Both were incubated for 10 min at 4 °C before the salt concentration of the latter was raised to 1.0 M by the addition of 5 M NaCl. The nucleoids that were diluted to 0.2 M NaCl were mixed by sucking them up and down 5 times through a pipette tip 1.0 mm wide. 200- $\mu\text{l}$  aliquots containing about  $2 \times 10^5$  nucleoids were applied to sucrose gradients (15–30%; 1 M NaCl) containing 0 or 16  $\mu\text{g/ml}$  ethidium. The gradients were then spun at 5000 rev/min for 25 min and analysed. The distance sedimented by the nucleoids is expressed as a ratio relative to the distance sedimented by nucleoids in gradient 1.

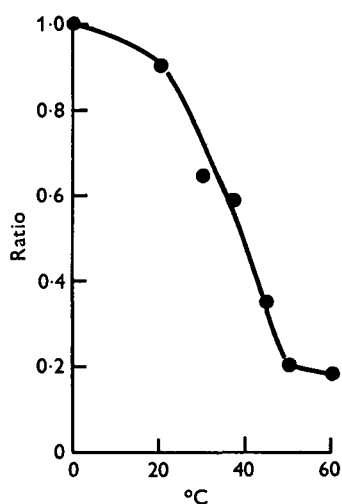


Fig. 12. The effect of incubation at different temperatures on the sedimentation of HeLa nucleoids.

The distance sedimented by nucleoids through gradients containing 16  $\mu\text{g/ml}$  ethidium after incubating them for 10 min at different temperatures is expressed as a ratio relative to nucleoids treated similarly but incubated on ice. HeLa nucleoids were prepared in 1.95 M NaCl on step gradients, counted, and diluted to 0.2 M NaCl with 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0). 200- $\mu\text{l}$  aliquots were incubated at different temperatures for 10 min before the addition of 80  $\mu\text{l}$  of 5 M NaCl. About  $2 \times 10^6$  nucleoids in 200- $\mu\text{l}$  aliquots were then applied to 4.6 ml sucrose gradients (15–30%; 1.0 M NaCl) containing 16  $\mu\text{g/ml}$  ethidium, spun at 7000 rev/min for 25 min at 20 °C before determination of the distance sedimented down the gradient as usual. Two reference gradients (cf. gradients 1 and 2 of Table 1) in each experiment were included to monitor the degree of supercoiling of untreated nucleoids.  $R^{16/0}$  was greater than 1.5.

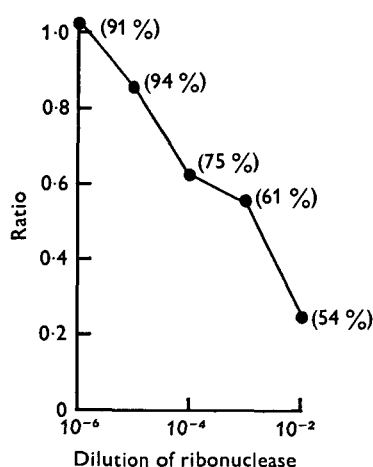


Fig. 13. The effect of ribonuclease on the sedimentation of HeLa nucleoids.

The distance sedimented by nucleoids through gradients containing  $16 \mu\text{g/ml}$  ethidium after incubating them for 10 min with ribonuclease is expressed as a ratio relative to the distance sedimented by untreated reference nucleoids sedimenting under the same conditions. HeLa nucleoids were prepared in  $1.95 \text{ M NaCl}$  on step gradients, counted, and diluted to  $2 \times 10^6/\text{ml}$  and  $0.2 \text{ M NaCl}$  using  $10 \text{ mM}$  tris-(hydroxymethyl)-aminomethane ( $\text{pH } 8.0$ ) supplemented with  $\text{NaCl}$  as required.  $50 \mu\text{l}$  of ribonuclease (see Materials and methods) were added to different  $400\text{-}\mu\text{l}$  aliquots of these nucleoids. The final dilution is given in the abscissa. Each was then incubated for 10 min at  $20^\circ\text{C}$  before the addition of  $80 \mu\text{l}$  of  $5 \text{ M NaCl}$ .  $200\text{-}\mu\text{l}$  aliquots were applied to gradients as described in the legend to Table 1.

Nucleoids were also prepared from HeLa cells which had been growing for 24 h in the presence of  $2 \mu\text{Ci/ml}$   $[5\text{-}^3\text{H}]\text{uridine}$ . Nucleoids were diluted and treated with ribonuclease as described above with the exception that aliquots of the reaction mixture were removed both before the addition of the enzyme and after the addition of  $5 \text{ M NaCl}$  to determine the amounts of radioactivity insoluble in trichloroacetic acid, as described in Materials and methods. The percentages of the radioactivity insoluble in trichloroacetic acid remaining after the 10-min incubation period are given in brackets. The integrity of these nucleoids was also monitored by fluorescence microscopy. At all dilutions, nucleoids remained intact, but at dilutions of  $10^{-3}$  and  $10^{-2}$  no nucleoli were visible after ribonuclease treatment.

Two reference gradients (cf. gradients 1 and 2 of Table 1) were included in each experiment to monitor the degree of supercoiling of untreated nucleoids.  $R^{16/0}$  was greater than 1.5.

removed, the nucleoids still appear intact in the fluorescence microscope but no longer contain visible nucleoli: they sediment slowly, like irradiated nucleoids.

#### *The protein content of nucleoids*

Nucleoid proteins were examined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Fig. 15 illustrates the proteins of HeLa nucleoids prepared in caesium chloride density gradients. Chromatin proteins, mixtures of marker proteins, and mixtures of mouse proteins that bind to DNA are included in Fig. 15 for comparison. Mouse and human nuclear proteins that bind to DNA

are very similar (Jost *et al.* 1975), so that mouse proteins serve as suitable markers for comparison with the proteins of HeLa nucleoids. Between 60 and 80 proteins are found in chromatin; the majority of these proteins are not present in appreciable quantities in nucleoids. The nucleoids do not contain any histones, but they do

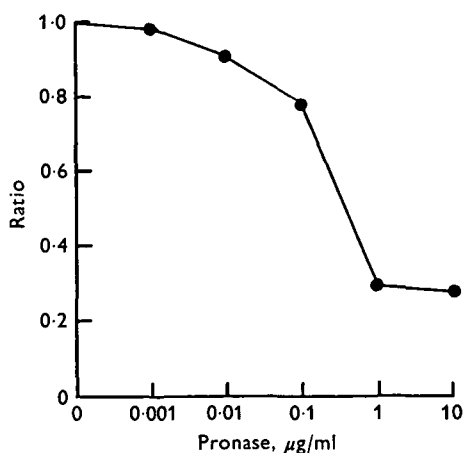


Fig. 14. The effect of pronase on the sedimentation of HeLa nucleoids.

The distance sedimented by nucleoids through gradients containing  $16 \mu\text{g/ml}$  ethidium after incubating them for 10 min with pronase is expressed as a ratio relative to the distance sedimented by untreated reference nucleoids sedimenting in similar gradients. HeLa nucleoids were prepared in  $1.95 \text{ M}$  salt on step gradients, counted, and diluted to  $10^7/\text{ml}$  using  $1.95 \text{ M}$  NaCl and  $10 \text{ mM}$  tris-(hydroxymethyl)-aminomethane (pH 8.0). Nucleoids were then diluted with  $10 \text{ mM}$  tris-(hydroxymethyl)-aminomethane (pH 8.0) to  $0.2 \text{ M}$  salt and  $2 \times 10^6/\text{ml}$ .  $50 \mu\text{l}$  of pronase (see Materials and methods) were added to different  $400\text{-}\mu\text{l}$  aliquots of these nucleoids. Each was then incubated for 10 min at  $20^\circ\text{C}$  before the addition of  $80 \mu\text{l}$  of  $5 \text{ M}$  NaCl.  $200\text{-}\mu\text{l}$  aliquots were then applied to  $4.6 \text{ ml}$  sucrose gradients ( $15\text{--}30\%$ ) containing  $1 \text{ M}$  salt and  $16 \mu\text{g/ml}$  ethidium. These were spun at  $5000 \text{ rev/min}$  for 25 min at  $20^\circ\text{C}$  before determination of the distance sedimented as usual. Two reference gradients (cf. gradients 1 and 2 of Table 1) were included to monitor the degree of supercoiling in untreated nucleoids.  $R^{16/0}$  was greater than 1.5.

contain 5–7 proteins in the molecular weight range of 45000–60000 and smaller quantities of 3 proteins in the range 60000–70000. The number of copies of these proteins in a nucleoid has been estimated at  $10^6\text{--}10^7$  by comparative densitometry of preparations stained by Coomassie brilliant blue with bovine serum albumin as a standard. The major proteins that bind to DNA and are released from it between  $0.1$  and  $0.4 \text{ M}$  NaCl are present in similar amounts per cell (cf. Fig. 15G). The protein content of nucleoids is not altered by irradiating them before isolation on the caesium chloride gradient (cf. Fig. 15B, C).

Proteins obtained from small numbers of nucleoids ( $< 10^5$ ) were labelled with  $^{125}\text{I}$ ; after electrophoresis, the labelled proteins were located in the gels (Fig. 16) by autoradiography. The nucleoid proteins may be compared with mouse proteins that bind to DNA (Fig. 16A, B). The proteins that remain associated with the

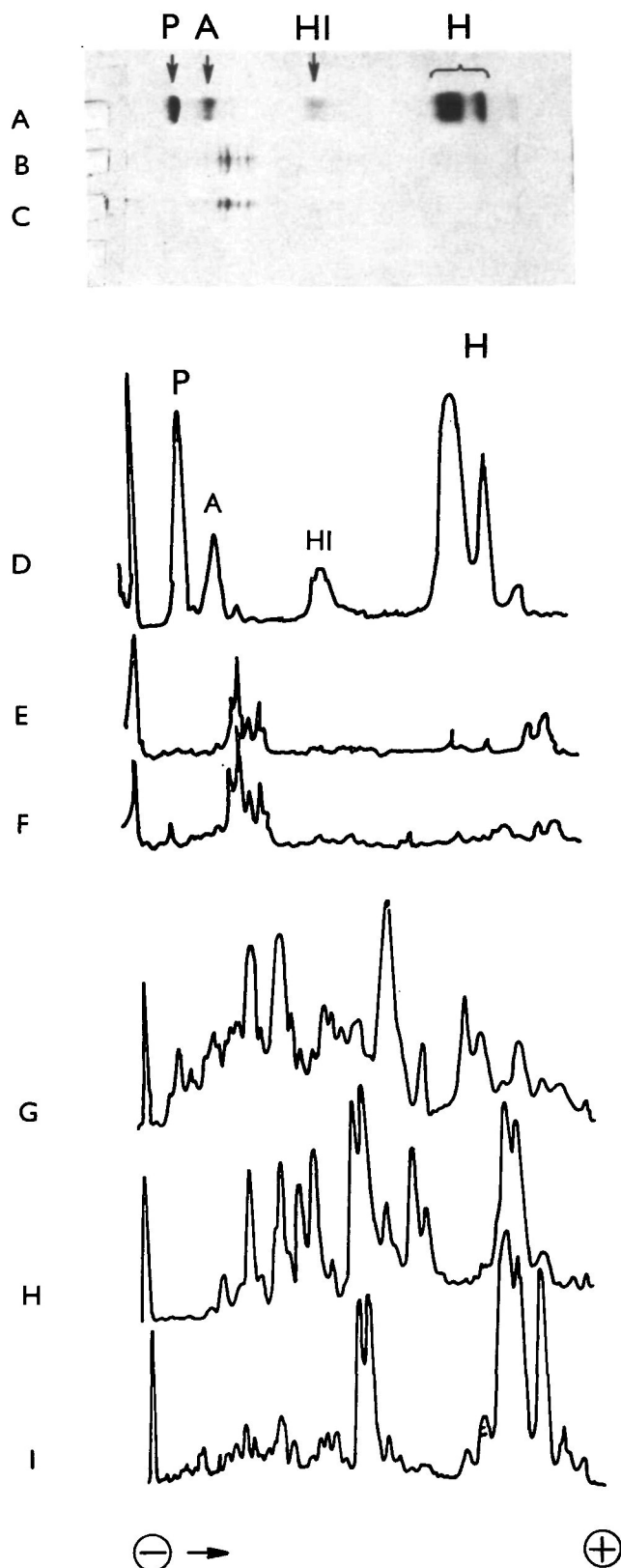


Fig. 15. For legend see opposite.



nucleoids prepared in caesium chloride gradients are also present in nucleoids washed with saturated sodium chloride (Fig. 16C) or prepared in 1.0 or 1.95 M salt (Fig. 16D, E, G). Nucleoids made in step gradients generally contain a wide variety of proteins in low quantities; fewer of these proteins are found in the nucleoids prepared in sucrose gradients (15–30%). These residual proteins may be detected by over-exposing the autoradiographs (cf. Fig. 16E, F). Whatever the method used to prepare the nucleoids, they are depleted of the histones and the major DNA-binding proteins characteristically found in chromatin. They do, however, contain a small number of proteins of high molecular weight.

When nucleoids are exposed to 1.0 M salt for shorter periods, they may be obtained containing larger amounts of histones. Fig. 17 illustrates such an experiment. HeLa cells were labelled with [ $^3\text{H}$ ]leucine and nucleoids quickly isolated in 1.0 M NaCl; their proteins were then analysed in gels. The radioactive profile of the gel indicates that the histones are present.

The proteins of the nucleoids from different Orders are found to be similar, but not identical, when analysed in polyacrylamide gels (Fig. 18).

#### DISCUSSION

By lysing HeLa cells and spinning the released nucleoids through 15% sucrose on to a shelf of 30% sucrose, HeLa nucleoids can be easily prepared in bulk. When viewed in the phase-contrast or fluorescence microscope, they resemble swollen nuclei. They contain the DNA and nearly all the RNA of the nucleus, but are depleted of nuclear proteins (Colman & Cook, 1976). When made in 1.95 M NaCl they contain no histones and lack most chromatin proteins. Between 10 and 50%

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Fig. 15. The protein content of nucleoids isolated in caesium chloride density gradients compared with chromatin proteins that bind to DNA, using polyacrylamide gels containing sodium dodecyl sulphate.

The proteins of nucleoids prepared in caesium chloride density gradients were analysed in polyacrylamide gels as described in Materials and methods. After electrophoresis, gels were stained with Coomassie blue, photographed, and tracings of the optical density made. Other protein samples, analysed under similar conditions, are included for comparison. A, a stained gel, which contained a mixture of reference proteins (P, phosphorylase A; A, bovine serum albumin; and calf thymus histones, HI and H). B, a stained gel containing the protein of nucleoids isolated in a caesium chloride density gradient. C, a stained gel containing the proteins of  $\gamma$ -irradiated nucleoids isolated as in B. The nucleoids were irradiated ( $0.6 \text{ J kg}^{-1}$ ;  $1.2 \text{ J kg}^{-1} \text{ min}^{-1}$ ) after addition of cells to the lysis mixture (1.0 M NaCl) floating on caesium chloride. D–F, densitometer tracings of the gels photographed in A, B and C respectively. G, densitometer tracing of a stained gel containing mouse proteins that bind to double-stranded DNA from calf thymus and which are released from the DNA by salt concentrations between 0.1 and 0.4 M NaCl. 0.1 optical density units (280 nm) of protein were applied to the gel. H, densitometer tracing of a stained gel containing mouse proteins that bind to double-stranded DNA from calf thymus and which are released by salt concentrations between 0.4 and 2.0 M NaCl. 0.1 optical density units (280 nm) of protein were applied to the gel. I, densitometer tracing of a stained gel containing the chromatin proteins of HeLa cells which are released by 2.0 M NaCl from chromatin. The proteins in A–C and G–I were run on separate slab gels.

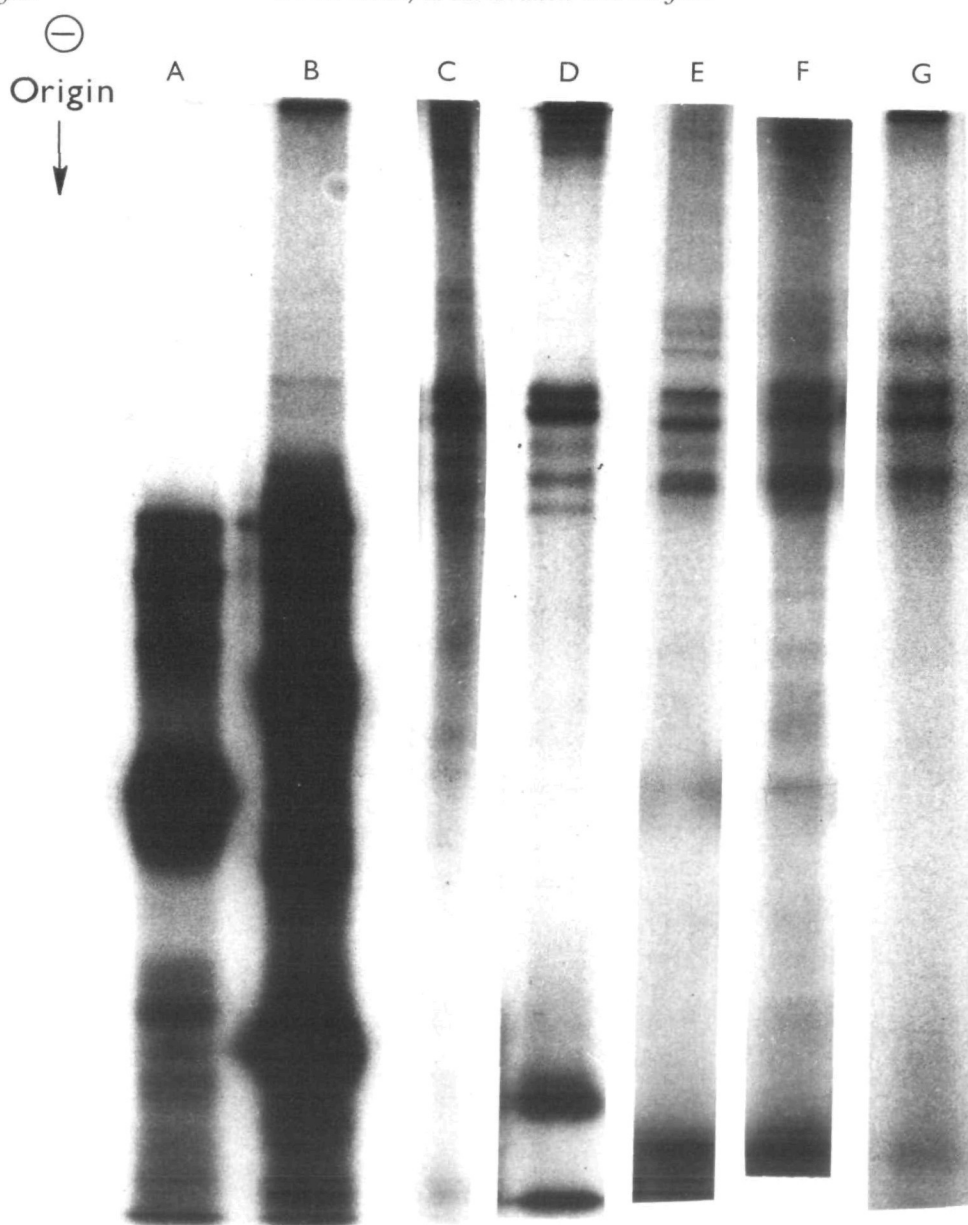


Fig. 16. Autoradiographs of gels containing  $^{125}\text{I}$ -labelled nucleoid proteins.

Different protein samples were labelled with  $^{125}\text{I}$ , subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate, and autoradiographs of the gels prepared. A, mouse proteins binding to double-stranded DNA of calf thymus and released from the DNA by salt concentrations between 0.1 and 0.4 M NaCl. B, mouse proteins binding to double-stranded DNA of calf thymus and released from the DNA by salt concentrations between 0.4 and 2.0 M NaCl. C, the proteins of HeLa nucleoids isolated in sucrose gradients (15–30%) containing 1.0 M NaCl, left for 15 min in saturated sodium chloride before pelleting the nucleoids by spinning at 40000 rev/min for 15 min. D, the proteins of HeLa nucleoids isolated in sucrose gradients (15–30%) containing 1.95 M NaCl. E, the proteins of HeLa nucleoids isolated in 1.95 M NaCl on step gradients. F, the proteins of HeLa nucleoids isolated in 1.95 M NaCl on step gradients. This is an autoradiograph of the same gel as that presented in E; the autoradiograph has been exposed for 4 times longer than that in E to reveal proteins present in low quantities. G, the proteins of HeLa nucleoids isolated in sucrose gradients (15–30%) containing 1.0 M NaCl. The proteins in A and B have been characterized (Jost *et al.* 1975) and serve as markers.

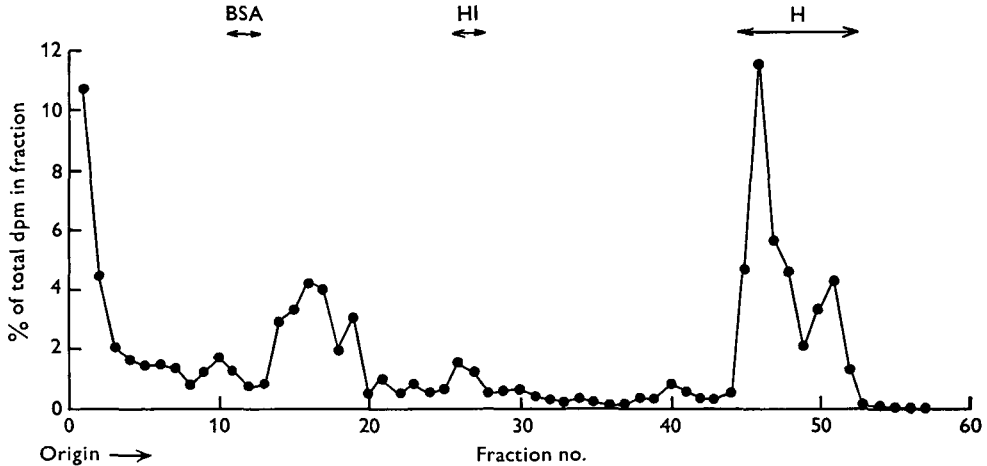


Fig. 17. The profile of radioactivity in a gel containing labelled proteins obtained from HeLa nucleoids prepared in 1.0 M NaCl.

HeLa cells were labelled by growing them in [ $^3\text{H}$ ]leucine (12  $\mu\text{Ci/ml}$ ) for 24 h, and nucleoids prepared in isokinetic gradients containing 1.0 M NaCl. When the gradients had been spun, the visible band of nucleoids derived from about  $8 \times 10^5$  cells was removed immediately through the side of the tube using a needle, and the salt concentration reduced to prevent further dissociation of the histones, by mixing with 4 vol. of ice-cold 10 mM tris-(hydroxymethyl)-aminomethane (pH 8.0) and 0.1 mM phenylmethylsulphonylfluoride. The nucleoids were then pelleted by spinning at 45000 rev/min for 15 min. The proteins in the pellet were analysed in gels and the radioactive profile of the gel determined as described in Materials and methods. There was a total of 35200 dpm in the gel. The percentage of the total radioactivity in each fraction of the gel is given. The position of marker proteins (bovine serum albumin, BSA; calf thymus histones, HI and H) run in adjacent gels is indicated.

of their proteins have molecular weights of 45000–60000; other minor constituents have molecular weights between 60000 and 70000. These proteins remain attached to nucleoids in saturating concentrations of NaCl. Nuclear envelopes contain some proteins in a similar molecular weight range (E. Jost, unpublished observations). Insect and amphibian nucleoids are also depleted of nuclear proteins.

Whereas high molecular weight DNA is easily sheared (Burgi & Hershey, 1961; Levinthal & Davison, 1961), the superhelical DNA packaged in nucleoids may be pipetted freely without loss of supercoils. Since about  $10^4$  single-strand breaks are required to remove supercoils from the DNA of a nucleoid (Cook & Brazell, 1975) the DNA packaged in a nucleoid is largely intact and very resistant to shear.

In 1.0 or 1.95 M NaCl, nucleoid DNA is supercoiled, but it has been argued (Cook & Brazell, 1975) that DNA *in vivo* is not in a high-energy conformation. After irradiation of living cells, the loss of supercoiling observed in the DNA of the isolated nucleoids is readily repaired when the cells are incubated at 37 °C; but this repair does not occur after irradiation of isolated nucleoids. The repair mechanisms *in vivo* must be capable not only of mending the breaks in sugar-phosphate bonds of the backbone strands, but also of re-introducing into the mended DNA the

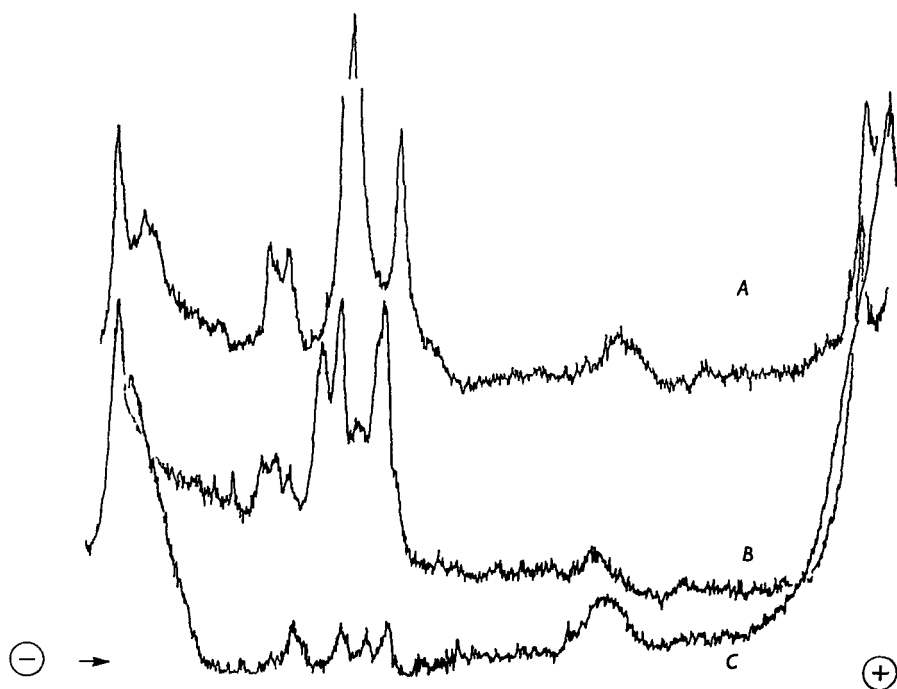


Fig. 18. Comparison of the major proteins in nucleoids made from *Xenopus*, HeLa and *Drosophila* cells.

Different protein samples were iodinated with  $^{125}\text{I}$ , subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate and autoradiographs of the gels made. Densitometer tracings made from the autoradiographs of the proteins of XTC-2 nucleoids prepared in sucrose gradients (15–30 %) containing 1.0 M NaCl (A), HeLa nucleoids isolated in 1.95 M NaCl in step gradients (B), and K85 nucleoids prepared in sucrose gradients (15–30 %) containing 1.0 M NaCl (C). The proteins of HeLa nucleoids (B) serve as markers (cf. Figs. 15 and 16).

correct configuration. The correct configuration may be restored if the supercoiled and nicked forms of the DNA *in vivo* are equally stable. Irradiation would therefore cut DNA strands without changing the number of times one strand of the DNA duplex winds round the other; repair would restore both topological constraints and the original configuration. This explanation requires that the torsional energy of supercoiling present in nucleoid DNA in 1.0 M NaCl is not present *in vivo*.

When a ligand that unwinds the duplex binds to supercoiled DNA, some of the free energy of supercoiling is released (Vinograd, Lebowitz & Watson, 1968; Davidson, 1972). [Intercalating agents like ethidium unwind DNA by reducing the number of duplex turns per unit length of the double helix. The duplex may also be unwound by denaturing or 'melting' the DNA; some, but not all, types of 'kink' also lead to unwinding (Crick & Klug, 1975).] Histones bound to DNA *in vivo* may act as unwinding agents. They unwind supercoiled circles of simian virus 40 DNA (Germond *et al.* 1975). They also dissociate from nucleoid DNA in the manner expected of unwinding agents (E. Jost & P. R. Cook, unpublished results).

Since the effects of salt on conformation are small, the supercoiling observed in nucleoid DNA may be explained as follows. DNA associated with histones *in vivo* is underwound relative to the DNA in nucleoids. On the removal of the histones, the hitherto relaxed DNA would adopt an increased number of double helical turns. As the DNA is subject to topological constraint, superhelical turns are necessarily formed. Results obtained earlier suggested that different regions of nucleoid DNA might be supercoiled to different degrees (Cook & Brazell, 1975). These differences in superhelical density in nucleoids could stem from differences in the amounts or types of unwinding ligands bound *in vivo*, and could be the basis of differential gene activity (Cook, 1973, 1974).

The effects of NaCl and various enzymes on nucleoids illuminate the mechanism that governs the packaging of their DNA. Constraints restricting rotation of one strand of the DNA duplex about the other are maintained in 1.95 M salt as well as in 1.0 M salt. Whereas the nucleoids remain intact in the presence of non-ionic detergents and saturating concentrations of salt, their integrity is destroyed by ionic detergents, high temperature, the effects of  $\gamma$ -irradiation, dithiothreitol, ribonuclease and pronase. Ribonuclease and trypsin unfold bacterial nucleoids, which suggests that bacterial DNA is condensed by both RNA and protein (Stonington & Pettijohn, 1971; Worcel & Burgi, 1972; Pettijohn & Hecht, 1973; Worcel, Burgi, Robinton & Carlson, 1973; Drlica & Worcel, 1975). As there may be a hierarchy of constraining mechanisms in the nuclei of higher cells, it is not yet clear which constraints are being removed by the different treatments. It also remains to be seen what relationship nucleoids have to the structures isolated by Ide, Nakane, Ansai & Andoh (1975).

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