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Spectrofluorometric Measurement of the Binding of Ethidium to Superhelical DNA from Cell Nuclei

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Structures retaining many of the morphological features of nuclei may be released by lysing HeLa cells in solutions containing non-ionic detergents and high concentrations of salt. These nucleoids contain few chromatin proteins. We have shown that the DNA of nucleoids is quasi-circular and supercoiled by measuring spectrofluorometrically the amount of the intercalating dye, ethidium, bound to unirradiated and γ-irradiated nucleoids. Ethidium binds to nucleoids in the manner characteristic of the binding to superhelical DNA: at low concentrations more ethidium binds to unirradiated nucleoids than to their γ-irradiated counterparts with broken DNA, and at higher concentrations less ethidium binds to the unirradiated nucleoids. The quasi-circles in nucleoids are 22 times less sensitive to γ-irradiation than are circles of pure PM2 DNA: they must contain about 2.2×10^5 base pairs.

The constraints that maintain the quasi-circularity of nucleoid DNA are very resistant to extremes of temperature and alkali; some remain under conditions in which the duplex is denatured. The constraints are destabilised by ethidium suggesting that they are stabilised by free energy of supercoiling. Proteolytic enzymes, but not ribonucleases, remove the constraints. Possible structures for the constraining mechanism are discussed.

Circular duplexes of DNA in which both strands of the duplex are covalently continuous possess one remarkable property not shared by their counterparts with broken strands: the two strands in the intact molecule are interlocked and can only be completely separated by severing one of the strands *i.e.* by breaking phosphodiester bonds (see [1–3] for a fuller discussion). We have shown that the DNA of higher cells is subject to the same kind of topological constraint as that found in circular molecules of DNA. Our approach was to lyse cells in solutions containing non-ionic detergents and high concentrations of salt to release structures that retain many of the morphological features of nuclei. These nucleoids contain few of the proteins characteristic of chromatin and sediment in sucrose gradients containing ethidium in the manner characteristic of superhelical DNA. Breaking the DNA by γ-irradiation abolished the characteristic behaviour [4–8] (see also [9–11]). We concluded that nucleoid DNA was made quasi-circular by organisation of linear duplexes into many loops so that a single-strand break in one loop released supercoiling (and the topological constraint) in that loop but not in adjacent loops. In the present paper we describe experiments that confirm that nucleoid DNA is constrained: we compared the binding of ethidium to unirradiated and γ-irradiated nucleoids. We have also investigated the nature of the forces that constrain the DNA.

Supercoiling is generally unfavoured thermodynamically [12–14]. At low concentrations the binding of the intercalating ligand, ethidium, to circular DNA unwinds the double helix, reduces the absolute number of negative superhelical turns and releases free energy of supercoiling. Therefore, ethidium binds more avidly to a negatively supercoiled DNA than to its broken and so relaxed counterpart containing no supercoils [12, 14, 15]. At higher concentrations, where binding induces the formation of supercoils of sense opposite to those initially present, less ethidium binds to the intact molecule. We have measured by fluorometry [16–20] the binding of ethidium to nucleoids derived from HeLa cells. Ethidium binds to nucleoids

Enzymes. Ribonuclease A (EC 3.1.4.22); ribonuclease T_1 (EC 3.1.4.8); trypsin (EC 3.4.21.4); deoxyribonuclease (EC 3.1.4.5).

in the manner characteristic of the binding to superhelical DNA.

MATERIALS AND METHODS

Reagents, Chemicals and Enzymes

The following radiochemicals were obtained from The Radiochemical Centre, Amersham: [5,6-^{3}H]uridine (48 Ci/mmol), L-[4,5-^{3}H]leucine (57 Ci/mmol).

Procedures for determining the radioactive content of samples insoluble in trichloroacetic acid have been described [7].

Chemicals were obtained from the following sources: Sarkosyl NL35, Ciba-Geigy (Simonsway, Manchester); sodium metrizoate solution 32.8% w/v, *i.e.* sodium salt of 3-acetamido-2,4,6-triiodo-5-(*N*-methylacetamido)-benzoic acid, Nyegaard and Co. A/S (Oslo, Norway).

DNA was prepared from HeLa nucleoids [8] and had melting temperatures of 86.5 °C and 63.5 °C in 0.2 M NaCl, 10 mM Tris (pH 8.0) and 0.2 M NaCl, 10 mM Tris (pH 8.0), 4 M $NaClO_4$ respectively.

Ribonuclease A from beef pancreas (grade RASE; 21.35 mg/ml; 3233 units/mg) was obtained from Worthington (Cambrian Chemicals Ltd, Croydon) and ribonuclease T_1 from *Aspergillus oryzae* (B grade) from Calbiochem (Bishops Stortford, Herts). Both were heated to 100 °C for 10 min to inactive any contaminating deoxyribonuclease. Pronase (B grade; 45000 units/g) was obtained from Calbiochem, dissolved in 10 mM Tris (pH 8.0) at 25 mg/ml and incubated at 20 °C for 10 min to inactive any contaminating nucleases prior to use. Trypsin from bovine pancreas was obtained from Calbiochem (A grade; 32000 units/mg) and Worthington (grade TRL3; 229 units/mg): both were dissolved in 10 mM Tris (pH 8.0) at 100 mg/ml and self-digested as above.

We have tried to destroy any contaminating deoxyribonuclease in our enzyme preparations by using the appropriate treatments. Nevertheless, we tested our enzyme preparations for any residual contamination by incubating them with closed circles of PM2 DNA: any circles nicked by deoxyribonuclease can be detected using agarose gels (see below). We incubated our enzyme preparations with PM2 DNA (40 μg/ml) using conditions of increasing stringency. Incubation with all enzymes induced no change in the relative proportions of the intact and nicked form using conditions similar to those used with nucleoids (*i.e.* incubation in 0.2 M NaCl, 10 mM Tris for 10 min at 30 °C). However, incubation for 60 min at 30 °C with 1.25 mg/ml pronase, the highest concentration used with the nucleoids, converted 10% of the intact circles to the nicked form. No nicking was observed under these conditions with trypsin (50 mg/ml), ribonuclease A (5 mg/ml) or ribonuclease T_1 (1250 units/ml). It should be borne in mind that the DNA in the quasi-circles of nucleoids is longer than that of PM2 (see later) so that a loss of supercoiling from nucleoid DNA provides a most sensitive test for any deoxyribonuclease activity that would be undetectable by any other means.

Preparation of Nucleoids

HeLa nucleoids were isolated using 'step' gradients containing 1.95 M NaCl [7]. Techniques for staining, counting and manipulating nucleoids have been described [7]. Nucleoid concentrations were adjusted with 10 mM Tris (pH 8.0) and the appropriate concentrations of NaCl. In some experiments unpurified nucleoids were used. In these cases 1 vol. of HeLa cells (80×10^6/ml) in phosphate-buffered saline was added to 3 vol. of lysis mixture (1.95 M NaCl): as ethidium fluoresces brightly in undiluted lysis mixture, after 5 min it was diluted with at least 100 vol. of the appropriate buffer before measuring the fluorescence of the sample. Nucleoids were γ-irradiated [7] before the addition of ethidium since high doses of irradiation reduce the fluorescence of ethidium. Nucleoid integrity was monitored by measuring the rate of sedimentation of nucleoids in sucrose gradients as described previously [4, 6].

Spectrofluorometry

Fluorescence measurements were made using a Farrand Mark 1 spectrofluorometer (Kontron Instruments, Watford). The excitation wavelength chosen was 510 nm since it is the isosbestic wavelength for bound and free ethidium [16] even though this excitation wavelength does not maximise the intensity of fluorescence. Fluorescence was measured at 590 nm, the wavelength of maximum emission. Slit widths of 10 nm were used throughout. Measurements were generally made on ice-cold samples.

Concentrations of stock solutions of ethidium were determined spectrophotometrically assuming an absorption coefficient of 5600 $M \cdot cm^{-1}$ at 480 nm and a molecular weight of 394.3 [21] (see [22] for a description of impurities in ethidium and their effect on the absorption coefficient).

The amounts of ethidium bound to pure DNA are generally estimated directly by reference to the fluorescence of a known concentration of ethidium under conditions where all the ethidium is bound (*i.e.* in the presence of excess DNA, or in our case, excess nucleoids). However, we cannot obtain concentrations of nucleoids great enough to provide an excess over the whole range of ethidium concentrations that we use. It would also be inappropriate to

determine the amounts of the dye bound to nucleoids by reference to binding isotherms constructed using an excess of pure DNA since the nucleoids also contain RNA [23]. The fluorescence of ethidium bound to nucleoids is therefore expressed as the fluorescence of an equivalent concentration of free ethidium which is determined as follows. The fluorescence of a mixture of ethidium and nucleoids is measured as the output (in μA) of a photomultiplier. Using a standard curve, the concentration of free ethidium giving the same output is determined. The total ethidium concentration and the concentrations of ethidium giving outputs equivalent to that of nucleoids and solvent alone are then substracted to yield the concentration of free ethidium which fluoresces as brightly as the bound ethidium (the equivalent concentration). At low ethidium concentrations where nucleoids are in excess the equivalent concentration is underestimated (results not given). At high concentrations (> 20 μg/ml) the ethidium absorbs strongly both the exciting and emitted light so complicating the analysis.

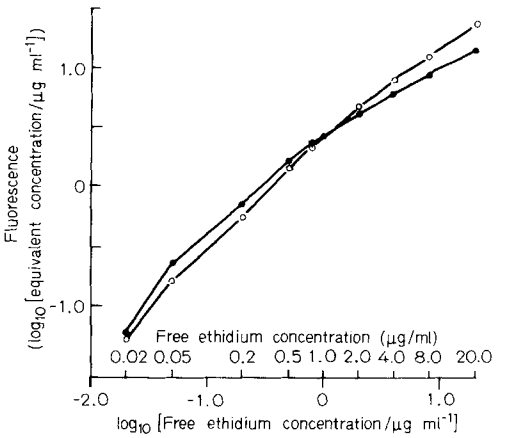

Fig. 1. *Ethidium binding to irradiated and unirradiated nucleoids in 2 M NaCl.* Nucleoids, isolated from 'step' gradients containing 1.95 M NaCl, were diluted to 0.2×10^6/ml using 2 M NaCl, 10 mM Tris (pH 8.0). Samples were γ-irradiated ($9.6\ J \cdot kg^{-1}$) before ethidium was added and the fluorescence measured. The fluorescence of the bound ethidium is expressed as the logarithm of the equivalent concentration, *i.e.* the concentration of free ethidium (μg/ml) which fluoresces as brightly as the bound ethidium (see Materials and Methods). (●——●) Unirradiated; (○——○) irradiated

Agarose Gels

The conversion of closed circles of PM2 DNA to the nicked form was detected using agarose gels [24]. Our sample of PM2 DNA contained 82% intact circles and 18% nicked circles. After electrophoresis gels were stained for 2 h with 0.5 μg/ml ethidium dissolved in electrophoresis buffer, destained for 1 h in electrophoresis buffer and photographed under short-wave illumination through an orange filter using Pan F film (Ilford). The negatives were traced with a Joyce-Loebl microdensitometer. The amount of DNA in the various bands was determined by cutting out the corresponding peaks in the microdensitometer tracing and weighing them. Photographic exposure times were chosen so that exposure for half or double the time did not alter the relative weights of the peaks corresponding to the supercoiled and nicked forms. We have neglected the effects of any differences in ethidium binding (and hence fluorescence) by equal weights of supercoiled and nicked DNA (see Fig. 4 for the size of such effects).

RESULTS

The Binding of Ethidium to Nucleoids

The fluorescence of ethidium is enhanced when it binds to both RNA and DNA [16]. Initially, we studied the binding of ethidium to HeLa nucleoids freed of most cytoplasmic RNA. Nucleoids were isolated using 'step' gradients, ethidium added and the fluorescence of the mixture measured in 2 M NaCl. The amount of ethidium bound to the nucleoids is determined from the fluorescence enhancement and is expressed as the equivalent concentration, *i.e.* the concentration of free ethidium (in μg/ml) which, on its own, fluoresces as brightly as the bound ethidium (see Materials and Methods). Ethidium is bound in rough proportion to the amount of free ethidium (Fig. 1). At very low concentrations (*i.e.* 0.02 μg/ml and below) the nucleoids are in excess (unpublished observations) and unirradiated and γ-irradiated nucleoids bind roughly equal amounts of ethidium. In the range 0.05 – 1.0 μg/ml the irradiated nucleoids bind less than their unirradiated counterparts; at about 1 μg/ml they bind an equal amount and at higher concentrations they bind more.

Small variations in nucleoid concentration markedly affect the fluorescence; increasing concentrations shift the two curves in Fig. 1 upwards but do not change the concentration of ethidium at which the irradiated and unirradiated nucleoids fluoresce similarly. Small differences in nucleoid concentration therefore complicate comparison of one experiment with another. This difficulty may be overcome by considering the binding capacity of unirradiated nucleoids relative to that of their irradiated counterparts (Fig. 2). The average ratios indicate that the irradiated and unirradiated nucleoids bind equal amounts of ethidium at about 1 μg/ml; at this concentration all superhelical turns have been removed from the DNA of unirradiated nucleoids. (Our studies on the sedimentation of unirradiated nucleoids in sucrose gradients containing ethidium showed that supercoiling was removed by 3 – 4 μg/ml ethidium [7]. We do not know

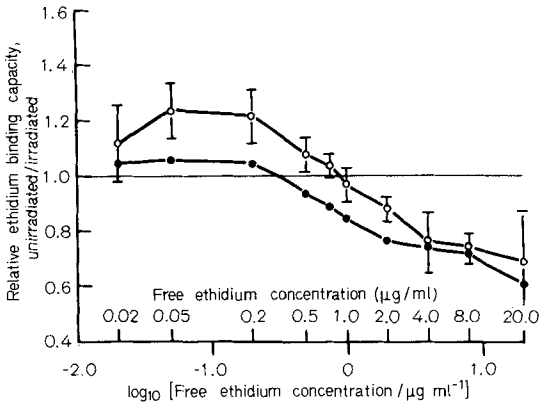

Fig. 2. *Ethidium-binding capacities of unirradiated nucleoids relative to those of γ-irradiated nucleoids.* The ethidium-binding capacity of unirradiated and γ-irradiated nucleoids was determined essentially as described in the legend to Fig. 1. Nucleoids, isolated from step gradients containing 1.95 M NaCl, were diluted to 0.2×10^6/ml and 2 M NaCl or 0.2 M NaCl using 10 mM Tris (pH 8.0) and the appropriate concentration of salt. Samples were irradiated (9.6 J · kg^{-1}) before ethidium was added and the fluorescence measured. (●——●) Binding in 0.2 M NaCl; (○——○) binding in 2 M NaCl. Error bars give the standard deviation of the means obtained from at least five different experiments

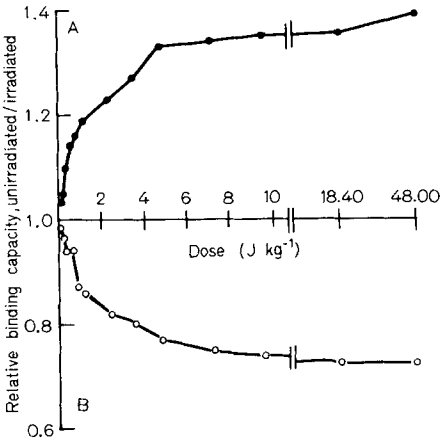

Fig. 3. *Ethidium-binding capacity of unirradiated nucleoids relative to that of nucleoids irradiated with different doses of γ-rays.* Nucleoids, isolated from 'step' gradients containing 1.95 M NaCl, were diluted to 0.2×10^6/ml using 2 M NaCl and 10 mM Tris (pH 8.0). Samples were irradiated (dose rate 1.2–6.1 J · kg^{-1}) with different doses of γ-rays, ethidium was added to a final concentration of 0.2 or 8 μg/ml and the amount of bound ethidium determined from the fluorescence as described in Materials and Methods. (A) 0.2 μg/ml ethidium; (B) 8.0 μg/ml ethidium

the reason for the difference given by the two methods.) In the experiments described in Fig. 1 and 2 we used nucleoids freed of cytoplasm. Essentially similar results are obtained with unpurified nucleoids.

A reduction in the salt concentration surrounding an intact circle of DNA unwinds the double helix, so reducing the number of (negative) superhelical turns [25,26]. Less ethidium is therefore required to remove supercoiling [20,27]. The DNA of nucleoids behaves like circular DNA in this respect; when the salt concentration is reduced from 2 M to 0.2 M NaCl the concentration of ethidium at which the irradiated and unirradiated nucleoids fluoresce similarly is roughly halved (Fig. 2).

A dose of 9.6 J · kg^{-1} removed nearly all supercoiling from nucleoid DNA so minimising the rate of sedimentation [4]; therefore the same dose should maximise the difference in ethidium-binding capacity of irradiated and unirradiated nucleoids. At concentrations of ethidium below the equivalence point (*e.g.* 0.2 μg/ml) the amount of ethidium bound by the irradiated nucleoids progressively decreases as the dose of radiation increases, whilst at concentrations above the equivalence point (*e.g.* 8 μg/ml) it progressively increases (Fig. 3). Small doses have a marked effect whereas increasing the dose above 9.6 J · kg^{-1} has relatively little further effect. The dose of γ-rays producing half the maximum effect is about the same as the dose that produced half the maximum reduction in sedimentation rate suggesting that the two methods detect targets of similar size.

We originally predicted that the quasi-circles might be the size of chromomeres or replicons [28]. Subsequently we estimated the length of DNA (the 'target') in one quasi-circle to be much greater than this by applying target theory to curves relating the dose of γ-rays to the sedimentation rate of nucleoids [4]. This estimate was necessarily a rough one because we applied data collected on the frequency of breaks induced by γ-irradiation under one set of conditions to the very different conditions that we use with nucleoids. We therefore compared the effects of γ-radiation on nucleoids and a circle of DNA of known molecular weight using the same conditions (Fig. 4).

We first examined the effects of irradiation on a sample of PM2 DNA containing 82% intact circles using agarose gels (Fig. 4C). The predominant forms resolved in such gels and obtained after irradiating the DNA with progressively increasing doses are the intact circle (below 6.5 J · kg^{-1}), nicked circle (6.5–550 J · kg^{-1}), linear duplex (550–1500 J · kg^{-1}) and fragments (above 1500 J · kg^{-1}), (results not shown).

The ethidium-binding capacity of irradiated PM2 DNA was also determined by fluorometry: it is expressed as a percentage relative to the amount bound to the unirradiated form (Fig. 4B). Irradiation increases the binding capacity. The dose of 82 J · kg^{-1} which produces 63% of the total increase in fluorescence is very similar to the dose of 91 J · kg^{-1} required to nick 63% of the circles and which was estimated using gels (cf. Fig. 4B, C). High doses (> 300 J · kg^{-1}) reduce binding to PM2 DNA: presumably the radia-

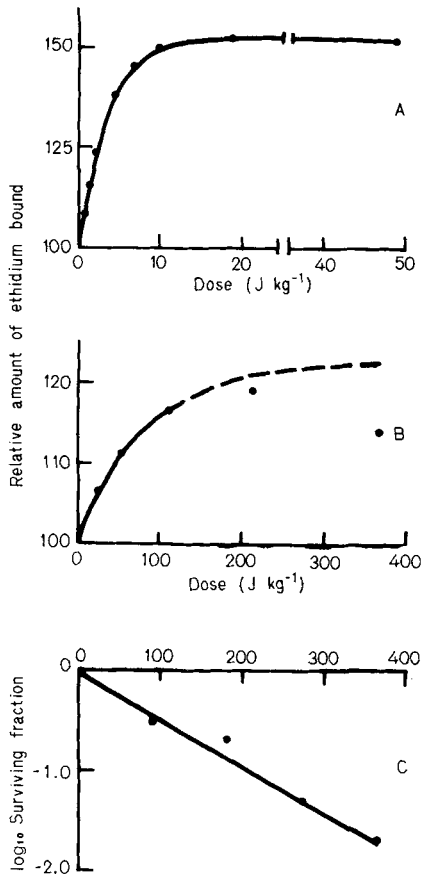

Fig. 4. *The effect of γ-irradiation on PM2 DNA and nucleoids.* (A) The ethidium-binding capacity of nucleoids irradiated with different doses of γ-rays is expressed as a percentage relative to the amount bound to unirradiated nucleoids. Nucleoids, isolated from 'step' gradients containing 1.95 M NaCl, were diluted to 0.4 $\times 10^6$/ml and 0.2 M NaCl, 10 mM Tris (pH 8.0). 1-ml samples were irradiated (dose rate 1.1 or 6.1 J · kg^{-1}), with different doses of γ-rays and then mixed with 1 ml 0.2 M NaCl, 10 mM Tris (pH 8.0) containing 16 μg/ml ethidium. The amount of ethidium bound to the nucleoids was determined from the fluorescence as described in Materials and Methods. (B) The ethidium-binding capacity of PM2 DNA irradiated with different doses of γ-rays is expressed as a percentage relative to the amount bound to unirradiated PM2 DNA. 1-ml samples of PM2 DNA (2 μg/ml in 0.2 M NaCl, 10 mM Tris, pH 8.0) were irradiated (dose rate 6.1 J · kg^{-1}) with different doses of γ-rays, mixed with 1 ml 0.2 M NaCl, 10 mM Tris (pH 8.0) containing 16 μg/ml ethidium and the amount of ethidium bound determined fluorometrically. (C) The fraction of PM2 DNA remaining intact and supercoiled following γ-irradiation is expressed as the logarithm. PM2 DNA (80 μg/ml in 0.2 M NaCl, 10 mM Tris, pH 8.0) was irradiated (dose rate 6.1 J · kg^{-1}) with different doses and the fraction of the DNA remaining intact determined using agarose gels as described in Materials and Methods. Analysis of the curve in (A) is simplified by assuming that the DNA of nucleoids is fully supercoiled or fully relaxed at values of the relative amounts of ethidium bound of 100% and 153% respectively. The corresponding values for PM2 DNA (B) are 100% and 122.5%. Analysis is further simplified by assuming that supercoiled targets (*i.e.* the circles or quasi-circles) of uniform size are converted independently to the relaxed form with first-order kinetics. The doses of γ-rays reducing the number of superhelical targets to 50% and 37% of the original number are (A) 2.5 and 3.5 (B) 55 and 82 (C) 62 and 91 J · kg^{-1} respectively. The quasi-circles are about 22.5 times larger than the circles of PM2 DNA

tion damages the DNA in a way that prevents intercalation. Irradiation has the same general effect on the ethidium bound by nucleoids, but smaller doses are required (Fig. 4A). (The doses used with nucleoids in these and subsequent experiments are very low and so are unlikely to have any effect on ethidium binding other than through effects on supercoiling.) Digestion with low concentrations of deoxyribonuclease 1 also increases the ethidium-binding capacity of unirradiated nucleoids: irradiation then has no additional effect (results not shown).

In principle the proportion of the DNA in nucleoids that is constrained and supercoiled can be estimated by comparing the relative increase in ethidium binding induced by irradiation in nucleoids and the pure PM2 DNA, which contains 82% superhelical DNA. Remarkably, irradiation has a substantially greater effect on the nucleoids than on the pure circles suggesting that essentially all the nucleoid DNA contains free energy of supercoiling. However we can offer no simple explanation of why the effect is so large. (Irradiation also has a much greater effect on the sedimentation rate of nucleoids: it halves the sedimentation rate of nucleoids whereas nicking only reduces the rate of sedimentation of pure circles by a quarter to a third [2,4].) As the doses that produce the same relative effects on nucleoids and the pure circles differ by about 22 times, the quasi-circles in nucleoids must be about 22 times larger than the circles of PM2 DNA. Assuming that PM2 DNA has a molecular weight of 6.5×10^6 and contains 9850 base pairs [29] the average quasi-circle in a nucleoid contains about 220000 base pairs (150×10^6 daltons).

Fluorescence Microscopy

Complexes of acridine orange with DNA and RNA fluoresce in the green and red respectively, so that nucleoids stained with this dye appear in the fluorescence microscope as green structures surrounded by a red rim; they also contain red nucleoli. When stained with ethidium, all parts of the nucleoid fluoresce in the orange, with the rim and the nucleoli fluorescing the brightest [7]. Before measuring the effects of various agents on supercoiling we monitored their effects on nucleoid integrity by fluorescence microscopy. After the nucleoids had been digested with deoxyribonuclease and stained with acridine orange, only the red rim and nucleoli remain. On the other hand the rim and nucleoli are not completely removed by ribonuclease digestion. Proteolytic enzymes (*i.e.* pronase and trypsin) and sodium dodecyl sulphate destroy the rim and this allows the DNA to disperse. Agents like sarkosyl, urea and potassium thiocyanate swell the nucleoids and increase the diameter of the rim whereas γ-irradiation disperses nucleoid DNA without affecting the rim. We therefore

Table 1. *The effect of ribonuclease on supercoiling in nucleoids*
HeLa nucleoids were isolated in 1.95 M NaCl from HeLa cells grown for 2 or 24 h in 4 or 1 μCi/ml [5,6-^{3}H]uridine respectively, counted and diluted to 0.2×10^6/ml in 0.2 M NaCl, 10 mM Tris (pH 8.0). Some of the nucleoids were γ-irradiated (9.60 J · kg^{-1}). Heat-inactivated ribonuclease A or T_1 was added to final concentrations of 53 μg/ml and 62.5 units/ml respectively. After incubation for 10 min at 30 °C, the amount of radioactivity insoluble in trichloroacetic acid in one portion of the sample was determined as described in Materials and Methods, and expressed as a percentage of the radioactivity found in undigested controls. Ethidium was added to a final concentration of 8 μg/ml to the remainder of the sample and the amount of dye bound to irradiated and unirradiated samples determined by fluorometry. The binding ratio is the amount of ethidium bound by unirradiated nucleoids divided by the amount bound by irradiated nucleoids. The absolute amounts of ethidium bound by irradiated nucleoids before digestion were between 5 – 10 % higher than those bound after digestion. Irradiated and unirradiated nucleoids were digested to similar extents

Ribonuclease	Binding ratio after labelling for					
	2 h			24 h		
	before digestion	after digestion	(^{3}H remaining undigested)	before digestion	after digestion	(^{3}H remaining undigested)
			(%)			(%)
A	0.66	0.65	(9)	0.72	0.68	(78)
T_1	0.66	0.7	(23)	0.72	0.66	(80)
A and T_1	–	–	–	0.72	0.66	(77)

believe that nucleoid DNA is encaged by RNA and protein.

Digestion of Nucleoids with Ribonucleases and Proteases

Protein or RNA might maintain the quasi-circular nature of nucleoid DNA by bridging the gap between two sites on one duplex to form a loop. Previously we have examined this possibility by digesting the nucleoids with ribonuclease or pronase: digestion with both enzymes reduces the rate of sedimentation of nucleoids [7]. The reduction might not result from the loss of supercoiling that would accompany the destruction of a molecular bridge maintaining the constraint but from digestion of a restraining cage surrounding the nucleoids: DNA released from its confinement might sediment more slowly even though it was still supercoiled. In principle we can distinguish between these two possibilities using the fluorometric assay: any loss of constraint on digestion of unirradiated nucleoids should affect their capacity to bind ethidium whether or not it is accompanied by a dispersion of the DNA.

We first digested nucleoids with ribonuclease. The results exemplify the difficulties introduced by contaminating deoxyribonucleases. We had earlier found that ribonuclease digestion reduced the sedimentation rate of unirradiated nucleoids to that of irradiated nucleoids [7]. We have repeated these experiments using a different batch of enzyme; this time we find that the rate of sedimentation is unaffected (results not shown). The results of a more detailed fluorometric analysis using the second batch of enzyme are presented in Table 1. Cells were labelled for 2 or 24 h with [^{3}H]uridine, and nucleoids were isolated and digested with ribonuclease A or T_1. Some labelled RNA in nucleoids cannot be digested by these enzymes, however high their concentration or however long the digestion period. The proportion of label resistant to digestion depends upon the labelling period, presumably reflecting the differential sensitivity of the various classes of nuclear RNA. After 2-h labelling, when heterogeneous nuclear RNA is richly labelled, about 10 % is resistant to ribonuclease A: after 24 h, when most of the label is found in ribosomal RNA and its precursors, 80 % is resistant. Digestion has little effect on the relative amounts of ethidium bound to irradiated and unirradiated nucleoids (Table 1). We conclude that the constraint is resistant to ribonuclease A and T_1.

We next digested the nucleoids using different concentrations of a broad spectrum protease, pronase (Fig. 5 A). (This enzyme preparation, which was free of detectable deoxyribonuclease activity under the conditions used, nevertheless did contain a very low level of contamination detectable under different conditions, see Materials and Methods.) Cells were labelled with [^{3}H]leucine, nucleoids isolated and their ethidium-binding capacity determined before and after digestion. Up to 40 % of the label may be removed without any effect on ethidium-binding, *i.e.* without any loss of supercoiling; some supercoiling remains even after 90 % of the label has been digested. Further digestion progressively removes more and more supercoiling. Digestion with a more specific protease, trypsin, which was free of contaminating deoxyribonuclease (see Materials and Methods), gave rather similar results; some supercoiling remained after removal of 50 % of the label (Fig. 5 B).

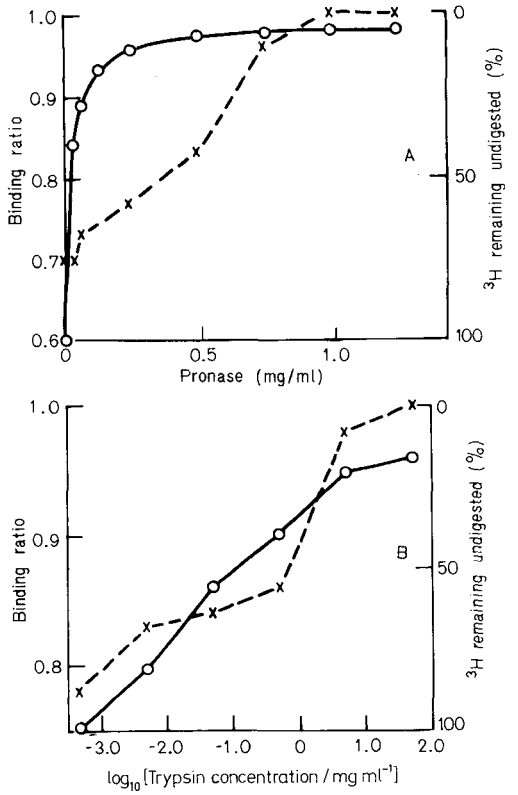

Fig. 5. *The effect of (A) pronase and (B) trypsin on supercoiling in nucleoids.* HeLa nucleoids were isolated in 1.95 M NaCl from HeLa cells grown for 2 h in L-[4,5-^{3}H]leucine (4 μCi/ml), counted and diluted to 0.2×10^6/ml in 0.2 M NaCl, 10 mM Tris (pH 8.0). Some of the nucleoids were γ-irradiated (9.6 J · kg^{-1}). Ethidium was added to a final concentration of 8 μg/ml and then (A) pronase or (B) trypsin to the final concentration indicated. After incubation for 10 min at 30 °C, the amount of radioactivity insoluble in trichloroacetic acid in one portion of each sample was determined as described in Materials and Methods, and expressed as a percentage of the radioactivity found in undigested controls incubated similarly. The amount of dye bound to irradiated or unirradiated nucleoids was determined by fluorometry using the remainder of the sample. The binding ratio is the amount of ethidium bound by unirradiated nucleoids divided by the amount bound by irradiated nucleoids. The absolute amounts of ethidium bound by irradiated nucleoids were unaffected by digestion: irradiated and unirradiated nucleoids were digested to similar extents. Ethidium was mixed with nucleoids before, and not after digestion, since such mixing might break DNA. Control experiments indicated that ethidium did not affect the extent of digestion. (O——O) ^{3}H remaining undigested; (×----×) binding ratio

A number of reasons prevent us from concluding that the constraining mechanism contains protein but not RNA. The first concerns the presence of contaminating deoxyribonuclease in our enzyme preparations; although we have attempted to destroy any such contamination we cannot be completely certain that we have done so. (This is discussed in Materials and Methods.) The second concerns the breaking of nucleoid DNA dispersed during digestion by the manipulations required for fluorometric analysis. DNA is very easily sheared [30,31] and as we are dealing with intact molecules of DNA whose sizes are much greater than those prepared by conventional procedures, supercoiling might readily be lost when the digested nucleoids are poured into the cuvette used for fluorometry. Third, protein or RNA might well be resistant to digestion by virtue of being so intimately associated with the DNA it is constraining.

The Effect of Heat on Ethidium Binding

Even gentle heating affects the rate of sedimentation of nucleoids, for example incubation at 50 °C for 10 min reduces their sedimentation rate to one-fifth [7]. This reduction might have several causes. If the constraining mechanism is very sensitive to heat, supercoiling would be released and the rate of sedimentation would fall. On the other hand, if it were insensitive a non-specific aggregation or temperature-induced unfolding or enlargement of the nucleoids might reduce the sedimentation rate. We therefore measured by spectrofluorometry the ethidium-binding properties of heated nucleoids. Initial experiments showed that the binding properties of nucleoids remained constant at 37 °C or below for at least 2 h, the time scale of our experiments. Irradiated and unirradiated nucleoids retained their differences in ethidium binding even after they had been heated to very high temperatures.

Nucleoids were heated and then cooled rapidly; ethidium was added and the amount of bound ligand determined by spectrofluorometric measurements on the cooled samples. In principle, at least four separate transitions could characterise binding to our superhelical template; three transitions would be shared by superhelical circles of pure DNA [25,32]. First, heating a circular duplex of DNA increases the pitch of the double helix and as the helix unwinds supercoils are lost. This transition should be reversed when the sample is cooled and so should remain undetected by our method. Second, at a temperature 20–30 °C higher than the melting temperature characteristic of the helix-coil transition of the nicked allomorph, the DNA of an intact circle denatures; it becomes single-stranded even though the two circular strands remain interlocked. Both the concentration of salts and chaotropic agents (*e.g.* $NaClO_4$) affect this transition temperature. On cooling rapidly, this transition is not reversed. As double strands of DNA bind less ethidium than single strands, this transition should be marked by a sharp drop in fluorescence and be common to both irradiated and unirradiated templates. (Since there are no experiments on DNA of molecular weights comparable to those in nucleoids, we do not yet know to what extent the rapid cooling completely prevents any reassociation of the highly interlocked and single-stranded DNA of heated nucleoids. However, it is clear that denaturation of the DNA in

nucleoids is not completely reversible.) A third transition, which is relatively insensitive to the salt concentration, occurs at about 100 °C when hydrolysis of phosphodiester bonds in a circular DNA leads to a loss of supercoiling. Hydrolysis should affect ethidium binding to a circle in a manner analogous to γ-irradiation. If the DNA of nucleoids is organised into loops then a fourth thermal transition will mark the loss of the topological constraint and should affect ethidium binding. In this series of experiments we have used an ethidium concentration of 8 μg/ml. At this concentration less ethidium binds to the unirradiated nucleoids and any loss of supercoiling on heating should be accompanied by an increased binding. The last two transitions should not be reversed by cooling. Of course, other thermal transitions may occur in nucleoids which affect ethidium binding, for example those involving RNA, but we can neglect these since they should be unaffected by radiation.

The results of the heating experiments are summarised in Fig. 6–8 and Table 2. We first determined the denaturation temperature (t_m) of pure HeLa DNA by measuring the hyperchromicity induced by heating. The absorbance at 259 nm was measured both at the denaturation temperature or at room temperature after the DNA had been heated and cooled. The t_m in 0.2 M NaCl, 10 mM Tris (pH 8.0) measured in the first way was 86.5 °C, the expected temperature [33]; the t_m measured after cooling the DNA was higher [34], denaturation being incomplete at 100 °C (Fig. 6A). The helix-coil transition in pure DNA can also be detected readily by fluorometry: denaturation reduces ethidium binding (Fig. 6B). The t_m is increased by high concentrations of salt or ethidium and decreased by 4 M $NaClO_4$ (Fig. 6B) [17, 33, 34].

Since heating aggregates nucleoids in the concentrated suspensions required for spectrophotometric analysis, we studied heated nucleoids using the more sensitive fluorometric method. Nucleoids in 0.2 M NaCl were heated for 10 min at different temperatures and then cooled rapidly; ethidium was added and the amount of bound ligand determined and expressed as a percentage relative to the amount bound by unirradiated nucleoids incubated on ice (Fig. 7A). Binding to irradiated nucleoids is relatively unaffected by incubating them at temperatures up to 90 °C. Above 90 °C the binding is reduced, as it is with pure DNA; an irreversible denaturation of double-stranded DNA in the irradiated nucleoids must reduce the binding. Heating unirradiated nucleoids to about 80 °C does not significantly affect ethidium binding but incubation between 85–95 °C increases it (Fig. 7A). We conclude that the topological constraint that maintains supercoiling is stable below 85 °C and is progressively destroyed at higher temperatures. Like intact and circular DNA which denatures at temperatures 20–30 °C higher than nicked DNA [32], the DNA of

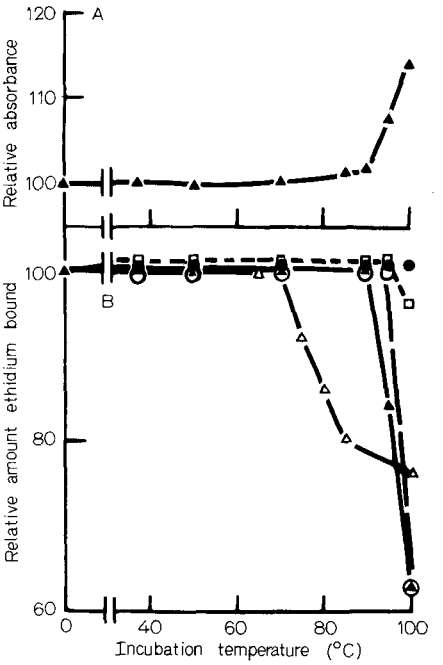

Fig. 6. *The thermal denaturation of pure HeLa DNA.* (A) The effect of heating on DNA measured using a spectrophotometer. The absorbance of DNA (12.5 μg/ml) heated in 0.2 M NaCl, 10 mM Tris (pH 8.0) is expressed as a percentage relative to the absorbance of an unheated sample. Samples were heated for 10 min, cooled by plunging them into ice-cold water and their absorbance measured at 259 nm. DNA which is completely denatured has a relative absorbance > 140. (B) The effect of heating on the binding of ethidium by DNA. The amount of ethidium bound to DNA incubated in various solvents is expressed as a percentage relative to the amount bound to DNA incubated on ice. 2-ml samples of DNA (0.6 μg/ml) were incubated for 10 min before they were plunged into ice-cold water; ethidium was added to a final concentration of 8 μg/ml either before or after incubation and the amount of ethidium bound determined by spectrofluorometry. No corrections have been made for any slight losses due to evaporation. Solvents: (▲——▲) 0.2 M NaCl, 10 mM Tris (pH 8.0); (○– – –○) 0.2 M NaCl, 10 mM Tris (pH 8.0), ethidium (8 μg/ml); (□– – –□) 2.0 M NaCl, 10 mM Tris (pH 8.0); (●) 2.0 M NaCl, 10 mM Tris (pH 8.0), ethidium (8 μg/ml); (△——△) 0.2 M NaCl, 10 mM Tris (pH 8.0), 4 M $NaClO_4$

unirradiated nucleoids also denatures at a temperature greater than the broken DNA of irradiated nucleoids; after heating above 95 °C, the unirradiated nucleoids bind more ethidium than their irradiated counterparts. The constraint cannot, therefore, be completely destroyed even at 95 or 100 °C. Little hydrolytic cleavage of phosphodiester bonds (which would also release supercoiling) can be occurring under these conditions. The sharp reduction in binding that accompanies the helix-coil transition must occur above 100 °C.

We use two parameters to describe the transition that marks the loss of the topological constraint. The $t_{1/2}$ is the temperature at which unirradiated nucleoids must be incubated to increase their ethidium-binding capacity to a value midway between that of unirradiated and irradiated nucleoids incubated on ice. In this

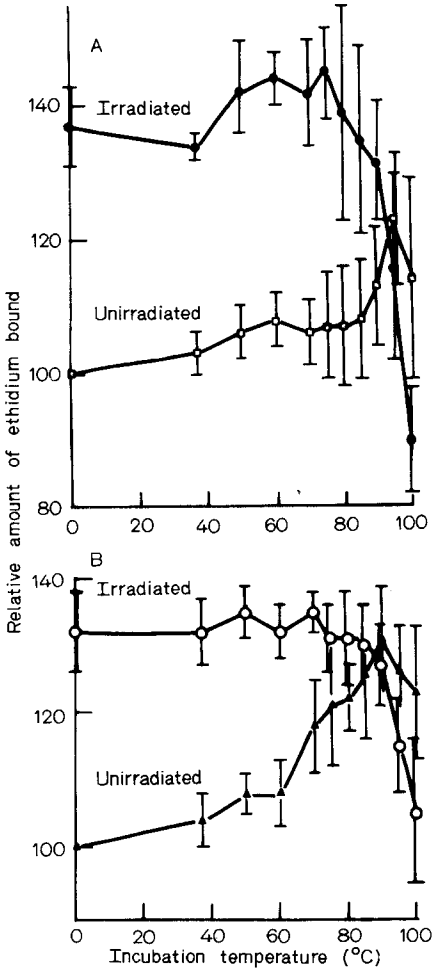

Fig. 7. *Effect of heating in 0.2 M NaCl on the binding of ethidium by nucleoids.* The amount of ethidium bound to irradiated or unirradiated nucleoids incubated in 0.2 M NaCl (in the presence or absence of ethidium) is expressed as a percentage relative to the amount bound to unirradiated nucleoids incubated on ice. Nucleoids were isolated in 1.95 M NaCl, counted and diluted to 5×10^4/ml in 0.2 M NaCl, 10 mM Tris (pH 8.0). 2-ml samples were dispensed into tubes and half the tubes were irradiated ($9.6\ J \cdot kg^{-1}$). Tubes were incubated for 10 min before they were plunged into ice-cold water. Ethidium was added to a final concentration of 8 μg/ml either before (B) or after (A) incubation and the amount of ethidium bound determined by spectrofluorometry. (A) (□——□) Ethidium bound to unirradiated nucleoids incubated in the absence of ethidium. (●——●) Ethidium bound to irradiated nucleoids incubated in the absence of ethidium. (B) (▲——▲) Ethidium bound to unirradiated nucleoids incubated in the presence of ethidium. (○——○) Ethidium bound to irradiated nucleoids incubated in the presence of ethidium. Error bars give the standard deviations of the means

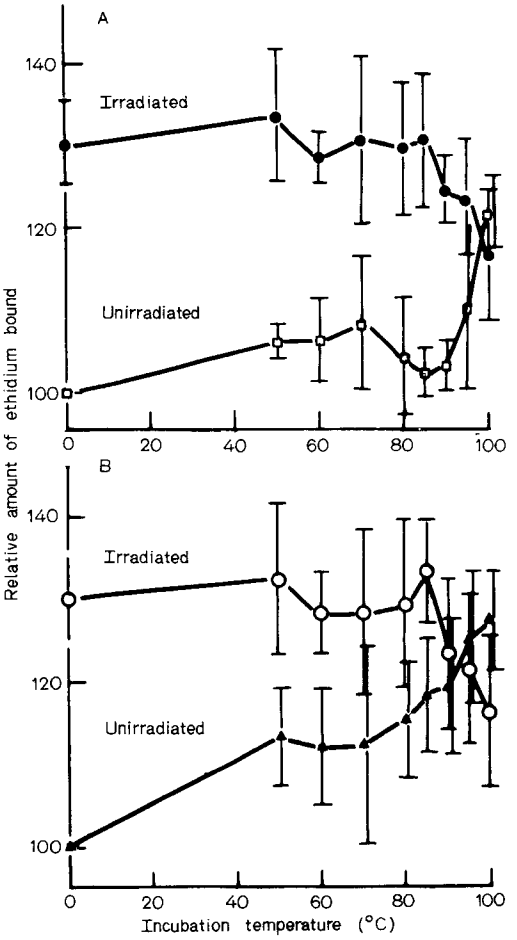

Fig. 8. *Effect of heating in 2 M NaCl on the binding of ethidium by nucleoids.* The amount of ethidium bound to irradiated or unirradiated nucleoids incubated in 2 M NaCl (in the presence or absence of ethidium) is expressed as a percentage relative to the amount bound to unirradiated nucleoids incubated on ice. Nucleoids were isolated in 1.95 M NaCl, counted and diluted to 5×10^4/ml with 2 M NaCl, 10 mM Tris (pH 8.0). Samples were dispensed, irradiated, incubated in the presence (B) or absence (A) of ethidium (8 μg/ml) and the amount of ethidium bound determined by spectrofluorometry as described in the legend to Fig. 7. Unirradiated nucleoids tended to aggregate after incubation at 70–80 °C in the presence of ethidium. Spectrofluorometric readings were therefore sometimes variable and where variation occurred the lowest reading was taken. (A) (□——□) Ethidium bound to unirradiated nucleoids in the absence of ethidium. (●——●) Ethidium bound to irradiated nucleoids in the absence of ethidium. (B) (▲——▲) Ethidium bound to unirradiated nucleoids in the presence of ethidium. (○——○) Ethidium bound to irradiated nucleoids in the presence of ethidium. Error bars give the standard deviations of the means. No corrections have been made for any slight losses due to evaporation

case it is 93 °C. The second is the equivalence temperature. After incubation at the equivalence temperature, the irradiated and unirradiated nucleoids have similar binding capacities (*i.e.* 94 °C). The similarities in the equivalence temperature, the $t_{1/2}$ and the t_m of the DNA in nucleoids suggests that both duplex structure and the topological constraint might be maintained by similar forces. If so, the parameters of the constraint should be increased in 2 M NaCl just like the t_m. We therefore heated nucleoids in 2 M NaCl and measured their ethidium-binding capacity; it is affected in much the same general way as it is in 0.2 M

Table 2. *The effect of temperature on supercoiling in nucleoids*
The amount of ethidium bound to irradiated and unirradiated nucleoids after incubation under different conditions was determined by spectrofluorometry (see Fig. 7 and 8). After incubation on ice, more ethidium binds to irradiated nucleoids than to unirradiated nucleoids, and after incubation at higher temperatures the amounts of ethidium bound to unirradiated nucleoids is increased. The $t_{1/2}$ is the temperature at which unirradiated nucleoids must be incubated to raise their ethidium binding to a value midway between that of unirradiated and irradiated nucleoids incubated on ice. The equivalence temperature is the temperature at which irradiated and unirradiated nucleoids must be incubated so that their ethidium binding capacity becomes the same. The t_m of pure DNA was determined as described in Fig. 6

Incubation conditions	$t_{1/2}$ of nucleoids	Equivalence temperature of nucleoids	t_m of pure DNA
	°C		
2 M NaCl	97	99	> 100
2 M NaCl + ethidium (8 μg/ml)	80	93	> 100
0.2 M NaCl	93	94	96
0.2 M NaCl + ethidium (8 μg/ml)	71	88	98

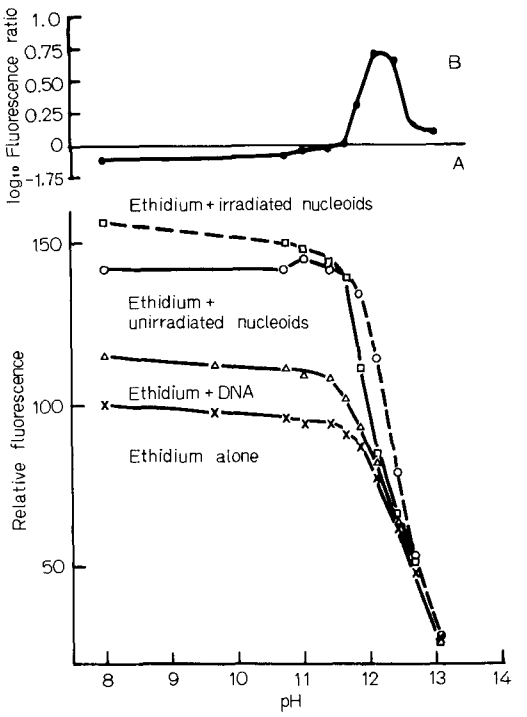

Fig. 9. *The effect of pH on the topological constraint.* The fluorescence of solutions of different pH containing ethidium and DNA or nucleoids was measured spectrofluorometrically. Solutions contained final concentrations of 8 μg/ml ethidium, 0.2 M NaCl, 5 mM Tris, and 0.025 M KH_2PO_4 in addition to various quantities of KOH. Buffers of different pH were made by titrating 0.05 M KH_2PO_4 with 0.5 M KOH, 0.05 M KH_2PO_4. 1 vol. of each of these buffers was mixed with 1 vol. of 0.4 M NaCl, 10 mM Tris (pH 8.0) and ethidium (16 μg/ml) containing unirradiated or irradiated nucleoids or HeLa DNA. The fluorescence of the mixture was determined and expressed as a percentage of the fluorescence of ethidium (8 μg/ml) in 0.2 M NaCl, 10 mM Tris (pH 8.0). The pH of similar solutions which lacked DNA or nucleoids was determined simultaneously. (A) The relative fluorescence of solutions containing ethidium alone (×——×), ethidium and 3 μg/ml HeLa DNA (△——△), ethidium and unirradiated nucleoids (5×10^4/ml) (○——○), ethidium and irradiated ($9.6\ J \cdot kg^{-1}$) nucleoids (5×10^4/ml) (□- - -□). (B) The fluorescence of the ethidium bound to irradiated nucleoids is divided by the fluorescence of the ethidium bound to unirradiated nucleoids to give a ratio. The fluorescence of the bound ethidium was obtained by substracting the fluorescence of ethidium alone from the fluorescence of the mixture of ethidium and nucleoids at each pH. The ratio is expressed as the logarithm

NaCl. The equivalence temperature, $t_{1/2}$ and t_m are all raised in 2.0 M NaCl (Fig. 8 A).

Free energy of supercoiling might affect the stability of the constraining mechanism. We therefore heated the nucleoids in the presence of a concentration of ethidium (*i.e.* 8 μg/ml) sufficient to reverse the sense of supercoiling (Fig. 7 B and 8 B). The t_m of pure DNA is increased by ethidium [17]. We confirmed that this was so under our experimental conditions; ethidium protects pure DNA from denaturation in both 0.2 M and 2.0 M NaCl (Fig. 6). In contrast to the t_m, the parameters of the constraint are decreased (Table 2): when nucleoids are heated to 60–90 °C in the presence of ethidium and then cooled, their ethidium-binding capacity is greater than that of their counterparts heated in the absence of the dye (Fig. 7 B and 8 B).

The results of the heating experiments are summarised in Table 2. When measured in the absence of ethidium, the two parameters of the constraint (*i.e.* the $t_{1/2}$ and equivalence temperature) are very similar to the t_m of pure DNA suggesting that the loops are maintained by forces similar to those that maintain the structure of the double helix. Their dissimilarity when measured in the presence of ethidium suggests that the naturally-occurring free energy of supercoiling stabilises the constraint.

The Effect of Alkali on Ethidium Binding

The remarkable thermal stability of the constraining mechanism prompted us to determine its stability to alkali, since alkali also denatures DNA (Fig. 9). The fluorescence of free ethidium is progressively quenched as the pH is raised above 11.5 and this falling background fluorescence complicates the titration experiments. The addition of pure DNA to the ethidium enhances its fluorescence, and the enhancement is lost between pH 11.5–12, the pH range in which the double helix denatures. Irradiated nucleoids also enhance ethidium's fluorescence and, as is the case with pure DNA, this enhancement is lost between pH 11.5–12.0. At pH 8.0 and in the presence of 8 μg/ml ethidium, unirradiated nucleoids bind less ethidium

Table 3. *Effect of various solvents on supercoiling in nucleoids*
The fluorescence of ethidium in various solvents and in the presence of unirradiated or irradiated (9.6 J · kg^{-1}) nucleoids was determined at room temperature by spectrofluorometry. The reference solvent contained a final concentration of 8 μg/ml ethidium, 0.2 M NaCl, 10 mM Tris (pH 8.0); other solvents contained in addition the agents at the final concentrations indicated. One volume of 0.4 M NaCl, 10 mM Tris (pH 8.0), with or without nucleoids (10^6/ml), was mixed with an equal volume of solvent plus ethidium and after careful mixing the fluorescence measured within 5 min. The fluorescence of ethidium bound to nucleoids was calculated by subtracting the fluorescence determined in the absence of the nucleoids from the fluorescence measured in their presence. Fluorescence ratio 1 is the fluorescence of ethidium in the solvent/fluorescence of ethidium in the reference solvent; fluorescence ratio 2 is the fluorescence of ethidium bound to unirradiated nucleoids in the solvent/fluorescence of ethidium bound to unirradiated nucleoids in the reference solvent; fluorescence ratio 3 is the fluorescence of ethidium bound to unirradiated nucleoids in solvent/fluorescence of ethidium bound to irradiated nucleoids in the solvent

Solvent		Fluorescence ratio		
		1	2	3
Reference		1.0	1.0	0.77
Metrizoate	(12.5%)	1.09	0.87	0.84
	(50%)	1.20	0.30	0.83
KSCN	(0.5 M)	1.01	0.82	0.88
	(1.0 M)	1.10	0.48	0.95
	(2.5 M)	1.29	0.24	1.0
Urea	(2.5 M)	1.38	0.76	0.85
	(5.0 M)	1.63	0.57	1.0
Sarkosyl	(0.625%)	1.28	0.52	0.81
	(1.25%)	1.35	0.42	1.0

than their irradiated counterparts. Around pH 11.0 the binding to unirradiated nucleoids increases almost to that of irradiated nucleoids. (This small increase, represented by only one point in the typical experiment presented in Fig. 9A, is quite reproducible.) We conclude that some, but not all, constraints are removed at pH 11.0. The sharp drop in fluorescence which accompanies the denaturation of the double helix in irradiated nucleoids and which occurs between pH 11.5 and 12.0 only occurs with unirradiated nucleoids in the pH range 12 – 12.5. These differences in ethidium binding are reflected in the relative amounts of ethidium bound to unirradiated and irradiated nucleoids (Fig. 9B). The DNA of unirradiated nucleoids is more stable to denaturation by alkali, as it is to thermal denaturation; therefore some constraints must remain even above pH 12.0. Again the similarities in the pH required to destroy the constraints and denature the duplex suggest that both constraining mechanism and duplex structure are stabilised by similar forces. Alkali affects the quasi-circles in nucleoids just like it affects circles of pure DNA [32, 35].

The Effect of Various Solvents on Ethidium Binding

The cage that surrounds the nucleoids might form the DNA into quasi-circles. We therefore investigated the effects on the constraining mechanism of a range of agents that distend the cage (Table 3). In the absence of nucleoids these agents enhance the fluorescence of ethidium (Table 3, fluorescence ratio 1) and reduce, but do not abolish, its affinity for the nucleoids (Table 3, fluorescence ratio 2). Using a concentration of 8 μg/ml of ethidium as before, we determined whether ethidium retained its higher affinity for irradiated nucleoids in the presence of the various agents (Table 3, fluorescence ratio 3). We have not studied the binding in great detail and since a variety of different effects might abolish any difference in binding only the retention of the difference is informative. (These effects include the breaking by the manipulation required for fluorometry of any supercoiled DNA released from its cage by the solvent and alterations in the degree of supercoiling induced by the solvent winding or unwinding the double helix.) Low concentrations of potassium thiocyanate, urea and sarkosyl (concentrations which nevertheless swell nucleoids) have little effect; higher concentrations abolish the difference. The difference is only maintained in metrizoate, a rather ineffective swelling agent. However, since we cannot obtain solutions containing higher concentrations of metrizoate, we cannot be certain that this agent differs from the others and that constraints can remain in highly swollen nucleoids.

DISCUSSION

Our experiments on the sedimentation of nucleoids in sucrose gradients containing intercalating agents originally suggested that the nuclear DNA of higher cells is quasi-circular [4]. In this paper we have confirmed this quasi-circularity using an independent method: nucleoids also bind ethidium in the manner characteristic of circular DNA. Although all our results can be simply explained if nucleoids contain many circles of DNA, they can be most easily reconciled with genetic evidence that suggests chromosomal DNA is linear if we assume that the DNA is organised into loops. What, then, ties the DNA in loops?

Fluorescence microscopy of nucleoids stained with ethidium or acridine orange suggests that nucleoid DNA is contained within a cage of protein and RNA. The packaging of the DNA within the cage makes the fragile DNA resistant to shear. The loops of DNA might be tied to this cage. The cage is probably related to the nuclear-pore complex and the nuclear envelope or matrix isolated by others [36 – 38]. It must be flexible so that the conformation of the DNA inside it can determine its shape and so the rate of sedi-

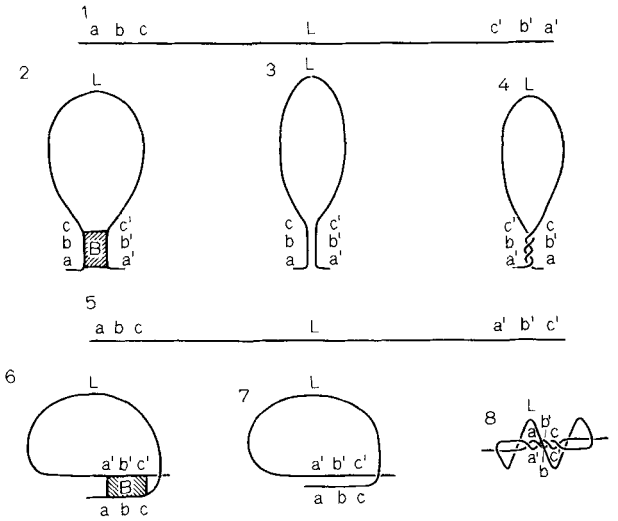

Fig. 10. *Some models of quasi-circles of nuclear DNA.* A line depicts one duplex bearing the repeated sequence abc...c'b'a' (1) or abc...a'b'c' (5). In (2) and (6) a molecular bridge, B, spans the gap between the repeated sequences forming a quasi-circle. B utilises free energy of supercoiling when it binds. The symmetry of B would determine whether form (2) could be derived from form (1) or (5) and similarly for form (6). Form (4), the simple eye-splice, is formed by looping (1) to give form (3) and then twisting the loop L through $2 \times 360°$. The stem forms a right-handed interwound superhelix. (A right-handed interwound superhelix is topologically equivalent to a left-handed toroidal superhelix [12]. The number of turns in the superhelix will, of course, depend on the length of the repeated sequence.) Form (4) is stabilised by specific interactions between base pairs. McGavin [41] has shown that G · C can pair specifically with G · C but not C · G (which is G · C inverted) and A · T with A · T and not T · A (which is A · T inverted). Similar specific interactions between base pairs to those involved in (4) stabilise the formation of (8) by twisting the looped form (7) of the linear duplex (5). Negative supercoiling in the loop, L, stabilises structures (2), (4), (6) and (8). The simple eye-splice (4) can only be derived from (1) but not from (5). Sequences like those in form (1) would be a palindrome if the internal sequence, L, were omitted. Complete palindromes are scattered throughout HeLa DNA [44,45] and might be formed from two contiguous half-palindromes, each from separate but adjacent loops

mentation of the nucleoid. Some agents (*e.g.* metrizoate) distend the cage and so reduce the sedimentation rate of the nucleoids without loss of supercoiling.

Most surprisingly the holding mechanism is stable at temperatures up to the denaturation temperature of the double helix. Like the t_m, it is destabilised by lowering the salt concentration. This suggests that the same forces maintain both duplex structure and the quasi-circularity. However, heating the nucleoids in the presence of ethidium reveals that forces other than those maintaining the structure of individual duplexes also constrain the DNA. Ethidium stabilises the double helix to denaturation but destabilises the constraint probably by binding and removing the free energy of supercoiling.

We can propose two kinds of constraining mechanism which utilise free energy of supercoiling (Fig. 10). One involves a molecular bridge between two sites on the duplex. The bridge might be a single molecule or part of a larger structure, for example the cage that surrounds the nucleoid. Whatever its precise nature, it must reduce the free energy of supercoiling when it binds to DNA. It must also remain tightly bound to DNA in 2 M NaCl, it must be thermostable and resistant to alkali and probably sensitive to proteases but not ribonucleases. Although proteins with some rather similar properties have recently been implicated in maintaining the circularity of linear molecules of viral DNA [39] there is a second, simpler, alternative. An eye-splice in a rope is maintained solely by interactions between strands. Perhaps specific interactions between identical sequences spaced along one duplex form the duplex into a series of eye-splices [28,40]. The close apposition of two identical duplexes to form a four-stranded structure in which two base pairs are themselves specifically paired (*i.e.* hydrogen-bonded) is stereochemically possible [41]. Pairing between two right-handed double helices is facilitated if the two double helices wind around each other in a right-handed interwound superhelix. Free energy of supercoiling would stabilise such a structure. The splice can be readily made and unmade without breaking covalent bonds by changing the degree of supercoiling in the loop (*i.e.* by changing the free energy of supercoiling so winding and unwinding the splice). Whatever its precise nature, the constraining mechanism in HeLa nucleoids differs from that in bacterial nucleoids where supercoiling is destroyed by ribonuclease or heating to 70 °C [42,43].

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