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 $2.2 \times 10^5$ 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

**Enzymes.** Ribonuclease A (EC 3.1.4.22); ribonuclease T1 (EC 3.1.4.8); trypsin (EC 3.4.21.4); deoxyribonuclease (EC 3.1.4.5).

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
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: [5,6-3H]uridine (48 Ci/mmol), L-[4,5-3H]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 NaClO4 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 T1 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; 45 000 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; 32 000 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 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·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 subtracted 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.

**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 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 × 10⁶/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.

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-irradiation on nucleoids and a circle of DNA of known molecular weight using the same conditions (Fig. 4).

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 × 10⁶/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 logarithm of the surviving fraction of the DNA. 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 × 10⁶ and contains 9850 base pairs [29] the average quasi-circle in a nucleoid contains about 220000 base pairs (150 × 10⁶ 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

<table>
<thead>
<tr>
<th>Ribonuclease</th>
<th>Binding ratio after labelling for 2 h</th>
<th>24 h</th>
<th>Binding ratio after labelling for 24 h</th>
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<tbody>
<tr>
<td></td>
<td>before digestion</td>
<td>after digestion</td>
<td>((^3)H remaining undigested)</td>
</tr>
<tr>
<td>A</td>
<td>0.66</td>
<td>0.65</td>
<td>(9)</td>
</tr>
<tr>
<td>T(_i)</td>
<td>0.66</td>
<td>0.7</td>
<td>(23)</td>
</tr>
<tr>
<td>A and T(_i)</td>
<td></td>
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</tbody>
</table>

believe that nucleoid DNA is encaged by RNA and protein.

Digestion of Nucleoids with Ribonuclease 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 \(^{3}\)HJuridine, and nucleoids were isolated and digested with ribonuclease A or T\(_i\). 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 conditions used, nevertheless did contain a very low level of contamination detectable under different conditions, see Materials and Methods.) Cells were labelled with \(^{3}\)Hleucine, 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).
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for 10 min at 30 °C, the amount of radioactivity insoluble in trichloroacetic acid in one portion of each sample was determined as nucleoids.

added to a final concentration of 8 µg/ml and then containing 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. NaClO₄) 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

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.
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 un-irradiated 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. We first determined the denaturation temperature (tₘ) 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 tₘ 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 tₘ 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 tₘ is increased by high concentrations of salt or ethidium and decreased by 4 M NaClO₄ (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 un-irradiated 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 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 t₁/₂ 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 × 10⁴/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·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 × 10⁴/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 θ₁,₂ and the θₚ 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 θₚ. 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 NaCl.
Table 2. The effect of temperature on supercoiling in nucleoids

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>$t_{1,2}$ of nucleoids</th>
<th>$t_{1,2}$ temperature of nucleoids</th>
<th>$t_m$ of pure DNA</th>
</tr>
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<tbody>
<tr>
<td>2 M NaCl</td>
<td>97</td>
<td>99</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>2 M NaCl + ethidium (8 μg/ml)</td>
<td>80</td>
<td>93</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>0.2 M NaCl</td>
<td>93</td>
<td>94</td>
<td>96</td>
</tr>
<tr>
<td>0.2 M NaCl + ethidium (8 μg/ml)</td>
<td>71</td>
<td>88</td>
<td>98</td>
</tr>
</tbody>
</table>

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

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. 7B and 8B). 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. 7B and 8B).

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...
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 (10⁶/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.

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<th>Solvent</th>
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<tr>
<td></td>
<td>1</td>
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<tr>
<td>Reference</td>
<td>1.00</td>
</tr>
<tr>
<td>Metrizoate</td>
<td>1.09</td>
</tr>
<tr>
<td>(12.5%)</td>
<td>1.20</td>
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<td>(50%)</td>
<td>1.62</td>
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<td>KSCN</td>
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<td>(0.5 M)</td>
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<td>(1.0 M)</td>
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<td>(2.5 M)</td>
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<td>Urea</td>
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<tr>
<td>Sarkosyl</td>
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<td>(0.625%)</td>
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**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 (10⁶/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.

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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 sarkosylin 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, 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-
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].

We thank Professor Henry Harris F. R. S. for his continued support and encouragement. Alan Jones for help with agarose gels and Dr R. Cotter (Searle Research Laboratories, High Wycombe) for kindly supplying us with PM2 DNA.

REFERENCES

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