Eur. J. Biochem. 74, 527–531 (1977)

The Superhelical Density of Nuclear DNA from Human Cells

Peter R. COOK and Iris A. BRAZELL

Sir William Dunn School of Pathology, University of Oxford

(Received October 9, 1976)

Structures resembling nuclei may be released by gently lysing human cells in solutions containing non-ionic detergents and high concentrations of salt. These structures, which we call nucleoids, sediment in sucrose gradients containing the intercalating agent, actinomycin D, in the manner characteristic of superhelical DNA. We have determined the concentration of actinomycin that minimises the rate of sedimentation of nucleoids. At this concentration, we have determined the amount of drug bound per base pair of DNA by means of a double-labelling procedure. Assuming that each molecule of actinomycin bound to nucleoid DNA unwinds the double helix by 26°, we calculate that there is one supercoil every 90–180 base pairs in nucleoid DNA. These values lie within the range found for the circular DNA molecules of plasmids and viruses.

Recent evidence suggests that chromatin is composed of subunits: sections of DNA about 200 base pairs in length are condensed by histones into roughly spherical particles to yield a flexible chain of repeating subunits [1–3]. It is not yet clear how the DNA is folded within the repeating unit. The three-dimensional structure of circular molecules of pure DNA has been described in some detail [4]. The description is based upon a distinction between the right-handed helical turns of the double helix and any superhelical turns that might be superimposed upon the duplex turns. The superhelical turns are lost spontaneously when phospho-diester bonds are broken in either of the backbone strands of the double helix. We have recently shown that the DNA of higher cells is supercoiled, like the circular DNA of viruses [5,6]. We have now determined the degree of supercoiling in the nuclear DNA of human cells.

When intercalating agents (*e.g.* ethidium bromide, actinomycin D) bind to an intact and circular molecule of DNA, the right-handed double helix is unwound and the number of superhelical turns is altered. The conformational changes associated with intercalation are reflected by alterations in the sedimentation coefficient of DNA. Intact circles of supercoiled DNA are compact and sediment more rapidly than their broken counterparts which have lost supercoils and so have become extended. The sedimentation coefficient of broken or nicked DNA changes little in the presence of increasing concentrations of intercalating agent, whereas that of supercoiled DNA changes dramatically. Initially intercalation removes supercoils; the now extended molecule sediments slowly like the nicked molecule. At high concentrations of intercalating agent, supercoils of the opposite sense to those initially present compact the molecule so that the sedimentation coefficient of the intact circle increases. The concentration of intercalating ligand which minimises the sedimentation coefficient of the intact circle gives an indication of the degree of supercoiling. The superhelical density (σ), the number of superhelical turns (τ) per 10 base pairs, may be determined using the relation

$$\sigma = -\frac{10\,\phi}{\pi}\,v$$

where ϕ is the angle in radians by which the double helix is unwound per bound intercalating molecule and v is the number of moles of intercalating agent bound per mole of nucleotide at the critical concentration at which intercalation has removed all superhelical turns [4,7,8]. The unwinding angle, ϕ, for the intercalating agents ethidium and actinomycin was until recently taken to be 12° [9,10] but now it is considered to be about twice as large [11–13].

Structures resembling nuclei may be released by gently lysing a wide variety of cells in solutions containing non-ionic detergents and high concentrations of salt [5,6,14]. These structures, which we call nucleoids, sediment in gradients containing intercalating agents in the manner characteristic of DNA that is intact, supercoiled and circular. Actinomycin D is an intercalating agent suitable for binding studies involving nucleoids. Nucleoids contain RNA [14a]

but actinomycin, unlike ethidium, binds very weakly to RNA [15–17]. We have determined the concentration of actinomycin D that reduces the rate of sedimentation of human nucleoids to a minimum. Using a double-labelling procedure we then determined the amount of drug bound per mole of nucleotide.

MATERIALS AND METHODS

Actinomycin C_1 (D) was obtained from Boehringer Mannheim GmbH. All solutions containing actinomycin D were kept in the dark as far as possible. Actinomycin concentrations were calculated assuming a molecular weight of 1255 and a molar absorption coefficient (at 441 nm) in 0.1 M phosphate buffer (pH 7.0) of 24600 M^{-1} cm^{-1}.

Human HeLa cells were grown in suspension as described [5].

Nucleoid conformation was analysed in isokinetic sucrose gradients [6]. Sucrose gradients (15–30% sucrose 4.6 ml; pH 8.0) contained 1.95 M sodium chloride, 0.01 M Tris and 0.001 M EDTA in addition to variable amounts of actinomycin D. 150 µl of lysis mixture (1.95 M NaCl) were layered on top of the gradient, followed by 50 µl of phosphate-buffered saline containing about 2×10^5 HeLa cells. The lysis mixture contained sodium chloride, EDTA, Tris and Triton X-100 to give final concentrations in the 200 µl on top of the gradient of 1.95 M, 0.1 M, 2 mM and 0.5% respectively. 15 min after the addition of the cells to the gradient, the tubes were spun (20 °C, SW 50.1 rotor, Beckman L2-65b ultracentrifuge) for 50 min at 5000 rev./min. The position of the nucleoids in the gradient was determined from their absorbance at 254 nm [5]. Six gradients were usually spun together in one rotor and at least one gradient of the six served as a reference and lacked actinomycin. The distances travelled by nucleoids in other tubes were expressed as ratios relative to that by nucleoids in the reference tube.

Labelled and unlabelled nucleoids were isolated in 'step' gradients and the conformation of the isolated nucleoids examined in isokinetic sucrose gradients containing 1.0 M NaCl and either 2 or 16 µg/ml ethidium bromide [14]. The isolated nucleoids in 1.95 M NaCl were diluted to 1.0 M NaCl with 10 mM Tris (pH 8.0) and 200-µl aliquots containing 5×10^5 nucleoids applied to isokinetic sucrose gradients (15–30%; 4.6 ml; pH 8.0) containing 1.0 M NaCl and 2 or 16 µg/ml ethidium bromide. Gradients were spun at 5000 rev./min for 25 min and the position of the nucleoids in the gradient determined as before.

DNA was purified from cells labelled with [^{14}C]-thymidine by first isolating nucleoids in 'step' gradients containing 1.95 M NaCl [14]. The salt concentration was reduced to 0.2 M by the addition of 10 mM Tris (pH 8.0) and nucleoid structure destroyed by adding sodium dodecyl sulphate to a final concentration of 0.2%. After 25 min at room temperature, the viscous solution of DNA was sheared by passing it ten times through a 19G needle. The solution was cooled to 0 °C and the precipitated sodium dodecyl sulphate removed by low-speed centrifugation. The density of the solution was adjusted to 1.7 g/cm^3 with solid caesium chloride and the DNA isolated by banding it in density gradients (3 ml) formed by spinning for 60 h at 35000 rev./min at 20 °C in the SW 50.1 rotor in a Beckman L2-65b ultracentrifuge. The gradients were fractionated, and fractions containing ^{14}C were pooled and dialysed at 4 °C against four changes of 10 mM Tris (pH 8.0), 0.1 M NaCl. The dialysate, which contained DNA of molecular weight $30-40 \times 10^6$ (determined by electrophoresis in agarose gels) had an absorbance at 260 nm 1.8 times greater than that at 280 nm.

Radioactive materials were obtained from the Radiochemical Centre, Amersham: [*methyl*-^{14}C]thymidine (59 Ci/mol); [^{3}H]actinomycin D (3300 Ci/mol). Procedures for fractionating gradients and for determining the radioactive content of the fractions insoluble in trichloroacetic acid have been described [14]. The radioactive content of fractions containing both ^{3}H and ^{14}C was also determined after oxidising the samples to carbon dioxide and water in a sample oxidiser (Packard). The specific activity of the DNA of HeLa cells grown in [^{14}C]thymidine for one generation was calculated assuming that each cell contained 11.9 pg DNA [14a]. Cell numbers were determined in a Coulter counter and their radioactive content determined after oxidising the samples.

RESULTS

We previously studied the sedimentation of HeLa nucleoids in sucrose gradients containing actinomycin D and 1.0 M NaCl [5]. Some histone remains bound to the nucleoids in 1.0 M NaCl but not in 1.95 M NaCl [14]. We chose to use 1.95 M NaCl in the present study since histones are unwinding agents [18] and their presence would therefore complicate the analysis. Actinomycin D affected the sedimentation of HeLa nucleoids in 1.95 M NaCl much as it did in 1.0 M NaCl (Fig. 1). As the concentration of the drug in the gradient increased, the distance travelled fell to a minimum and then increased again. The sedimentation rate was smallest in the presence of 2–4 µg/ml actinomycin D: in subsequent experiments we used gradients containing 3.5 µg/ml actinomycin.

The amount of DNA in the gradients which binds actinomycin D was determined with cells labelled with

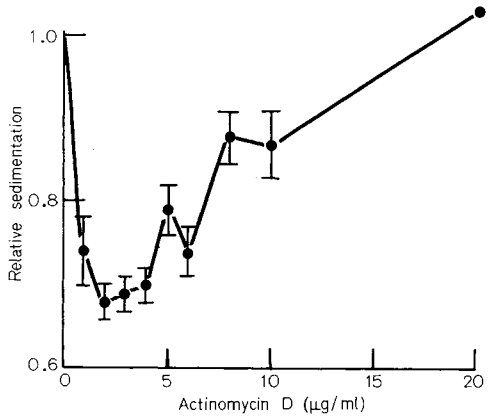

Fig. 1. *The effect of actinomycin D on the sedimentation of HeLa nucleoids.* The distance sedimented by nucleoids in isokinetic sucrose gradients (4.6 ml, 15–30% sucrose, pH 8.0) containing 1.95 M NaCl and different concentrations of actinomycin D is expressed as a ratio relative to nucleoids sedimenting in the absence of the drug. Error bars give the standard error of the mean

Table 1. *Labelling nucleoids with [^{14}C]thymidine affects their conformation*

HeLa cells were grown for 24 h in [^{14}C]thymidine (0.05 μCi/ml). Nucleoids were prepared from these cells and from other unlabelled cells using 'step' gradients and their conformation analysed in sucrose gradients containing ethidium bromide (see Materials and Methods). The distance sedimented by the nucleoids is expressed as a ratio relative to that sedimented by the unlabelled nucleoids in gradients containing 2 μg/ml ethidium bromide (EtdBr)

Nucleoids	Relative sedimentation in EtdBr	
	2 μg/ml	16 μg/ml
Unlabelled	1.0	1.56
^{14}C-labelled	0.86	1.37

[^{14}C]thymidine. This approach is not entirely satisfactory since the labelling alters the conformation of the nucleoids. (Presumably radioactive decay breaks nucleoid DNA and thus releases supercoils.) This effect is illustrated in Table 1. The conformation of labelled nucleoids was compared with that of unlabelled nucleoids using an assay described previously [14]. Like nucleoids containing DNA broken by γ-irradiation the ^{14}C-labelled nucleoids sediment slightly more slowly than untreated nucleoids [14]: nevertheless they sediment at least 1.5 times further in the presence of 16 μg/ml ethidium than they do in the presence of 2 μg/ml indicating that although the labelling slightly alters the conformation of the DNA in the nucleoids, the alterations are small.

The binding of [^{3}H]actinomycin D to nucleoids labelled with [^{14}C]thymidine is illustrated in Fig. 2. Labelled cells were applied to sucrose gradients containing [^{3}H]actinomycin distributed uniformly

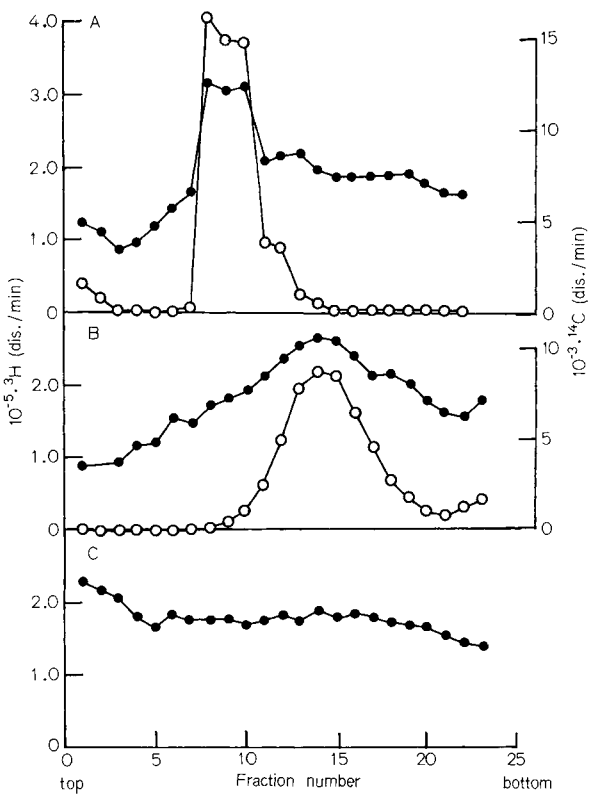

Fig. 2. *Binding [^{3}H]actinomycin D to [^{14}C]DNA.* HeLa cells were grown for 24 h in [^{14}C]thymidine (0.05 μCi/ml); the cells were washed three times in phosphate-buffered saline, resuspended, counted and their radioactive content insoluble in trichloroacetic acid determined: (●) ^{3}H; (○) ^{14}C. (A) Binding of [^{3}H]actinomycin D to ^{14}C-labelled nucleoids. 50-μl aliquots containing about 2.5×10^{5} cells were added to 150 μl lysis mixture (1.95 M NaCl) floating on a sucrose gradient (15–30% sucrose, 4.6 ml, pH 8.0) containing 1.95 M NaCl. Both the lysis mixture (1.95 M NaCl) and the sucrose gradient contained [^{3}H]actinomycin D (3.5 μg/ml, 1.33 μCi/ml). 15 min after the addition of cells to the lysis mixture, gradients were spun and then 6-drop fractions were collected from the gradient and the radioactive content of 50-μl aliquots from each fraction determined directly or after sample oxidation. (B) Binding of [^{3}H]actinomycin D to [^{14}C]DNA. DNA was purified from the ^{14}C-labelled cells used in (A) as described in Materials and Methods. 50-μl aliquots of the dialysed sample of DNA which contained about the same amount of ^{14}C as that in the 50-μl aliquots applied to gradients in (A), were applied to sucrose gradients containing [^{3}H]actinomycin D and overlaid with lysis mixture (1.95 M NaCl) as in (A). Gradients were spun at 20000 rev./min for 16 h at 20 °C in the SW 50.1 rotor. 6-drop fractions were collected and the radioactive content of aliquots determined as before. (C) The content of [^{3}H]actinomycin D in the gradients. Control gradients, to which no [^{14}C]DNA was added, were included in each experiment. These gradients were spun, fractionated and analysed as above

throughout the gradient (Fig. 2C). After the gradient had been spun, the [^{3}H]actinomycin is reduced in amount at the top of the gradient and concentrated in the region containing the nucleoids (Fig. 2A). The nucleoids (labelled with ^{14}C), as they pass down the gradient, bind actinomycin thus reducing the concentration at the top (Fig. 2A). The extra actinomycin

Table 2. *The superhelical density of nucleoid DNA*
Cells were grown for 24 h in [^{14}C]thymidine and then applied to sucrose gradients containing 3.5 μg/ml [^{3}H]actinomycin. The gradients were spun, fractionated and the radioactive content in aliquots from each fraction determined. (Gradients in experiments 1 and 2, 3 and 4–7 were spun at 5000, 6000 and 7500 rev./min respectively). The superhelical density is calculated assuming that each molecule of actinomycin D bound to the nucleoids unwinds the double helix by 26°

Expt	[^{3}H]Actinomycin/[^{14}C]DNA	[^{14}C]Radioactivity applied to gradient	$10^{-4}\times$ Number of cells applied to gradient	Binding ratio (v)	Superhelical density (σ)	Number of base pairs per supercoil
	dis. min^{-1}/dis. min^{-1}	dis./min				
1	22	38000	34	0.061	−0.088	110
2	18	45000	28	0.077	−0.11	91
3	13	50000	34	0.049	−0.071	140
4	11	88000	35	0.070	−0.10	100
5	22	58000	41	0.082	−0.12	83
6	11	66000	28	0.068	−0.099	100
7	14	74000	37	0.076	−0.11	91
			Mean	0.069	−0.10	103
			Range	(0.082 to 0.049)	(−0.12 to −0.07)	(83 to 140)

found in the fractions containing the nucleoids can be expressed as the extra radioactivity due to ^{3}H relative to the radioactivity of [^{14}C]DNA (Table 2). Nucleoids do not sediment very far into the gradients when the gradients are spun at 5000 rev./min (Fig. 2A). However, if the gradients are spun faster (at 6000 or 7500 rev./min) the nucleoids sediment further. This does not affect the [^{3}H]actinomycin/[^{14}C]DNA ratio (Table 2). The results of seven experiments are summarised in Table 2.

In order to calculate the superhelical density of nucleoid DNA we assume that every molecule of actinomycin that binds to the nucleoids contributes to the unwinding of the double helix. However, nucleoids are complex structures: they contain RNA and protein [14, 14a] so that some of the actinomycin D might be bound to constituents of nucleoids other than DNA. Moreover, some regions of nucleoid DNA might be inaccessible to the drug. We therefore compared the binding of actinomycin to pure DNA and to nucleoids containing an equivalent amount of DNA. Cells were grown in [^{14}C]thymidine and divided into two samples. One was applied to a sucrose gradient containing [^{3}H]actinomycin and the ratio of extra ^{3}H radioactivity to ^{14}C radioactivity determined as described above (Fig. 2A). DNA in the other sample was purified and the actinomycin-binding capacity of the pure DNA determined in a sucrose gradient containing [^{3}H]actinomycin under conditions almost identical to those used for the nucleoids (Fig. 2B). Since the DNA (molecular weight $\approx 30-40\times10^{6}$) sediments so much more slowly than the nucleoids, gradients containing pure DNA were spun much faster for longer times. The DNA (labelled with ^{14}C) sediments as a broad peak and binds actinomycin. As the [^{14}C]DNA samples used in the experiments illustrated in Fig. 2A and 2B had the same specific activity, their relative capacity to bind the drug may be compared. In three such comparisons, we found that nucleoids bound 93%, 96% and 82% of the actinomycin bound to DNA. The simplest interpretation of this comparison is that nucleoids and pure DNA possess roughly similar numbers of binding sites.

DISCUSSION

Intercalating agents affect differently the sedimentation of nucleoids and intact circles of pure DNA. Intercalation decreases the sedimentation rate of intact and circular DNA to a minimum rate which is indistinguishable from the rate of nicked circles sedimenting under the same conditions. This is not so for nucleoids; the minimum sedimentation rate of unirradiated nucleoids is greater than that of their γ-irradiated counterparts that have lost their supercoils. We have argued elsewhere [5] that there might be regions of DNA in the nucleoids with differing superhelical densities so that at a level of intercalation that reduced the number of superhelical turns in one region to zero other regions might remain supercoiled. The rate of sedimentation of the nucleoid would then reflect the 'average' conformation of all the different regions so that we would then calculate an 'average' superhelical density.

We assume that equilibrium is established in the gradients between free and bound actinomycin. Although actinomycin complexes slowly with pure DNA, much of the binding occurs within the time used in our experiments [19]. Furthermore, we assume that the labelling of nucleoid DNA does not alter its

supercoiling (Table 1). As high salt concentrations suppress the non-intercalative binding of other intercalating ligands [20,21], and as nucleoids and pure DNA have roughly the same number of binding sites we also assume that all the actinomycin bound to nucleoids in 1.95 M NaCl is intercalated.

The average superhelical density, calculated from data obtained from seven different experiments (Table 2), corresponds to one supercoil every 103 base pairs of DNA. A range of drug concentrations (*i.e.* 2–4 μg/ml) minimises the rate of sedimentation of nucleoids. For trivial reasons we chose to use a concentration of 3.5 μg/ml in our experiments. We can evaluate the effect of our choice since, under non-saturating conditions, actinomycin is bound to pure DNA in rough proportion to the concentration of the free drug [19]. The limits to our estimate then become one supercoil every 90–180 base pairs (at 4 and 2 μg/ml actinomycin respectively). These values lie within the range found for the circular DNA molecules of plasmids and viruses [22].

Breaks in DNA induced by γ-rays are readily repaired *in vivo*; repair restores the original superhelical configuration [5,23]. We have argued elsewhere [5,14] that this suggests that the torsional energy of supercoiling present in nucleoid DNA in 1.95 M NaCl is dissipated *in vivo*. (Both temperature and the concentration of salts will affect the degree of supercoiling [13] so that the superhelical density of nucleoid DNA under physiological conditions will be about half that in 1.95 M NaCl at 20 °C.) The histones may be the agents that dissipate the free energy of supercoiling and unwind the DNA [18]. When histones, and perhaps other nuclear proteins, are removed from the DNA during the formation of nucleoids, the double helix spontaneously increases its number of turns. As free rotation about the ends of stretches of nucleoid DNA is restricted, superhelical turns are necessarily formed. The conformation of the DNA in chromatin therefore resembles that of the 'mini-chromosome' formed from the circular DNA of Simian virus 40 and cellular histones [3,13,18]. *In vivo*, the double helix is unwound by about 0.5–1 turn in each chromatin sub-unit.

We thank Professor H. Harris F.R.S. for his continued support and encouragement and A. Jones for determining the sizes of samples of DNA.

REFERENCES

1. Kornberg, R. D. (1974) *Science (Wash. D.C.) 184*, 868–871.
2. Senior, M. B., Olins, A. L. & Olins, D. E. (1975) *Science (Wash. D.C.) 187*, 173–175.
3. Griffith, J. D. (1975) *Science (Wash. D.C.) 187*, 1202–1203.
4. Bauer, W. & Vinograd, J. (1968) *J. Mol. Biol. 33*, 141–171.
5. Cook, P. R. & Brazell, I. A. (1975) *J. Cell Sci. 19*, 261–279.
6. Cook, P. R. & Brazell, I. A. (1976) *J. Cell Sci. 22*, 287–302.
7. Bauer, W. & Vinograd, J. (1970) *J. Mol. Biol. 47*, 419–435.
8. Upholt, W. B., Gray, H. B. & Vinograd, J. (1971) *J. Mol. Biol. 61*, 21–38.
9. Fuller, W. & Waring, M. T. (1964) *Ber. Bunsenges Phys. Chem. 68*, 805–808.
10. Waring, M. J. (1970) *J. Mol. Biol. 54*, 247–279.
11. Wang, J. C. (1974) *J. Mol. Biol. 89*, 783–801.
12. Pulleyblank, D. E. & Morgan, A. R. (1975) *J. Mol. Biol. 91*, 1–13.
13. Shure, M. & Vinograd, J. (1976) *Cell, 8*, 215–226.
14. Cook, P. R., Brazell, I. A. & Jost, E. (1976) *J. Cell Sci. 22*, 303–324.
14a. Colman, A. C. & Cook, P. R. (1977) *Eur. J. Biochem.* in the press.
15. Waring, M. J. (1965) *J. Mol. Biol. 13*, 269–282.
16. Hamilton, L. D., Fuller, W. & Reich, E. (1963) *Nature (Lond.) 198*, 538–540.
17. Haselkorn, R. (1963) *Science (Wash. D.C.) 143*, 682–684.
18. Germond, J. E., Hirt, B., Oudet, P., Gross-Bellard, M. & Chambon, P. (1975) *Proc. Natl Acad. Sci. U.S.A. 72*, 1843–1847.
19. Müller, W. & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251–290.
20. Peacocke, A. R. & Skerrett, J. N. H. (1956) *Trans. Faraday Soc. 52*, 261–279.
21. Li, H. J. & Crothers, D. M. (1969) *J. Mol. Biol. 39*, 461–477.
22. Gray, H. B., Upholt, W. B. & Vinograd, J. (1971) *J. Mol. Biol. 62*, 1–19.
23. Cook, P. R. & Brazell, I. A. (1976) *Nature (Lond.) 263*, 679–682.

P. R. Cook and I. A. Brazell, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, Great Britain, OX1 3RE