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SUPERCOILS IN HUMAN DNA

P. R. COOK AND I. A. BRAZELL
Sir William Dunn School of Pathology,
South Parks Road, Oxford, England

SUMMARY

The three-dimensional structure of a double-stranded DNA molecule may be described by distinguishing the helical turns of the DNA duplex from any superhelical turns that might be superimposed upon the duplex turns. There are characteristic changes in the hydrodynamic properties of superhelical DNA molecules when they interact with intercalating agents. The hydrodynamic properties of nuclear structures released by gently lysing human cells are changed by intercalating agents in this characteristic manner. The characteristic changes are abolished by irradiating the cells with γ-rays but may be restored by incubating the cells at 37 °C after irradiation. These results are interpreted as showing that human DNA is supercoiled. A model for the structure of the chromosome is suggested.

INTRODUCTION

The three-dimensional structure of a double-stranded molecule of DNA may be described by distinguishing the helical turns of the DNA duplex from any superhelical turns that might be superimposed upon the duplex turns. This distinction has proved useful in interpreting the effects of the intercalating dye ethidium bromide on the hydrodynamic properties of DNA (Bauer & Vinograd, 1968, 1970; Wang, 1969). Circular molecules of DNA in which the duplex strands are covalently continuous are found in a variety of viruses, in *Escherichia coli* and in the mitochondria of higher cells. Studies *in vitro* on the binding of intercalating dyes to these molecules have shown that all, when intact, contain superhelical turns. The superhelical turns are lost spontaneously when phospho-diester bonds are broken in either of the backbone strands of the duplex so that linear derivatives of these circular DNA molecules, or those bearing one single strand scission or nick, contain no superhelical turns. It has been suggested that superhelical turns might be a general feature of the DNA of higher organisms and that they might play a role in controlling gene expression (Cook, 1973, 1974). The present paper shows that the hydrodynamic properties of nuclear structures released by gently lysing human cells are similar to those of circular DNA molecules that contain superhelical turns. Irradiation of DNA with γ-rays is known to induce single-strand breaks and if the cells are irradiated after lysis the nuclear structures no longer behave as if they contained superhelical DNA; instead they behave like nicked DNA. These results are interpreted as showing that human DNA is supercoiled. A model for the structure of the chromosome is suggested. A supercoil model for nucleohistone has been proposed based upon X-ray diffraction data (Pardon & Wilkins, 1972).

Superhelical turns are maintained in closed and circular DNA molecules by the chemical forces that hold the duplex together (Vinograd *et al.* 1965). Topological considerations alone require that superhelical turns cannot be added or removed without either simultaneously changing the average number of base pairs per duplex turn or by breaking sugar-phosphate bonds. Expressed formally

$$\alpha = \beta + \tau,$$

where α (the topological winding number) is the number of complete revolutions made by one strand about the duplex axis when the axis is constrained to lie in a plane. α is constant in the absence of strand scission. It is convenient to regard α as being composed of 2 winding numbers, β and τ, although higher order windings are possible. β (the duplex winding number) is defined as the number of revolutions made by either strand about the duplex axis in the unconstrained molecule; τ (the superhelix winding number) is equal to the number of revolutions made by the duplex axis about the superhelix axis in the unconstrained molecule. The superhelix density, σ, is an intensive quantity equal to the number of superhelical turns per 10 base pairs. By convention, right hand duplex turns are considered to be positive: when naturally occurring and circular DNA molecules have been examined *in vitro*, all have been found to be characterized by $\tau < 0$, i.e. the superhelical turns in the non-interwound molecule are left-handed (cf. Bauer & Vinograd, 1968, for a discussion of the sign convention). Since α is a constant, changes in τ and β are related by

$$\Delta\tau = -\Delta\beta.$$

It is not known whether or not $\tau < 0$ *in vivo*, since the conformation of the duplex *in vivo* (and so β and hence τ) might be different from that *in vitro*.

When the intercalating dye, ethidium bromide, binds *in vitro* to an intact circle of DNA the helical DNA duplex is unwound (i.e. β decreases; Wang, 1974) and in consequence τ increases. Intercalation of increasing amounts of dye causes a continuous and reversible reduction in β so that τ, which in the absence of dye is less than 0, increases to zero and then becomes positive (i.e. superhelical turns of opposite sense appear). The conformational changes associated with the binding of the dye are reflected by changes in the sedimentation coefficient of DNA. Intact DNA molecules which are circular and supercoiled, being compact, sediment more rapidly than their nicked counterparts having no supercoils. The sedimentation coefficient of nicked DNA changes little in the presence of increasing concentrations of ethidium, whereas that of supercoiled DNA changes dramatically. It first decreases by about a quarter to a minimum value, which is indistinguishable from that of nicked circles at a similar dye concentration. At this equivalence value, the molecules contain no superhelical turns (i.e. $\tau = 0$). With further intercalation, the sedimentation coefficient of the intact form increases as supercoils of the opposite sense appear (τ becomes positive, i.e. in the non-interwound molecule, the superhelix is right-handed) and the molecule becomes more compact again. The concentration of ethidium at which the sedimentation coefficient of the intact circle is a minimum, gives an indication of the superhelix density, and, if the length of the molecule is known,

the number of superhelical turns in the molecule (Bauer & Vinograd, 1968; Upholt, Gray & Vinograd, 1971): the more left-handed superhelical turns there are in the non-interwound molecule, the greater the concentration of ethidium required to produce the minimum sedimentation coefficient. At this minimum point, intercalation has led to unwinding of the duplex so that all superhelical turns have been removed. When one ethidium molecule intercalates the duplex is unwound by about 26–28° (Wang, 1974) and the product of the number of moles bound and this unwinding angle is related to the number of superhelical turns. Intercalation of a variety of agents (e.g. aminoacridines, propidium di-iodide, actinomycin D) causes the sedimentation coefficient of supercoiled DNA to vary in the biphasic manner, whereas non-intercalating compounds (e.g. streptomycin sulphate, berenil) have little effect (Waring, 1970).

If, in the presence of intercalating agents the sedimentation coefficient of DNA varies in this biphasic manner, and if nicking agents abolish the biphasic response, it can be inferred that the DNA is subject to topological restraints that prevent free rotation of one strand of the duplex about the other. During the course of a study on the sedimentation of chromatin through sucrose gradients containing ethidium, it was found that the velocity of sedimentation of nuclei varied in this manner.

MATERIALS AND METHODS

Cells

Human HeLa cells, obtained initially from Dr R. T. Johnson (Department of Zoology, Cambridge), were grown as suspension cultures at 37 °C in minimal essential medium supplemented with 2·5 % foetal calf serum, non-essential amino acids (all from Biocult, Glasgow), sodium pyruvate (2 mM) and the antibiotics neomycin (30 μg/ml), kanamycin (30 μg/ml) and streptomycin (200 μg/ml). Cultures were diluted with an equal volume of fresh medium daily. The same cells were grown as monolayers under the conditions described by Cook (1975). Samples of cells for lysis were prepared by spinning down cells in logarithmic growth, washing them in phosphate-buffered saline and resuspending them in phosphate-buffered saline at room temperature.

Cells at a concentration of about 4×10^5/ml were labelled for 24 h by the addition of [*Me*-^{3}H]-thymidine (The Radiochemical Centre, Amersham, Bucks; 56 Ci/mmol) or L-[4,5-^{3}H]leucine (The Radiochemical Centre; 53 Ci/mmol) to a final concentration of 0·5 or 5 μCi/ml, respectively.

Chemicals

Chemicals were obtained from the following sources: sucrose, RNase free – Cambrian Chemicals Ltd., Croydon, Surrey; ethidium bromide (3,8-diamino-6-phenyl-5-ethylphenanthridium bromide) – Sigma, Kingston upon Thames, Surrey; propidium di-iodide (3,8-diamino-5-diethylmethyl-aminopropyl-6-phenylphenanthridinium di-iodide) – Calbiochem Ltd, London; actinomycin D – Merck, Sharpe and Dohme Research Lab., West Point, Pa., U.S.A.; streptomycin sulphate (streptomycin sesquisulphate) – Glaxo Laboratories Ltd, Greenford, Middlesex; berenil (4,4′-diazoamino-dibenzamidine diaceturate), a gift from Dr H. Loewe from Farbwerke Hoechst, Frankfurt-am-Main, Germany; the dye Hoechst 33258 ([2-[2-(4-hydroxy-phenyl)-6-benzimadazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol 3HCl]), a gift from Dr H. Loewe; dexamethosone (9α-fluoro-16α-methyl prednisolone acetate) Sigma; Triton X-100 – BDH Chemicals Ltd, Poole, Dorset.

Sucrose gradients

Sucrose gradients (4·6 ml) were prepared in centrifuge tubes for the Spinco SW 50·1 rotor by the method of Noll (1967). All manipulations were carried out at room temperature using sterile solutions. Sucrose gradients (15–30%; pH 8·0) generally contained sodium chloride (1 M), tris-(hydroxymethyl)-amino-methane (0·01 M), ethylenediaminetetra-acetic acid (0·001 M) in addition to variable amounts of ethidium or other agents; 150 μl of lysis mixture (pH 8·0) were layered carefully on to the top of the gradient, followed by 50 μl of phosphate-buffered saline containing between 1 and 5×10^5 cells. The lysis mixture contained sodium chloride, ethylenediamine-tetra-acetic acid, tris-(hydroxymethyl)-amino-methane and Triton X-100 to give final concentrations in the 200 μl on top of the gradient of 1 M, 0·1 M, 2 mM and 0·5%, respectively. Fifteen minutes after the addition of cells to the gradient, tubes were spun at 20 °C in the SW 50·1 rotor in a Beckman L2-65b centrifuge at 5000 rev/min. Within 20 s, the rotor reached 5000 rev/min and 25 or 80 min after the beginning of the spin the rotor was allowed to coast to a halt without braking. The rotor revolved 130000 and 405000 times during the 25- and 80-min spins, respectively. Gradients were analysed immediately using an Isco density gradient fractionator (Shandon Southern Instruments Ltd, Camberley, Surrey) to pass the gradient upwards through an absorbance monitor (Isco) operating at a wavelength of 254 nm. Light scattering contributed to the optical density.

The position of the nuclei in the gradient was indicated by a peak in the $O.D._{254}$ trace – this was confirmed by phase-contrast microscopy. The size of the peak obtained with unirradiated nuclei in gradients containing no ethidium was always smaller than that obtained with irradiated nuclei or in the presence of ethidium. This might have resulted from variations in light scattering. The distance the nuclei travelled down the gradient was determined by measuring the distance on the trace from the meniscus to peak. In many experiments the optical density trace revealed some heterogeneity in sedimentation of nuclei, and in this case measurements were made from the vertical line at the median value which divides the area under the optical density trace into halves. This heterogeneity perhaps arose due to variations in size of nuclei at different stages in the cell cycle. In all experiments, one tube of the six in the SW 50·1 rotor contained nuclei which served as a reference and the distances travelled by nuclei in the other tubes were expressed as a ratio relative to this tube. Two kinds of reference tubes (containing 0 or 5 μg/ml ethidium in the gradient) were used. Initially tubes were spun for 25 min and the reference tube contained unirradiated cells and no ethidium. However, nuclei subjected to 9·6 J kg^{-1} of γ-irradiation travelled a little way down the gradient and not far enough for accurate resolution of their optical density peak from that of the lysis mixture at the top of the gradient (cf. Fig. 8; the experimental scatter of data increases at doses above 9·6 J kg^{-1}). For this reason, spins of 80 min were also used with a reference tube containing unirradiated cells and 5 μg/ml ethidium in the gradient. Data from both 25- and 80-min spins are presented in some cases in the same graph and where this is so a ratio of 1·0 obtained with an 80-min spin is aligned on the ordinate with the average ratio obtained with unirradiated nuclei spun for 25 min in 5 μg/ml ethidium bromide (i.e., a ratio of 0·47). The scale of the ordinate referring to 80-min spins is half that referring to 25-min spins. This mode of presentation allows a rough extrapolation of the ordinate scale beyond the observed ratios and permits some comparability between the ratios obtained in the 25- and 80-min spins. It should be stressed, however, that nuclei did not behave as ideal particles in the 'isokinetic' gradients (Noll, 1967) so that such comparisons are necessarily inexact.

Gradients containing radioactive material were analysed by allowing fractions of 6 drops to fall, after passing through the ultraviolet-monitor, on to 2·1-cm glass-fibre disks (Whatman, GF/A) carried on an Ultrorac 7000 fraction collector (LKB-Produkter AB, Sweden). The disks were allowed to dry, submerged successively in tanks of 5% trichloroacetic acid and ethanol, dried, placed in a glass vial containing 2·5 ml Unisolve 1 (Koch-Light, Colnbrook, Bucks) and counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 3390 with absolute activity analyser).

The following control experiments showed that the bulk of the nuclei applied to the gradient co-sedimented with the optical density peak. Cells labelled with tritiated thymidine were applied in the usual way to gradients that had been underlaid with 0·5 ml of a very dense solu-

tion of 60% sucrose saturated with caesium chloride. This underlay prevents any rapidly sedimenting material from reaching the bottom of the centrifuge tube. Fig. 1 shows the optical density and radioactive profiles of such gradients. The bulk of thymidine-labelled material co-sediments with the nuclei, although some material always sediments on to the underlay and a much lesser amount (about 5%) remains at the top of the gradient. When [^{3}H]leucine was used as a label, little label co-sedimented with the nuclei, most (about 70–80%) remaining at the top of the gradient. Subjecting the cells to irradiation or the presence of different amounts of ethidium in the gradient did not grossly influence the proportions of radioactive material recoverable in the optical density peak (Table 1, p. 267).

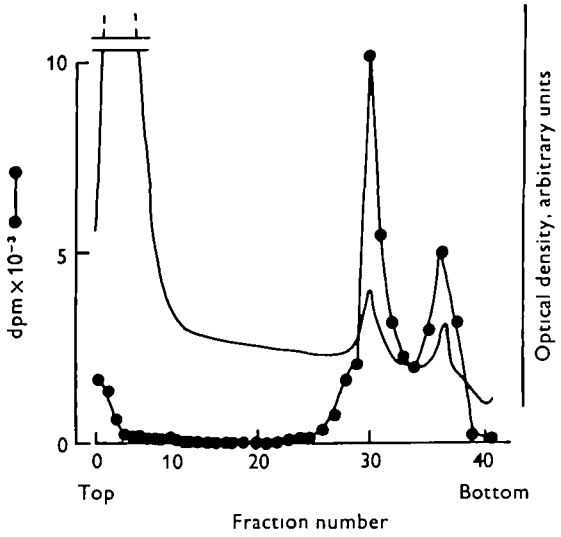

Fig. 1. Optical density and radioactivity profiles of a sucrose density gradient. Thymidine-labelled cells were applied to the gradient which contained an underlay of 60% sucrose saturated with caesium chloride, spun at 5000 rev/min for 25 min, and optical density and radioactivity profiles determined. Conditions were those described in Materials and methods. There are 3 peaks in the optical density profile: at the top of the gradient the lysis mixture absorbs strongly, near the bottom some rapidly sedimenting material was caught on the caesium shelf, and in between the two is the peak due to the nuclei. The bulk of the labelled thymidine co-sediments with the middle peak.

Caesium chloride density gradients

Three millilitres of a 2:3 (w/v) solution of caesium chloride in distilled water in a centrifuge tube were overlayed with 150 μl lysis mixture followed by 50 μl of phosphate-buffered saline containing cells. After 15 min the tube was spun at 38000 rev/min for 48 h in the SW 50.1 rotor on a Beckman L2-65b ultracentrifuge to establish the density gradient. Densities were determined using an Abbé refractometer.

γ irradiation

A Gamma Cell 40 (Atomic Energy of Canada Ltd) containing 2 caesium 137 sources in opposition was used to deliver 1·2 J kg^{-1} min^{-1} over a uniform field. For measurement of the sedimentation properties of γ-irradiated nuclei, nuclei were irradiated at room temperature in the lysis mixture on top of the sucrose gradient immediately after the addition of cells to the lysis mixture. As usual, spinning was begun 15 min after the addition of the whole cells to the lysis mixture. In the lysis mixture little, if any, repair took place.

The effect of irradiation on the colony-forming ability of single HeLa cells was studied by irradiating, in suspension, defined numbers of HeLa cells that had hitherto been grown as a monolayer culture. Cells in 5 ml of medium contained in bottles (Falcon Plastics) were irradiated for different periods, then allowed to attach and grow, and 12 days later colonies were fixed, stained and counted. The cloning efficiency of unirradiated cells varied between 15 and 20% in different experiments. The number of colonies arising from unirradiated cells was defined as 1 and numbers from irradiated cells expressed as a fraction of this.

Recovery of nuclei following γ irradiation was investigated by irradiating cells in growth medium and then subsequently incubating them for different periods of time at 4 or 37 °C before mixing them with 5 volumes of ice-cold phosphate-buffered saline. Cells were then spun down at 4 °C, resuspended in chilled phosphate-buffered saline and applied to gradients and spun as usual. These manipulations took about 15 min during which some recovery took place (compare Figs. 8 and 11).

RESULTS

Conditions for lysis

The experiments reported below resulted from a study on supercoiling in chromatin, and this study required a method for gently lysing cells and nuclei to release chromatin without applying excessive shearing forces that would break DNA, so leading to loss of supercoils. A wide range of ionic and non-ionic detergents (e.g. sodium dodecylsulphate, sarkosyl, cetyltriethyl ammonium chloride, sodium deoxycholate, Triton X-100, Triton N-101, Triton WR 1339, Brij, Nonidet P-40, Tween-80, Lubrol W) and phospholipases (A and D) was screened to see whether these agents would lyse cells and nuclei. Lysis of cells was monitored by phase-contrast microscopy. Although the cell membrane was easily lysed, only the ionic detergents proved effective in lysing nuclei. These, however, are known to eliminate supercoiling from the chromosomes of *Escherichia coli* (Worcel & Burgi, 1972). Although a satisfactory lysis mixture has been obtained that releases supercoiled DNA-protein complexes from cells and nuclei (Brazell & Cook, unpublished), it seemed likely that the sedimentation coefficient of nuclei containing supercoiled DNA might reflect changes in the conformation of the DNA within the nucleus: this proved to be the case.

Triton X-100 and Brij at final concentrations of 0·5 and 1% proved suitable for lysing cells to release nuclei. Cytoplasmic material and membrane blebs could be seen adhering to the nuclei by phase-contrast microscopy. The nuclei did not behave as osmometers – in 1 M sodium chloride they were larger in size than in living cells, and they swelled slightly in 1·95 M sodium chloride. These nuclei formed an aggregate in caesium chloride density gradients, banding at equilibrium at a density of about 1·42 g cm^{-3}. Examination of the aggregate by phase-contrast microscopy after removal from the caesium chloride gradient showed that it contained nuclei that were still intact and swollen with visible nucleoli, and with some adherent cytoplasm and membrane.

Much of the nuclear protein is lost from the nuclei; only about 7% of the total acid-insoluble material is recoverable in the nuclear fraction from the gradients after labelling with [^{3}H]leucine (Table 1). When viewed with phase-contrast optics, the nuclei appeared much paler on lysis of the cells which suggests that many nuclear proteins had been lost.

The conditions used to lyse the cells critically affect the sedimentation of nuclei – omission of ethylenediaminetetra-acetic acid from the lysis mixture results in nuclei sedimenting 79% as far as those from cells lysed in the presence of 0·1 M EDTA (i.e. ratio of 0·79). Variation of time of lysis from 5 to 30 min had little effect on the ratio, but longer periods led to its reduction. These results are consistent with the notion that nicks accumulate rapidly in DNA in the presence of magnesium ions (see later).

Table 1. *Effect of various treatments on recovery of label co-sedimenting in gradients with nuclei*

	Dose, J kg^{-1}	Ethidium bromide, μg/ml	Duration of spin, min	Recovery, % of dpm applied to gradient in nuclear peak
[^{3}H]thymidine	0	0	25	74
	0	3	25	74
	0	5	25	72
	0	5	80	73
	9·6	3	80	73
	9·6	5	80	74
[^{3}H]leucine	0	0	25	7·2
	0	4	25	7·0
	0	6	25	7·5
	0	5	80	7·6
	9·6	4	80	7·6
	9·6	6	80	7·1

Cells were labelled, added to the gradients, spun, fractionated and the number of dpm co-sedimenting with the nuclear peak determined. The number of trichloroacetic acid-insoluble dpm was determined for an aliquot of cells, and the recovery of dpm in the nuclear peak expressed as a percentage of the number of dpm applied to the gradient. Conditions were those described in Materials and methods; the dose of γ-rays, ethidium bromide concentrations in the gradients, and duration of spin are given in the Table.

Effects of intercalating agents on the sedimentation properties of nuclei

The distance sedimented by nuclei in sucrose gradients was described as a ratio relative to that of nuclei under standard conditions. This ratio is related in a complex way to the sedimentation coefficient. 'Isokinetic' gradients (Noll, 1967) were used for this study: the velocity of sedimentation of an ideal particle in such gradients is expected to be constant throughout the gradient, but this was not so of nuclei since halving the sedimentation time reduced the distance sedimented by less than half. Care should, therefore, be taken in interpreting the results.

The presence of the intercalating agents ethidium, propidium or actinomycin D in the gradients markedly affected the sedimentation properties of nuclei (Figs. 2–5). As the concentration of intercalating agent in the gradient was increased the distance travelled by the nuclei fell to a minimum and then rose again. The 3 curves are very similar; for example, the minimum in each of the 3 cases occurred at a concentration

of about 10^{-5} M. There is, however, one noteworthy difference: nuclei sedimented further in the presence of higher concentrations of actinomycin D than at equivalent molarities of ethidium and propidium.

Differences in sedimentation rates of nuclei in the presence of different amounts of ethidium might result from variations in DNA or protein content of the nuclei. Studies on labelled nuclei showed that such variations, if they occur at all, are small and are unlikely to account for the large variations in sedimentation rate (Table 1). Fig. 6 shows that the non-intercalating agents streptomycin and berenil have little effect on the sedimentation of the nuclei. The dye Hoechst 33258 (cf. Latt, 1973) and dexamethosone also had little effect when tested between concentrations of 2–20 and 2–25 μg/ml respectively.

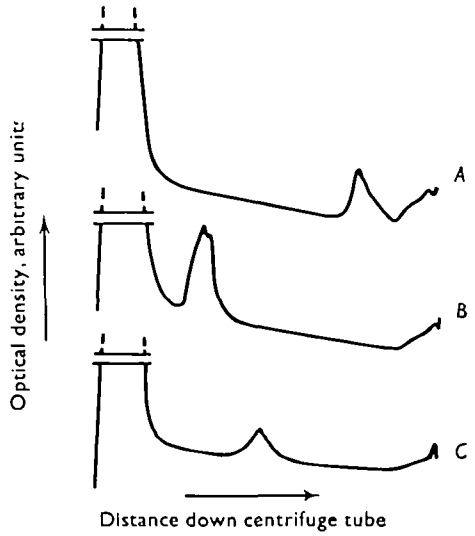

Fig. 2. The effect of ethidium bromide on the sedimentation of nuclei. Cells were applied to gradients containing 0, 5 or 15 μg/ml ethidium, spun at 5000 rev/min for 25 min and optical density profiles (*A*, *B*, *C*, respectively) determined as described in Materials and methods. The absorption at the top of the gradient is due to the lysis mixture. The nuclei are detected as a peak in the profile.

Effects of γ irradiation on the sedimentation properties of nuclei

Low doses of γ-rays are known to induce single-strand breaks in DNA, so that irradiation of superhelical DNA should lead to loss of supercoils. Figs. 7–9 illustrate the effect of γ-rays on the sedimentation of nuclei. As the dose of γ-rays is increased, the distance sedimented falls. Small doses have a marked effect on the sedimentation of the nuclei, whereas increasing the dose above about 10 J kg^{-1} has little further effect. This presumably reflects a limiting velocity, where all supercoils have been lost. The effects of low doses of γ-rays on the sedimentation of the nuclei can be compared with effects of similar doses on the ability of HeLa cells to form colonies (Fig. 10). As expected, the logarithm of the number of colonies formed decreases linearly with dose, with an LD_{60} of 1·35 J kg^{-1} (cf. Puck & Marcus, 1956). In contrast

the logarithm of the distance sedimented by nuclei does not decrease linearly with dose to the limiting velocity, but the dose that gives a 50% effect is roughly similar to the LD_{50} (Figs. 9, 10).

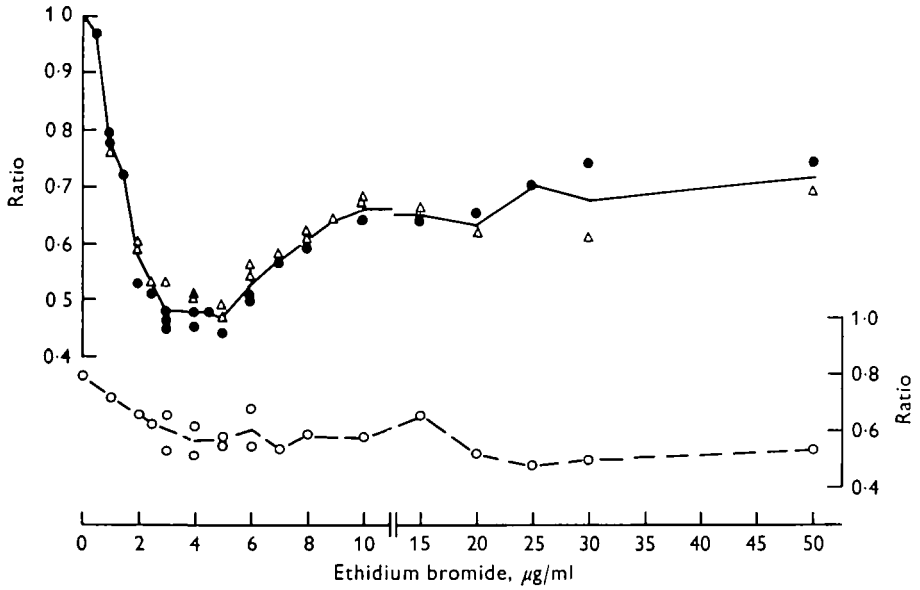

Fig. 3. The effect of ethidium bromide on the sedimentation of nuclei. The distance sedimented by nuclei in gradients containing different concentrations of ethidium is expressed as a ratio relative to nuclei sedimenting under standard conditions. The ratios (a measure of the sedimentation rate) were determined from optical density profiles similar to those presented in Fig. 2. The upper and lower curves relate to unirradiated and irradiated (9·60 J kg^{-1}) nuclei spun for 25 and 80 min respectively. A ratio of 1 refers to unirradiated nuclei sedimenting in gradients containing no ethidium (left-hand ordinate), or 5 μg/ml ethidium (right-hand ordinate). A ratio of 1 on the right-hand ordinate is equivalent to a ratio of 0·47 on the left-hand ordinate. The alignment and scales of the ordinates permit a rough comparison of the sedimentation rates of irradiated and unirradiated nuclei. △—△, unirradiated nuclei released from cells lysed with Brij; ●—●, unirradiated nuclei released from cells lysed with Triton X-100; ○—○, irradiated nuclei released from cells lysed with Triton X-100.

Variations in the sedimentation rate of nuclei induced by γ irradiation might arise from loss of protein or DNA from the nuclei. Experiments reported in Table 1 using labelled cells, indicate that if such effects occur, they are very small, and so are unlikely to account for the large variations in sedimentation rate.

These effects of γ-rays are consistent with the notion that single-strand breaks are induced in DNA, with consequent loss of supercoils. Changes in sedimentation rate of the nuclei may reflect an internal transition from a compact supercoiled DNA to the more open and nicked molecule. If irradiated DNA is indeed nicked, then the sedimentation of nuclei should no longer show the biphasic effect in response to different concentrations of intercalating agents. Figs. 3–5 show that this is indeed so. The presence of ethidium or propidium leads to a monotonous and slight decrease

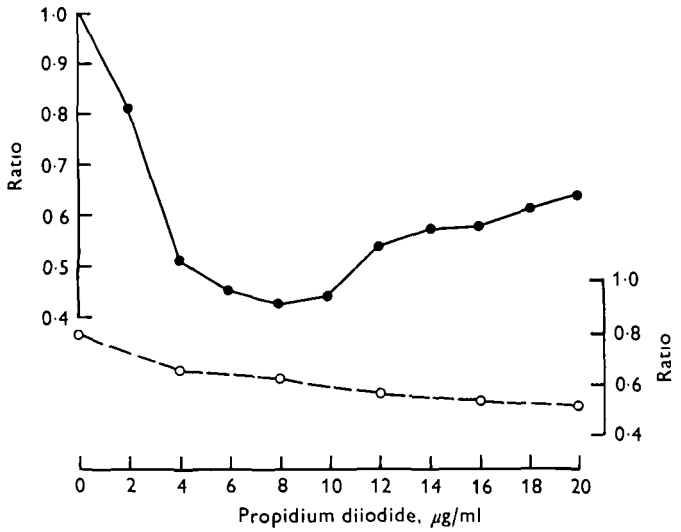

Fig. 4. The effect of propidium di-iodide on the sedimentation of nuclei. The distance sedimented by nuclei in gradients containing different concentrations of propidium is expressed as a ratio relative to nuclei sedimenting under standard conditions. The standard conditions are described in Materials and methods and in the legend to Fig. 3. The upper (●—●—●) and lower curves (○—○—○) relate to unirradiated and irradiated (9·60 J kg^{-1}) nuclei respectively.

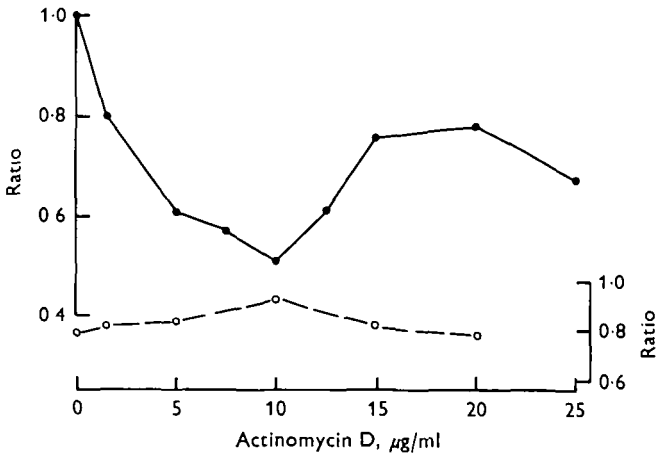

Fig. 5. The effect of actinomycin D on the sedimentation of nuclei. The distance sedimented by nuclei in gradients containing different concentrations of actinomycin is expressed as a ratio relative to nuclei sedimenting under standard conditions. The standard conditions are described in Materials and methods and in the legend to Fig. 3. The upper (●—●—●) and lower (○—○—○) curves relate to unirradiated and irradiated (9·60 J kg^{-1}) nuclei respectively.

in rate of sedimentation of irradiated nuclei, without suggestion of a minimum at concentrations of about 10^{-5} M, whereas actinomycin D has little effect (see Discussion). As expected, the presence of non-intercalating agents hardly affects the rate of sedimentation of irradiated nuclei (Fig. 6).

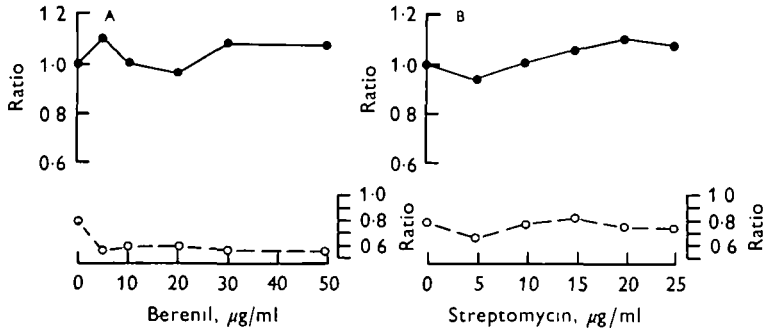

Fig. 6. A, B, the effects of berenil and streptomycin, respectively, on the sedimentation of nuclei. The distances sedimented down gradients containing different concentrations of berenil and streptomycin are expressed as ratios relative to nuclei sedimenting under standard conditions. The standard conditions are described in Materials and methods and in the legend to Fig. 3. The upper (●—●—●) and lower (○—○—○) curves relate to unirradiated and irradiated (9·60 J kg^{-1}) nuclei respectively.

Repair

Fig. 11 shows the change in the sedimentation properties when cells were irradiated and then incubated at 4 or 37 °C for different periods, before applying them to the gradient. Even though the dose of irradiation was large enough to reduce the fraction surviving to less than 0·01 the irradiated nuclei quickly regained the properties of unirradiated nuclei, e.g. within about 60 min at 37 °C the process was complete. This process does not take place in lysed cells and proceeds very much more slowly at 4 °C. It has the properties expected of the processes known to take place in irradiated cells when single-strand breaks in DNA are repaired.

DISCUSSION

When viewed under phase-contrast the structures released by lysing HeLa cells with Triton X-100 in 1 M salt have the appearance of nuclei depleted of protein. Nuclei such as these should prove useful in studies on the conformation of DNA, since the DNA within them should be very resistant to shearing forces.

Intercalating agents have a profound effect on the rate of sedimentation of the nuclei, the rate appears to reflect the conformation of the DNA within the nucleus. The rate of sedimentation depends upon the concentration of intercalating agent in the gradient in a way characteristic of supercoiled DNA. Three different intercalating agents (ethidium, propidium and actinomycin D) affected the sedimentation of nuclei in this way, whereas the non-intercalating agents berenil and streptomycin had little effect. Berenil is of interest in this context because it is a trypanocidal drug like many inter-

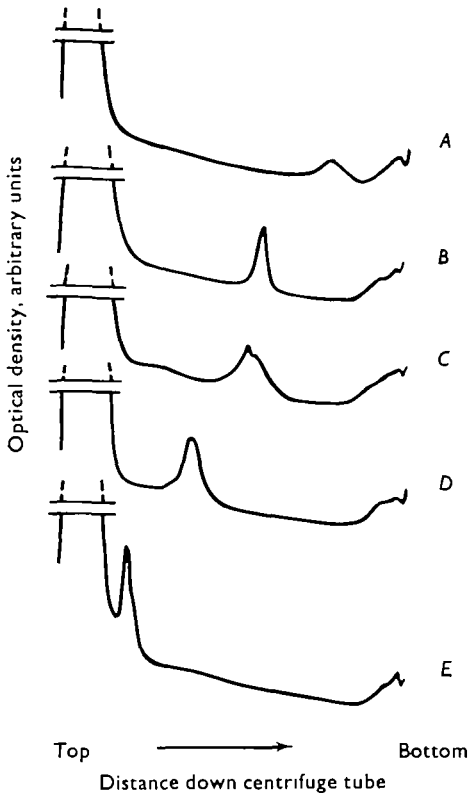

Fig. 7

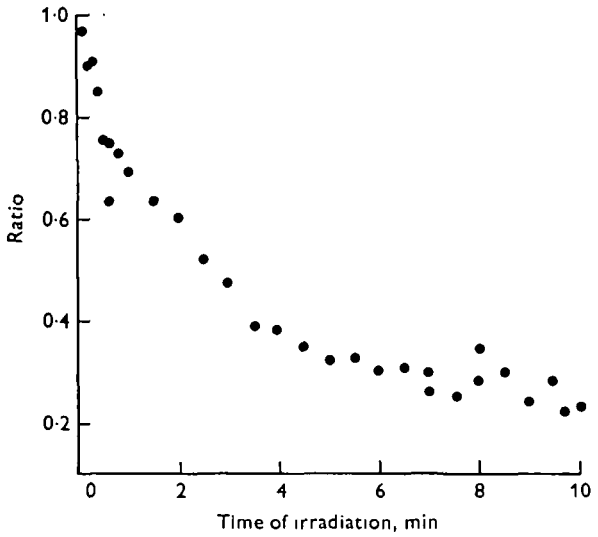

Fig. 8

Fig. 7. The effect of irradiation on the sedimentation of nuclei. Curves *A*, *B*, *C*, *D*, *E* correspond to doses of 0, 0·6, 1·2, 3·6, and 10·8 J kg^{-1}, respectively. Cells were applied to the gradients as described in Materials and methods, irradiated with different doses of γ-rays, the gradients were then spun at 5000 rev/min for 25 min, and their optical density profiles determined. There are 2 peaks in the optical density profile. At the top of the gradient the lysis mixture absorbs strongly and lower down is the peak of absorption due to the nuclei.

Fig. 8. The effect of irradiation on the sedimentation of nuclei. The distance sedimented by irradiated nuclei in gradients lacking ethidium is expressed as a ratio relative to unirradiated nuclei sedimenting under standard conditions. Nuclei were irradiated for different times, the time of irradiation being proportional to dose. (Dose rate 1·20 J kg^{-1} min^{-1}.) The ratios (a measure of the sedimentation rate) were determined from optical density profiles similar to those presented in Fig. 7. A ratio of 1 refers to unirradiated nuclei, sedimenting under the standard conditions (see Materials and methods). Gradients were spun for 25 min.

calating agents. It also inhibits specifically the synthesis of supercoiled mitochondrial DNA and interacts *in vivo* with existing mitochondrial DNA in such a way that the mitochondrial DNA may be isolated as a covalently closed and circular DNA molecule free of superhelical turns (Rastogi & Koch, 1974).

There are not only general, but also detailed similarities between the effects of intercalating agents on the sedimentation of circular DNA and nuclei. In contrast to the effects of other intercalating agents, the binding of actinomycin to linear DNA *in*

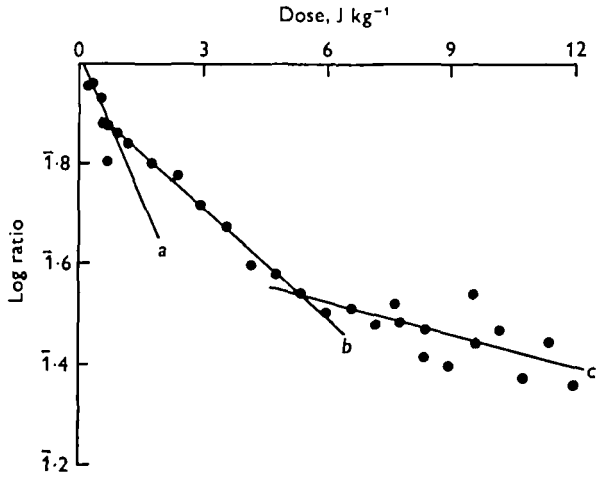

Fig. 9. The effect of irradiation on the sedimentation of nuclei. The distance sedimented by irradiated nuclei in gradients lacking ethidium is expressed as a ratio relative to unirradiated nuclei sedimenting under standard conditions. The data are the same as those presented in Fig. 8; the logarithm of the ratio is plotted against dose of γ-rays. Interpretation is simplified by assuming that nuclei sedimenting with rates characterized by ratios of 1·0 and 0·25 contain DNA all of which or none of which is supercoiled. Intermediate ratios are taken as reflecting in direct proportion the fraction of supercoiled 'targets' in a nucleus. The curve implies that nuclei contain a continuous range of target lengths of DNA in which rotation of one strand about the other is restricted. Analysis is further simplified by assuming that nuclei contain supercoiled targets of 3 different lengths (*a*, *b* and *c*), each being converted independently to the nicked form with first-order kinetics (i.e. lines *a*, *b* and *c*). The doses of γ-rays reducing the number of intact targets to 37 % (i.e. $\frac{1}{e} \times 100$) of the original number are 1·50, 3·96 and 13·86 J kg^{-1} for targets *a*, *b* and *c* respectively. Roughly similar target sizes of 3×10^9, 10^9 and 3×10^8 Daltons of DNA for *a*, *b* and *c* respectively may be calculated if it is assumed either that the ionizing radiation has a 'direct' effect (Method I of Lea (1955); cf. Meredith & Massey (1972)) or that one single-strand break is induced by deposition of the experimentally determined value of 50 eV (Dean, Ormerod, Serianni & Alexander, 1969).

vitro increases its rate of sedimentation, but the increase only occurs in DNA of high molecular weight. It is thought that this results from intramolecular looping of DNA facilitated by the peptide rings of bound actinomycin (Müller & Crothers, 1968). At high levels of intercalation, and due to this looping, circular and supercoiled molecules of DNA show higher rates of sedimentation in the presence of actinomycin, than do similar molecules in the presence of other intercalating agents (Waring, 1970). Actinomycin also differs in this respect from other intercalating agents in its effect on the sedimentation of nuclei. In the presence of high levels of actinomycin both irradiated and unirradiated nuclei sediment faster than their counterparts in the presence of equivalent molarities of ethidium. The detailed similarities in the behaviour, *in vitro*, of pure supercoiled DNA and nuclei suggests that the DNA in the nuclei is supercoiled. This DNA is subject to the same kinds of topological constraints restricting rotation of one strand of the duplex about the other as those found in circular supercoiled DNA.

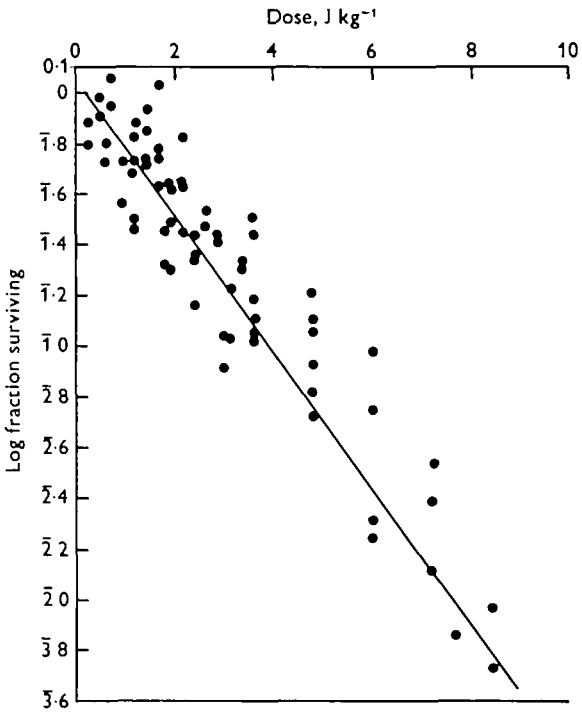

Fig. 10

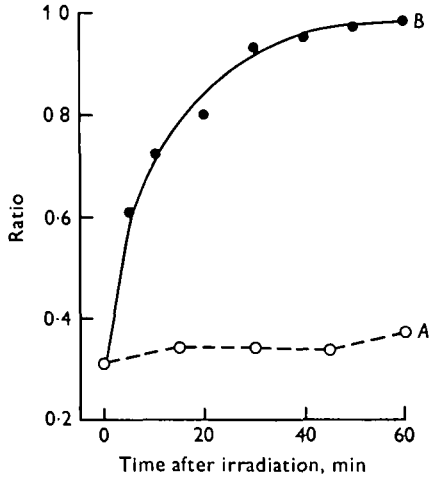

Fig. 11

Fig. 10. The effect of γ irradiation on the colony-forming ability of HeLa cells. Suspensions of single HeLa cells were irradiated with different doses of γ-rays, and 12 days later surviving colonies counted. The numbers of colonies arising from unirradiated cells was defined as 1, and numbers from irradiated cells expressed as a fraction of this. The line is that of best fit. Doses of 1·35 and 1·85 J kg^{-1} reduce the surviving fraction to 0·5 and 0·37 respectively.

Fig. 11. The effect on nuclear sedimentation of incubating irradiated cells at 4 and 37 °C (curves *A* and *B*, respectively). The distance sedimented by nuclei in gradients (in the absence of ethidium) is expressed as a ratio relative to nuclei sedimenting under standard conditions. A ratio of 1 refers to unirradiated nuclei sedimenting in gradients containing no ethidium. Cells were irradiated with 9·60 J kg^{-1} and then incubated for different times at 4 or 37 °C before applying them to the gradients as described in Materials and methods. Spins were for 25 min at 5000 rev/min.

There is, however, one major difference between the behaviour of circles of pure DNA and that of nuclei. 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 of unirradiated nuclei, whose minimum sedimentation rate is greater than that of irradiated nuclei that have lost their supercoils. This could arise for a number of reasons. For example, some regions of the nucleus might be inaccessible to the intercalating agent – but not to γ-rays – so that they remained supercoiled. Alternatively, there might be various populations of DNA molecules in the nucleus with differing superhelical densities, so that at a level of intercalation that reduced the number of superhelical turns in one population to zero (i.e. $\tau = 0$), other populations might remain supercoiled (i.e.

$\tau \neq 0$). The rate of sedimentation of the nucleus will reflect the 'average' conformation of all the different populations, so that whilst γ-irradiation can effectively reduce the average number of superhelical turns to zero, intercalation cannot.

In circular molecules, the maintenance of the high energy and supercoiled state is dependent upon the restriction of net rotation of the one backbone strand of the duplex about the other. This topological constraint exists in intact molecules but is lost on nicking, since the cut ends of the one strand are able to rotate freely about the sugar-phosphate bonds in the other. It is possible that the DNA of human cells might be organized into covalently continuous circles; alternatively supercoils might be maintained in linear molecules if rotation of one strand about the other was restrained (cf. Cook, 1973, 1974). The experiments involving the γ irradiation of cells provide some information as to the length of DNA over which the topological constraint acts. The doses of γ-rays required to eliminate supercoiling are very low, suggesting that a single-strand scission at any place in a considerable length of DNA eliminates the topological constraint.

Irradiation of nuclei affects their rate of sedimentation; as the dose increases, the rate continuously decreases. In this, nuclei behave differently from single circles of supercoiled viral DNA, where irradiation (or nicking) induces a single step transition from the rapidly sedimenting supercoiled form to the slower sedimenting and nicked form; no forms sedimenting at intermediate rates are seen (Boyce & Tepper, 1968). Instead, nuclei behave more like catenated circles of DNA, where, for example, nicking of one of the supercoiled circles in a dimer results in sedimentation at a rate between those of the fully supercoiled or fully nicked dimers (Brown & Vinograd, 1971). An intermediate rate of sedimentation is also seen in DNA molecules from *Escherichia coli* which have accumulated a number of nicks, so losing some, but not all, of their superhelical turns (Worcel & Burgi, 1972). The presence of various concentrations of ethidium in the gradient has little effect on the sedimentation of nuclei irradiated with a dose of 9·6 J kg^{-1} – the nuclear DNA has the characteristics of nicked DNA. As there is no evidence for the induction by γ-rays of a continuous reduction in superhelical density of supercoiled circles of viral DNA, one interpretation of the present results is that an unirradiated nucleus contains a population of supercoiled DNA molecules, and that a proportion of these become nicked on irradiation. After irradiation with low doses, some of the DNA molecules will be unchanged and supercoiled, the remainder being nicked and without any supercoils, so that the nucleus sediments at a rate between that of a nucleus containing fully supercoiled DNA molecules and that in which all such molecules have lost their supercoils.

The doses of γ-rays required to produce these effects are very small, smaller by orders of magnitude than the doses generally used to detect physico-chemical changes (e.g. single-strand breaks in DNA) in irradiated cells. About 1·90 J kg^{-1} are required to produce half the maximal effect on the sedimentation. Repair of irradiated nuclei is readily detectable and rapid. Irradiation of lysed cells with 9·60 J kg^{-1} abolishes the biphasic response of the sedimentation rate to ethidium. Irradiation of living cells with the same dose presumably induces about the same number of single-strand

cuts, but mending of the cuts then restores the sedimentation to normal. This could arise for two reasons. First, the DNA might exist *in vitro* in a high-energy (e.g. supercoiled) state; irradiation might then lead to strand scission and an alteration in configuration as a result of a relaxation to the lower-energy (i.e. nicked) state. If this

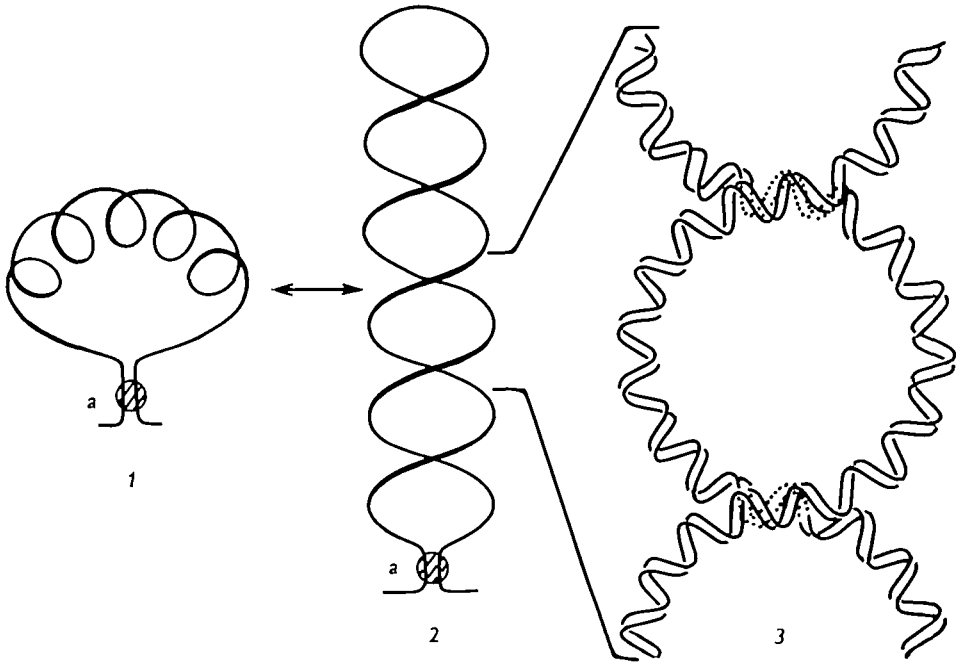

Fig. 12. The first-order structures for one unit of the human chromosome *in vitro*. Structures *1* and *2* represent toroidal and interwound models for one superhelical duplex characterized by $\tau = -5$. The inset (*3*) shows the double helical structure of the duplex in the region where duplex crossover occurs in the interwound form *2*. The backbone strands of the double helix are, of course, covalently continuous – here they are drawn in *3* as if they are discontinuous to illustrate the sense of double and superhelical helices. A topological constraint acts at point *a*, which holds the 2 duplexes so that rotation about their ends is restricted (i.e. supercoils are maintained in the loop). A single-strand nick at any point in the loop in either structure *1* or *2* would eliminate supercoiling. Structures *1* and *2* are interconvertible without removing the topological constraint, but it seems likely for mechanical reasons that in the absence of stabilizing protein or RNA molecules, structure *2* would be more stable. Each structure contains far fewer superhelical turns than the 7000–17000 (i.e. one every 90–230 base pairs) that would be expected in a loop of 10^9 Daltons of DNA. (These figures are calculated by assuming that the superhelical density of the loop falls within the range found in naturally occurring circles (Gray, Upholt & Vinograd, 1971) and that ethidium unwinds the duplex helix by 26° (Wang, 1974).) There would be about 10000 units of the size of 10^9 Daltons in a HeLa nucleus.

is so, the repair mechanism must not only be capable of subsequently mending the breaks in the sugar-phosphate bonds of the backbone strands but also of re-introducing into the mended DNA a higher energy configuration. Although enzymes that alter the superhelical properties of DNA have recently been isolated from both

animal and bacterial cells (Alberts & Frey, 1970; Wang, 1971; Champoux & Dulbecco, 1972; Sigal *et al.* 1972; Molineux, Friedman & Gefter, 1974) a second explanation seems more likely. In the living cell the DNA might not be supercoiled, or rather its conformation might be as stable as that of the nicked form. (It must, nevertheless, be subject to the topological constraints that α is invariant, since when it is placed in the new environment of the sucrose gradient it behaves like a population of supercoiled DNA molecules and unlike that of nicked ones.) Irradiation of living cells would, therefore, induce single-strand scissions without causing any resultant change in the conformation of the DNA. Repair of broken sugar-phosphate bonds would restore both the topological constraints and the original configurations. If variety of supercoiling exists within the cell nucleus, it may be that not all variants are as stable as the nicked form, so that after irradiation all cut sugar-phosphate bonds would be repaired without complete restoration of the original superhelical properties. The lethal effects of γ-rays might then not result from the primary effect of single-strand scissions which are readily repaired, but from the introduction of an incorrect configuration into the DNA during repair.

The length of DNA (the 'target') in which net rotation of one strand of a duplex about the other is restricted may be determined by applying target theory to the curves relating dose of γ-rays to the sedimentation of nuclei (cf. Fig. 9). As a number of assumptions were made in the calculation, the estimate of the target size – about 10^9 Daltons of DNA – is necessarily approximate. Whatever the exact size, the topological constraint acts over great lengths of DNA, and one likely way this might be achieved is by the organization of the linear duplex into loops. A specific pairing between base sequences – perhaps involving repetitive base sequences – at different regions along one duplex could give rise to loop formation. Alternatively the pairing might involve a molecular bridge between the 2 regions, for example of the type that maintain loops in the DNA of *Escherichia coli* (Worcel & Burgi, 1972; Worcel, Burgi, Robinton & Carlson, 1973). Fig. 12 illustrates two possible first order structures for one unit of the human chromosome *in vitro*. (Higher order structures are, of course, possible.) It is attractive to suppose that supercoils will be the basis of any sub-unit structure of the chromosome involving interactions of RNA or proteins with DNA which are independent of specific base sequences (cf. Kornberg, 1974; Senior, Olins & Olins, 1975). It remains to be seen what relationship the supercoil model of Pardon & Wilkins (1972) might have to the structure discussed in this paper.

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