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CONFORMATIONAL CONSTRAINTS IN NUCLEAR DNA

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SUMMARY

We have investigated DNA superstructure in a wide range of nuclei of higher cells by gently lysing cells to release structures that resemble nuclei but are depleted of nuclear proteins. The sedimentation properties of these structures, which we call nucleoids, have been examined in sucrose gradients containing the intercalating agent, ethidium. The sedimentation rate of nucleoids derived from the growing cells of mammals, birds, amphibians and insects varies in the manner characteristic of circular and superhelical molecules of DNA. These characteristic changes in sedimentation rate are abolished by irradiating the nucleoids with low doses of γ-rays, a procedure known to introduce single-strand scissions into DNA. We have also investigated by similar means DNA superstructure in nucleoids derived from a variety of different chick cells. Nucleoids derived from adult hen erythrocytes differ from the other nucleoids studied in that their sedimentation rate does not vary in the manner characteristic of supercoiled DNA.

INTRODUCTION

Whereas very little is known of DNA superstructure in nuclei of higher cells, the superstructures of the circular DNA molecules found in viruses have been analysed in some detail (Bauer & Vinograd, 1968, 1971). This analysis is based upon the distinction between the helical turns of the DNA duplex and any superhelical turns that might be superimposed upon the duplex turns. The superhelical turns are maintained by the forces that hold the duplex together (Vinograd *et al.* 1965); they are generally 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 generally contain no superhelical turns. The topological winding number, α, is an important parameter used in the description of this kind of superstructure. α is defined as the number of complete revolutions made by one strand about the duplex axis, when the axis is constrained to lie in a plane. In the intact circle, α is constant and can only be changed by breaking covalent bonds in either of the backbone strands of the DNA duplex followed by rotation of one strand about the other. When α is invariant, a topological constraint is said to act, and a decrease in the duplex turns leads to a corresponding increase in the number of superhelical turns (see Bauer & Vinograd, 1968, for a formal discussion). Binding of the intercalating dye, ethidium, to DNA unwinds the DNA duplex, so changing the number of superhelical turns. As the sedimentation rate of a circular molecule depends on its number of superhelical turns, molecules subject to the

topological constraint (i.e. α is invariant) exhibit characteristic changes in sedimentation coefficient when they interact with ethidium. These characteristic changes are abolished when the molecule is nicked and the topological constraint lost (i.e. α may now vary).

We are currently investigating DNA superstructure in the nuclei of higher cells. Our approach has been to lyse human cells gently to release structures that resemble nuclei but are depleted of nuclear protein, and then to measure the rate of sedimentation of these structures – which we call 'nucleoids' – in gradients containing ethidium (Cook & Brazell, 1975). The rate of sedimentation of the nucleoids varies in the biphasic manner characteristic of DNA in which α is invariant. Furthermore, these characteristic changes are abolished by irradiation with γ-rays – a procedure known to induce single-strand breaks in DNA, so destroying constraints. We interpret these results as showing that human nucleoids contain DNA that is subject to the same kind of topological constraint restricting rotation of one strand of the duplex about the other as that found in intact and circular DNA molecules (i.e. α is invariant).

In the present paper, nucleoids prepared from dividing avian, amphibian and insect cells have been investigated; the sedimentation rates of these nucleoids in the presence of ethidium vary in the biphasic manner characteristic of intact and circular DNA. It seems likely, therefore, that nuclear DNA from all actively dividing cells is constrained so that α is invariant. Nucleoids have also been prepared from a range of chicken cells differing in their synthetic activities. Mature hen erythrocytes are nucleated, unlike their mammalian counterparts, and during their development there is a gradual loss of synthetic ability culminating in the complete inactivity of the mature erythrocyte. As the nucleus becomes less active it becomes more condensed. The peripheral blood of 5-, 12- and 18-day-old chick embryos contains predominantly erythroblasts, reticulocytes and erythrocytes respectively (Romanoff, 1960). Inactive erythrocytes were also obtained from the peripheral blood of the adult hen.

MATERIALS AND METHODS

Cells

Human HeLa cells were grown in suspension as described (Cook & Brazell, 1975). Chick embryo fibroblasts were obtained by trypsinization of 12-day-old chick embryos, maintained as monolayer cultures in minimal essential medium supplemented with 10 % foetal calf serum and antibiotics (Cook, 1975) at 37 °C and used between 2 and 4 weeks later. Adult hen erythrocytes were obtained from peripheral blood (Harris, Watkins, Ford & Schoefl, 1966) and embryonic red blood cells from embryos of different ages by cutting their blood vessels and allowing them to bleed into the allantoic cavity (Bolund, Ringertz & Harris, 1969). The XTC-2 cell-line (Pudney, Varma & Leake, 1973) of the South African clawed toad, *Xenopus laevis* (kindly supplied by Dr K. Smith) was grown in a monolayer culture at 25 °C in minimal essential medium supplemented as above. Monolayer cultures (kindly supplied by Dr D. B. Roberts) of the *Drosophila melanogaster* cell-line K85 (Echalier & Ohanessian, 1970; Dolfini, 1971) were grown at 25 °C in Schneider's *Drosophila* medium (Gibco-Biocult Ltd., Paisley, Scotland) supplemented with antibiotics and 15 % foetal calf serum which had been heat inactivated by incubation for 30 min at 65 °C.

Samples of cells for lysis were prepared by spinning down the cells in suspension, washing them in phosphate-buffered saline (Dulbecco & Vogt, 1954) and resuspending them in phos-

phate-buffered saline at room temperature. Monolayer cultures of chick embryo and XTC-2 cells were removed by incubation in the presence of phosphate-buffered saline supplemented with 0·02 % ethylenediaminetetra-acetic acid; K85 cells were removed from monolayers by vigorous washing with phosphate-buffered saline.

Sucrose gradients

Isokinetic sucrose gradients were prepared, spun and analysed as described by Cook & Brazell (1975). All manipulations were carried out at room temperature using sterile solutions. Sucrose gradients (15–30 % sucrose: pH 8·0; 4·6 ml) generally contained sodium chloride (1·0 M), tris-(hydroxymethyl)-amino-methane (0·01 M), ethylenediaminetetra-acetic acid (0·001 M) in addition to variable amounts of ethidium. 150 μl of lysis mixture were layered on to the top of the gradient, followed by 50 μl of phosphate-buffered saline containing cells. Fifteen minutes after the addition of the cells to the gradient, tubes were spun at 20 °C in the SW50.1 rotor in a Beckman L2–65b centrifuge at speeds and times indicated in Tables 1 and 2 (pp. 290, 297) and in the legends to the figures. Gradients were analysed by passing them through an absorbance monitor operating at a wavelength of 254 nm.

The position of nucleoids in the gradient was indicated by a peak in the $O.D._{254}$ trace. The distance the nucleoids travelled down the gradient was determined by measuring the distance on the trace from the meniscus to the peak. Six gradients were generally run simultaneously in one rotor, and one gradient contained nucleoids which served as a reference. The distances travelled by nucleoids in other tubes were expressed as a ratio relative to nucleoids in this tube. The contents of the reference tubes are indicated in the legends. Data from spins of different speeds and duration are sometimes presented on the same graph, and ordinate scales have been selected to permit rough extrapolations beyond the observed ratios (Cook & Brazell, 1975).

γ-irradiation

A Gamma Cell 40 (Atomic Energy of Canada Ltd.) containing 2 caesium 137 sources in opposition was used to deliver low doses of 1·20 J kg^{-1} min^{-1} over a uniform field. High doses were obtained from one caesium 137 source contained in a Gravitron RX30 machine (Gravatom Ind., Gosport, Hampshire) delivering doses between 2·48 and 17·21 J kg^{-1} min^{-1}. Dose rates were determined using a Nuclear Enterprises Ionex 2500 dosimeter (Bath Road, Beenham, Reading) equipped with a 0·6-ml thimble ionization chamber. In all cases cells were added to gradients, and the contents of the whole gradient irradiated at room temperature for the appropriate time before spinning as usual 15 min later.

RESULTS

Lysis procedure

A method was developed in an earlier study (Cook & Brazell, 1975) for lysing human HeLa cells to release nucleoids. This method involves the use of the non-ionic detergent Triton X-100 to disrupt membranes and 1·0 M NaCl to reduce protein binding to DNA. It is applicable for the preparation of nucleoids from cells of insects, amphibians, birds and mammals. The properties of this range of nucleoids are characterized further in the accompanying paper (Cook, Brazell & Jost, 1976). Nucleoids differ from nuclei in that they are depleted of nuclear protein.

Effects of intercalating agents on the sedimentation properties of nucleoids derived from cells of different Orders

The effect of the intercalating agent, ethidium, on the rate of sedimentation of nucleoids was studied by spinning nucleoids through gradients containing ethidium.

Table 1. *Relative sedimentation rates of nucleoids from different species*

Species	Unirradiated			Irradiated, 9·60 J kg^{-1}			Unirradiated rate* / Irradiated rate
	Centrifuge speed, rev/min	Centrifuge time, min	Relative sedimentation rate*	Centrifuge speed, rev/min	Centrifuge time, min	Relative sedimentation rate*	
Human (HeLa)	5000	25	1·0	5000	80	0·20	5·0
Frog (XTC-2)	7000	25	0·41	12000	25	0·10	4·1
Chick (embryonic fibroblast)	7000	40	0·24	12000	40	0·06	4·0
Fly (K85)	10000	25	0·17	10000	25	0·09	1·9

* The rates of sedimentation of nucleoids are expressed as a fraction of the rate of sedimentation of HeLa nucleoids, assuming that the rate is directly proportional to (i) the distance travelled by nucleoids down the gradient, (ii) the square of the centrifuge speed, and (iii) duration of spin. It should be pointed out, however, that although the gradients are 'isokinetic', nucleoids do not behave as ideal particles in them (cf. Cook & Brazell, 1975).

The distance sedimented is expressed as a ratio relative to that of nucleoids sedimenting under standard conditions. The ratio is related in a complex way to the sedimentation coefficient, since the nucleoids do not behave as ideal particles in these gradients (cf. Cook & Brazell, 1975).

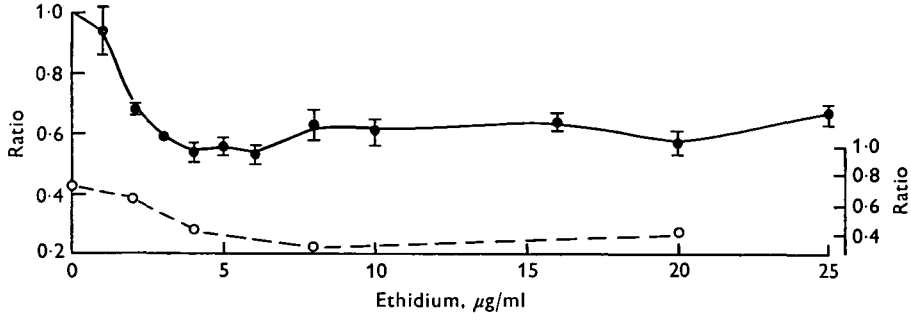

Fig. 1. Effect of ethidium on the sedimentation of nucleoids derived from embryonic chicken fibroblasts. The distance sedimented by nucleoids in gradients containing different concentrations of ethidium is expressed as a ratio relative to reference nucleoids sedimenting under the same conditions. The upper and lower curves relate to unirradiated and irradiated (9·60 J kg^{-1}) nucleoids spun for 40 min at 7000 and 12000 rev/min respectively. Between $0·5–2 \times 10^5$ cells were applied to each gradient. A ratio of 1 refers to unirradiated reference nucleoids 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 0·56 on the left-hand ordinate. The alignment and scales of the ordinates permit a rough comparison of the sedimentation rates of irradiated and unirradiated nucleoids. Error bars give the standard error of the mean.

Unirradiated nucleoids derived from HeLa cells, chick fibroblasts, epithelial cells of the *Xenopus* cell-line (XTC-2) and cells from a *Drosophila* cell-line (K85) sediment at very different rates in sucrose gradients in the absence of ethidium (Table 1) – their relative sedimentation rates roughly reflecting their relative DNA contents. Each, however, exhibits a similar dependence of sedimentation rate on ethidium concentration (Figs. 1–3). As the concentration of ethidium in the gradient increases, the distance travelled by the nucleoids falls to a minimum and then rises again. In sharp contrast, irradiated nucleoids have a much lower sedimentation rate in the absence of ethidium, and their sedimentation rate is little affected by various concentrations of ethidium in the gradient. The concentration of ethidium giving the lowest sedimentation rate is roughly the same in all cases (about 4 μg/ml), suggesting a similarity in the average degree of supercoiling in these nucleoids of the different Orders.

Low doses of γ-rays are known to induce single-strand breaks in DNA, so removing topological constraints (i.e. α need no longer remain constant). Figs. 1–3 show the effects of irradiation on the sedimentation rate of nucleoids derived from the different Orders. In all cases, irradiated nucleoids sediment more slowly than their unirradiated counterparts (Table 1). Increasing the concentration of ethidium in the gradient leads to a slight decrease in sedimentation rate, in marked contrast to the biphasic response obtained with unirradiated nucleoids (Figs. 1–3).

In the absence of ethidium, increasing the irradiation dose decreases the sedimentation rate (Figs. 4, 5). Small doses have a marked effect on the sedimentation of the nucleoids, larger doses having a progressively smaller effect. Nucleoids from chicken, frog and insect cells, therefore, behave like those from human cells (Cook & Brazell,

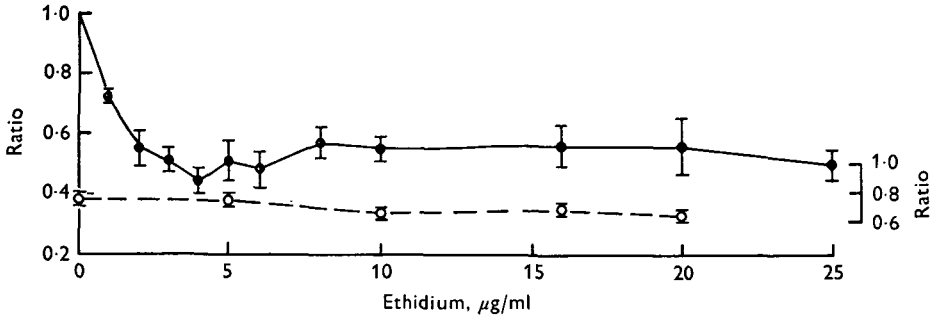

Fig. 2. Effect of ethidium on the sedimentation of amphibian nucleoids (cf. legend to Fig. 1). The upper and lower curves relate to unirradiated and irradiated (9·60 J kg^{-1}) nucleoids spun for 25 min at 7000 and 12000 rev/min respectively. Between 0·5–1 × 10^5 cells were applied to each gradient. A ratio of 1 on the right-hand ordinate is equivalent to a ratio of 0·51 on the left-hand ordinate.

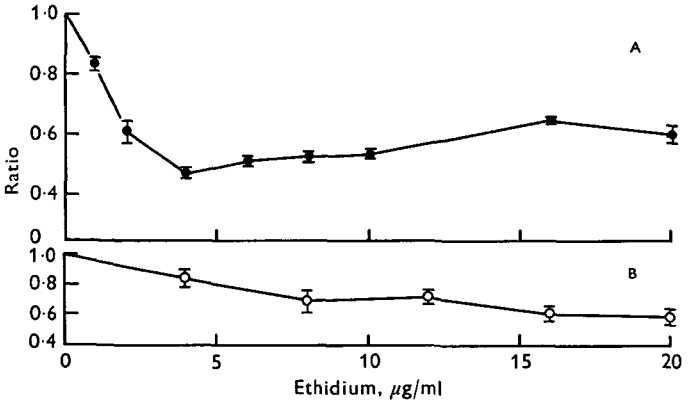

Fig. 3. Effect of ethidium on the sedimentation of insect nucleoids (cf. legend to Fig. 1). A, unirradiated; B, irradiated. The upper and lower curves relate to unirradiated and irradiated (34·70 J kg^{-1}) nucleoids spun for 25 min at 10000 and 20000 rev/min respectively. Between 2·5–5 × 10^5 cells were applied to each gradient. A ratio of 1 refers to unirradiated nucleoids (upper graph) or irradiated nucleoids (lower graph) sedimenting in gradients containing no ethidium.

1975). The doses required to halve the sedimentation rate are in each case approximately the same, although there is some indication that reduction of the sedimentation rate of insect nucleoids to a minimum value requires larger doses than those required for the other nucleoids.

The conformation of DNA in different types of chicken nucleoid

The conformation of DNA in a range of cells of one species was investigated by sedimenting chick nucleoids derived from cells with differing synthetic activities through sucrose gradients. The peripheral blood of 5-, 12- and 18-day-old embryos contains predominantly erythroblasts, reticulocytes and erythrocytes respectively. Inactive erythrocytes are also found in the peripheral blood of the adult hen. As can be seen from Table 2, the different chicken nucleoids sediment at very different rates. For example, nucleoids from red blood cells of 5-day-old embryos sediment 5 times more slowly than those from embryonic fibroblasts; those from adult hen erythrocytes do so 30 times more slowly. This is a remarkable result, since the most highly condensed nucleus of the adult hen erythrocyte gives rise to a nucleoid with the slowest rate of sedimentation. As the embryonic red blood cells mature, the sedimentation rate of their derivatives becomes more like those of the adult hen erythrocyte.

Nucleoids from the different embryonic red cells all give, to some degree, biphasic curves; those of the hen erythrocyte do not (Fig. 6). In the presence of high levels of ethidium in the gradient (> 10 μg/ml) nucleoids from the red cells of 5-day-old embryos sediment at rates considerably greater than the minimum rate (at 4 μg/ml). whereas nucleoids from adult hen erythrocytes sediment more and more slowly as the ethidium concentration is progressively increased.

As we found with the cell-types of the different Orders, γ-irradiation of chick nucleoids made from embryonic red cells reduces their sedimentation rate, similar doses being required to reduce their sedimentation to the limiting rate (Fig. 7). Irradiation of nucleoids derived from chick fibroblasts and embryonic red cells abolishes the biphasic variation of sedimentation rate in gradients containing ethidium (Figs. 1, 6). On the other hand, nucleoids from mature hen erythrocytes show no such biphasic variation, but rather a decrease in rate as the ethidium concentration is increased: irradiation induces no change in this behaviour.

The relative sedimentation rates of nucleoids from different sources are summarized in Tables 1 and 2. Nucleoids from the rapidly dividing cells of the different Orders vary no more than 5-fold in sedimentation rate, and irradiation in each case reduces this rate; on the other hand bird nucleoids show a 30-fold variation and γ-irradiation reveals an interesting effect. Whatever the sedimentation rate of the unirradiated embryonic nucleoids, their irradiated counterparts all sediment at roughly the same rate as those of hen erythrocytes. In other words, nucleoids from the different embryonic cells can be induced to sediment like those from adult hen erythrocytes simply by irradiation. An alternative way of expressing these results is to compare the relative rates of sedimentation of irradiated and unirradiated nucleoids. For example, irradiation reduces by factors of 4, 3 and 2 the sedimentation rates of the nucleoids from 5-day-old, 12-day-old and 18-day-old embryos respectively, and the rates of hen erythrocyte nucleoids are unaffected.

One possible explanation of the slow rate of sedimentation of the hen erythrocyte nucleoids, and of their behaviour in gradients containing ethidium, is that covalent bonds in their DNA are cut by nucleases released after lysis of the erythrocyte. This

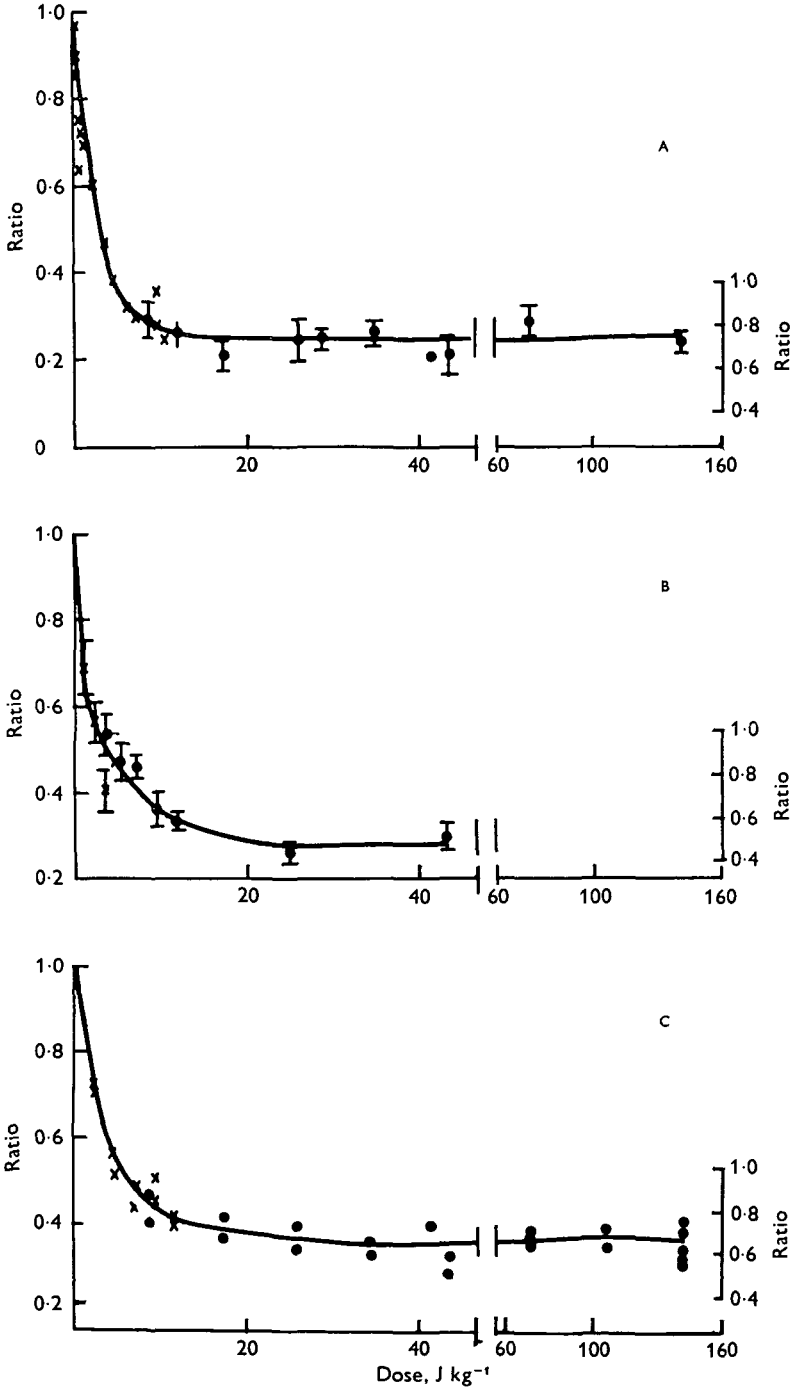

Fig. 4 A–C.

explanation is examined in the mixing experiments reported in Table 3. Hen erythrocytes were added to the lysis mixture floating on top of a sucrose gradient, and nucleoids then removed from the lysis mixture by high-speed centrifugation. The lysis mixture, now pink due to the haemoglobin released from the erythrocytes, was removed from the gradients and mixed with freshly prepared HeLa nucleoids. The integrity of the HeLa nucleoids was then examined by spinning them in gradients containing ethidium. As can be seen from Table 3, the pink mixture derived from the

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Fig. 4. Effect of irradiation dose on sedimentation of human, frog and insect nucleoids. The distance sedimented by γ-irradiated nucleoids in gradients lacking ethidium is expressed as a ratio relative to reference nucleoids sedimenting under the same conditions. Graph A, B and C refer to HeLa, XTC-2 and K85 cells respectively.

The data presented in each curve result from 2 sets of experiments; one (×—×) involving irradiation at a low dose rate (1·2 J kg^{-1} min^{-1}) relates to the left-hand ordinate, the other (●—●) involves higher dose rates (between 2·48 and 17·21 J kg^{-1} min^{-1}) and relates to the right-hand ordinate. In each case the scales and alignment of the ordinates permit a rough comparison of the sedimentation rates of nucleoids irradiated at the different dose rates. Error bars give the standard error of the mean.

A, between 0·5 – 1·0 × 10^5 HeLa cells were applied to each gradient, irradiated for different times, the time of irradiation being proportional to dose, before spinning for 25 min at 5000 or 10000 rev/min. Left-hand ordinate: a ratio of 1 refers to unirradiated nucleoids sedimenting under standard conditions (25 min, 5000 rev/min) in a reference tube; the distance sedimented under the same conditions by irradiated nucleoids (dose rate 1·20 J kg^{-1} min^{-1}) is expressed as a ratio relative to the distance sedimented by the unirradiated nucleoids in the reference tube. Right-hand ordinate: a ratio of 1 refers to irradiated nucleoids (4·80 J kg^{-1} at a dose rate of 1·20 J kg^{-1} min^{-1}) sedimenting under standard conditions (25 min, 10000 rev/min) in a reference tube; the distance sedimented under the same conditions by irradiated nucleoids (dose rates between 2·48 and 17·21 J kg^{-1} min^{-1}) is expressed as a ratio relative to the distance sedimented by the irradiated nucleoids in the reference tube.

B, between 0·5 – 1 × 10^5 XTC-2 cells were applied to each gradient, irradiated for different times before spinning for 25 min at 7000 or 12000 rev/min. Left-hand ordinate: a ratio of 1 refers to unirradiated nucleoids sedimenting under standard conditions (25 min, 7000 rev/min) in a reference tube; the distance sedimented under the same conditions by irradiated nucleoids (dose rate 1·20 J kg^{-1} min^{-1}) is expressed as a ratio relative to the distance sedimented by the unirradiated nucleoids in the reference tube. Right-hand ordinate: a ratio of 1 refers to irradiated nucleoids (2·40 J kg^{-1} at a dose rate of 1·20 J kg^{-1} min^{-1}) sedimenting under standard conditions (25 min, 12000 rev/min) in a reference tube; the distance sedimented under the same conditions by irradiated nucleoids (dose rate 2·48–4·16 J kg^{-1} min^{-1}) is expressed as a ratio relative to the distance sedimented by the irradiated nucleoids in the reference tube.

C, between 2·5–5 × 10^5 K85 cells were applied to each gradient and irradiated for different times before spinning for 25 min at 10000 or 15000 rev/min. Left-hand ordinate: a ratio of 1 refers to unirradiated nucleoids sedimenting under standard conditions (25 min, 10000 rev/min) in a reference tube; the distance sedimented under the same conditions by irradiated nucleoids (dose rate 1·20 J kg^{-1} min^{-1}) is expressed as a ratio relative to the distance sedimented by the unirradiated nucleoids in the reference tube. Right-hand ordinate: a ratio of 1 refers to irradiated nucleoids (4·80 J kg^{-1} at a dose rate of 1·20 J kg^{-1} min^{-1}) sedimenting under standard conditions (25 min, 15000 rev/min) in a reference tube; the distance sedimented under the same conditions by irradiated nucleoids (dose rates between 2·48 and 17·21 J kg^{-1} min^{-1}) is expressed as a ratio relative to the distance sedimented by the irradiated nucleoids in the reference tube.

hen erythrocytes has no significant effect on the sedimentation of HeLa nucleoids. Treated nucleoids sediment like untreated control nuclecids. It seems unlikely that any diffusible agent which affects nucleoid integrity is released from the hen erythrocytes after lysis.

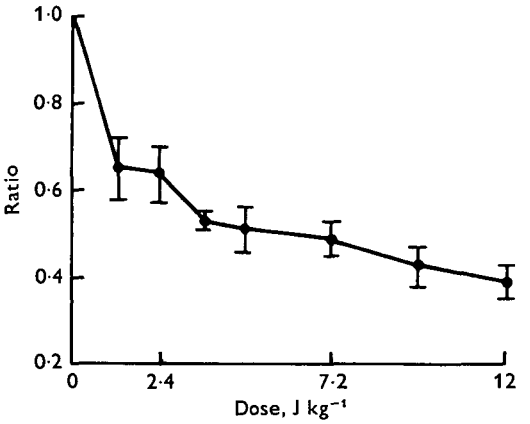

Fig. 5. Effect of irradiation dose on sedimentation of chicken nucleoids derived from embryonic fibroblasts. The distance sedimented by irradiated nucleoids in gradients lacking ethidium is expressed as a ratio relative to unirradiated reference nucleoids sedimenting under the same conditions. Between $0{\cdot}5$–1×10^5 cells were applied to each gradient, irradiated (dose rate $1{\cdot}20$ J kg^{-1} min^{-1}) for different times – the time of irradiation being proportional to dose – before spinning for 40 min at 7000 rev/min.

DISCUSSION

The procedure described in a previous paper (Cook & Brazell, 1975) for making nucleoids from human cells is generally applicable to the cells of insects, birds and amphibia. (These nucleoids are characterized further in the accompanying paper: Cook, Brazell & Jost, 1976.) The intercalating agent, ethidium, profoundly affects the rate of sedimentation of these nucleoids in sucrose gradients. The rate of sedimentation of nucleoids derived from dividing cells depends upon the concentration of intercalating agent in the gradient in a way characteristic of pure viral DNA that is circular and covalently continuous, suggesting that rotation of one strand of the DNA duplex about the other is similarly restricted in both nucleoid and viral DNA. In both kinds of DNA, some constraint keeps the topological winding number (α) constant.

Nucleoids derived from the different kinds of dividing cells all show a similar biphasic response of sedimentation rate to ethidium concentration, suggesting that all have the same average degree of supercoiling in their DNA. Irradiation reduces the sedimentation rate and abolishes the biphasic response. (The shape of one of these curves – that given by HeLa nucleoids – has been discussed earlier: Cook & Brazell, 1975.) A rough estimate of 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 nucleoids (cf. Figs. 4, 5 and 7; fig. 9 of Cook & Brazell, 1975). The higher doses of γ-irradiation used in the present work reveal targets of correspondingly smaller size than those found

Table 2. *Relative sedimentation rates of nucleoids derived from different chicken cells*

Chicken cell	Unirradiated			Irradiated, 9·6 J kg^{-1}			Unirradiated rate* / Irradiated rate
	Centrifuge speed, rev/min	Centrifuge time, min	Relative sedimentation rate*	Centrifuge speed, rev/min	Centrifuge time, min	Relative sedimentation rate*	
Fibroblast	7000	40	1·0	12000	40	0·24	4·2
Red blood cell from 5-day-old embryo	15000	40	0·2	20000	40	0·047	4·3
Red blood cell from 12-day-old embryo	20000	40	0·13	30000	40	0·041	3·2
Red blood cell from 18-day-old embryo	25000	40	0·08	30000	40	0·042	1·9
Red blood cell from adult hen	30000	60	0·034	30000	60	0·034	1·0

* See Legend to Table 1.

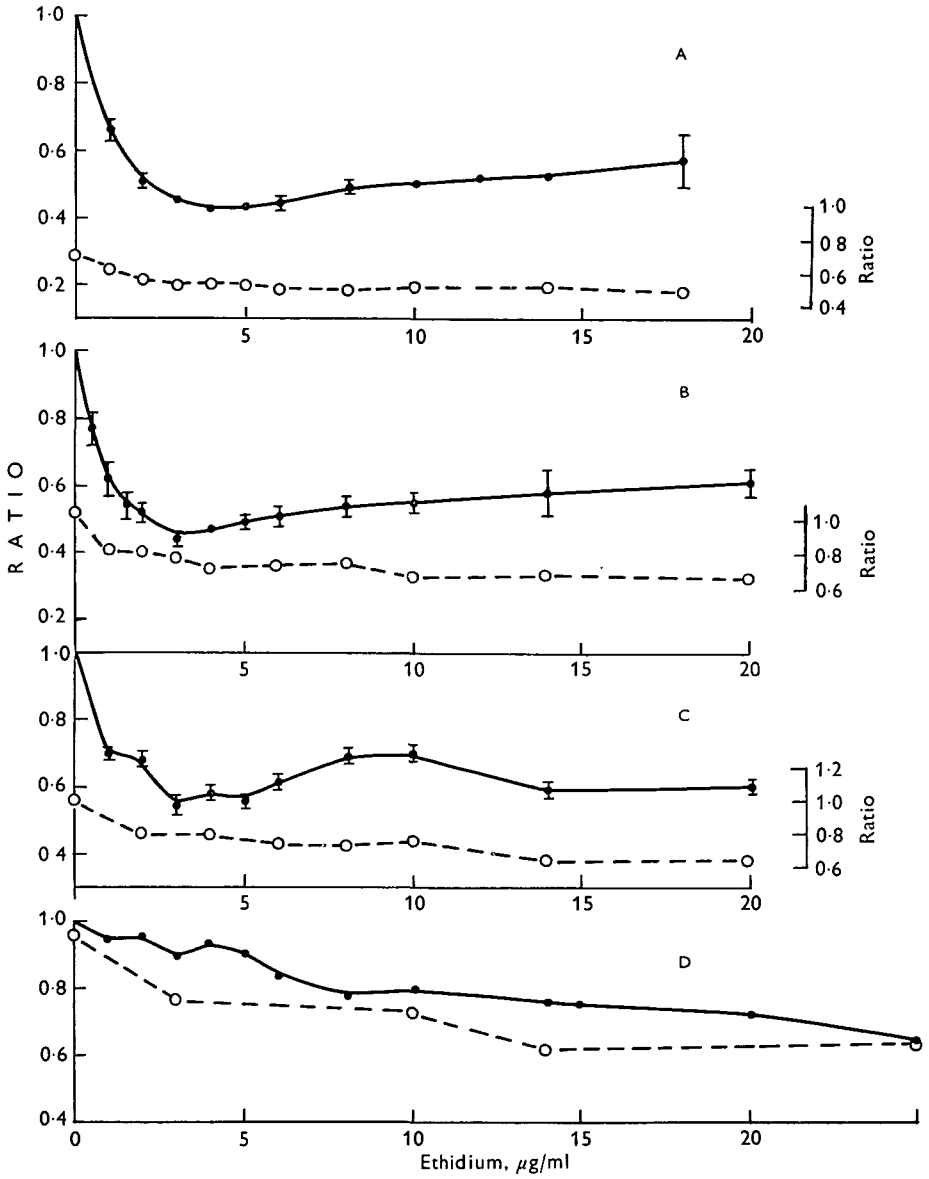

Fig. 6. Effect of ethidium on sedimentation of nucleoids derived from different red blood cells of the chicken. The distance sedimentated in gradients containing different concentrations of ethidium by nucleoids derived from various red blood cells is expressed as a ratio relative to reference nucleoids sedimenting under the same conditions. Graphs A, B, C and D refer to nucleoids derived from red blood cells of 5-day-old, 12-day-old, 18-day-old embryos and of adult hens, respectively. In each case the upper (●—●) and lower (○—○) curves relate to unirradiated and irradiated (9·60 J kg^{-1} at a dose rate of 1·20 J kg^{-1} min^{-1}) nucleoids. Between 0·5 – 2 × 10^5 cells were put on to each gradient. A ratio of 1 refers to unirradiated reference nucleoids sedimenting in gradients containing no ethidium (left-hand ordinate) or 5 μg/ml ethidium (right-hand ordinate). The alignment and scales of the ordinates permit a rough comparison of the sedimentation rates of irradiated and unirradiated

in the previous work. As higher doses are required to reduce the sedimentation rate of insect nucleoids to a minimum than are required with nucleoids from the other Orders, insect nucleoids contain correspondingly smaller targets. Whatever the exact size – one estimate is about 10^9 Daltons of DNA (Cook & Brazell, 1975) – the topological constraint acts over great lengths of DNA. Whether or not these targets correspond to any unit of function remains to be seen.

The topological constraint might result from the action of non-specific forces, in the sense that one ball of wool corresponding to one duplex strand might contain parts of that strand that are constrained by frictional forces, whilst another ball, composed of a strand of similar length but wound differently, might also be constrained, but in different places. However, this simple kind of non-specific mechanism is unlikely to be involved in constraining the DNA in nucleoids, for it would be expected that the more tightly wound the ball of wool, the more likely that sections of its strand would be constrained. Presumably nuclei of the different diploid chick cells contain similar strands of DNA, yet the more condensed the nucleus of the red cell, the less the DNA of its nucleoid is constrained; the most highly condensed nucleus of the hen erythrocyte yields a nucleoid with unconstrained DNA. Whatever the means by which the DNA is constrained, the mechanism is probably common to the range of cells studied and specific in the sense that the mechanism acts on restricted sections of the DNA.

The DNA of the different Orders studied here, of *Escherichia coli* (Worcel & Burgi, 1972) and of various viruses and plasmids (Bauer & Vinograd, 1971 has now been shown to be subject to topological constraints that maintain α invariant. It would seem likely that all genetically active DNA will be constrained. A wide variety of the linear DNA molecules of viruses have a base sequence at one end complementary to a base sequence at the other, such that hydrogen bonding between the ends would cause circle or loop formation concurrently with the imposition of a topological constraint (Thomas, 1967; Garon, Berry & Rose, 1975). This kind of circle or loop formation involving repetitive sequences of DNA and stabilized perhaps by the action of specific proteins or membrane attachment, might be the means whereby the linear molecules of eukaryotic DNA are constrained (Cook, 1973, 1974).

Nucleoids prepared from a range of chick cells having different biosynthetic activities, ranging from dividing and genetically active fibroblasts and erythroblasts to inert erythrocytes, have strikingly different properties. The integrity of nucleoids stained

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nucleoids. Error bars give the standard error of the mean. All gradients were spun for 40 min except those containing nucleoids derived from red cells of the adult hen – these were spun for 60 min.

The left-hand ordinates refer to unirradiated nucleoids spun at 15000 rev/min (A), 20000 rev/min (B), 25000 rev/min (C) or 30000 rev/min (D). Reference tubes contained unirradiated nucleoids sedimenting in the absence of ethidium under the same conditions.

The right-hand ordinates refer to irradiated nucleoids spun at 20000 rev/min (A) or 30000 rev/min (B, C). Reference tubes contained unirradiated nucleoids sedimenting in the presence of 5 μg/ml ethidium under the same conditions.

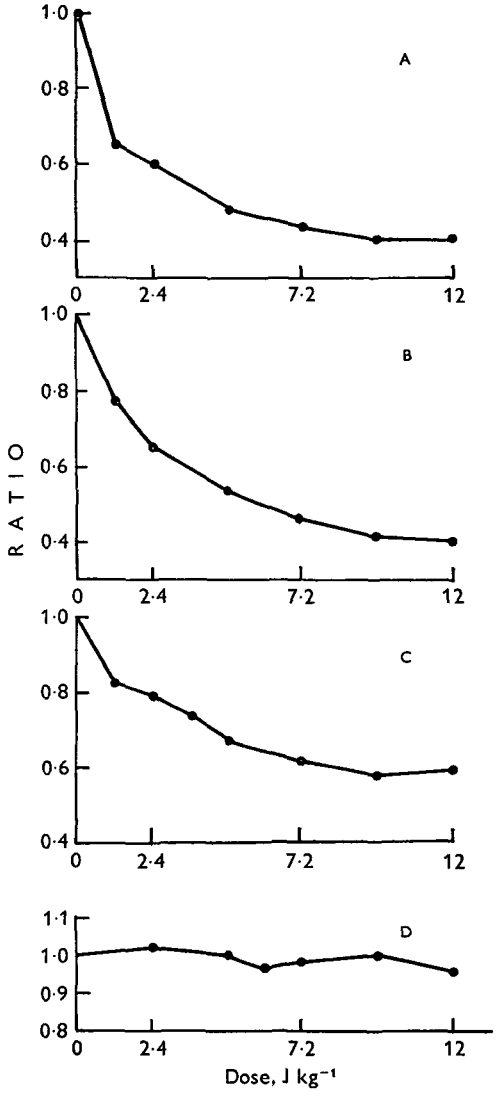

Fig. 7. Effect of irradiation dose on sedimentation of different chicken nucleoids. The distance sedimented by irradiated nucleoids derived from different red blood cells (in gradients lacking ethidium) is expressed as a ratio relative to unirradiated reference nucleoids sedimenting under the same conditions. Between $0{\cdot}5 - 2 \times 10^5$ cells were applied to each gradient, irradiated (dose rate $1{\cdot}20$ J kg^{-1} min^{-1}) for different times – the time of irradiation being proportional to dose – before spinning. A, nucleoids from red blood cells of 5-day-old embryos, spun at 15000 rev/min for 40 min. B, nucleoids from red blood cells of 12-day-old embryos, spun at 20000 rev/min for 40 min. C, nucleoids from red blood cells of 18-day-old embryos, spun at 25000 rev/min for 40 min. D, nucleoids from adult hen erythrocytes spun at 30000 rev/min for 60 min.

Table 3. *Relative distance sedimented by HeLa nucleoids in gradients containing different concentrations of ethidium*

	Ethidium bromide concentration, μg/ml		
	0	4	16
Untreated	1·0	0·54	0·78
Treated	0·94	0·50	0·66

The distance sedimented by HeLa nucleoids after treatment with extracts of lysed hen erythrocytes in gradients containing ethidium is expressed as a ratio relative to untreated reference nucleoids. 50-μl aliquots containing 20×10^6 hen erythrocytes in phosphate-buffered saline were added to 150 μl of lysis mixture floating on top of sucrose gradients. After 15 min, the gradients were spun at 50000 rev/min for 15 min at 4 °C, and then 150 μl of the pink solution at the top of the gradient removed. 300 μl of this pink solution which contained no nucleoids were added to 225 μl of fresh lysis mixture followed by 75 μl containing about 3×10^5 HeLa cells in phosphate-buffered saline. 200-μl aliquots of this mixture were added to 3 sucrose gradients containing 0, 4 and 16 μg/ml ethidium; 15 min after the addition of the HeLa cells to the lysis mixture the gradients were spun at 5000 rev/min for 25 min at 20 °C before determination of the position of the treated nucleoids in the gradients as usual. Suspensions of untreated nucleoids were prepared by mixing 450 μl of fresh lysis mixture with 150 μl of phosphate-buffered saline containing about 3×10^5 HeLa cells. 200-μl aliquots of this mixture were added to 3 sucrose gradients containing 0, 4 and 16 μg/ml ethidium and spun as before.

with ethidium may be monitored using a fluorescent microscope since DNA–ethidium complexes fluoresce brightly (Cook *et al.* 1976). Stained nucleoids derived from fibroblasts and embryonic red cells appear as discrete entities; these are relatively stable in the presence of 1·0 M NaCl. Their DNA seems to be compactly packaged within the nucleoid, since solutions of nucleoids are not viscous. On the other hand, when hen erythrocytes are added to a lysis mixture containing the non-ionic detergent Triton X-100 and 1·0 M NaCl, nucleoids are released which are initially visible as discrete entities. These then become progressively more diffuse so that within 15 min the intensity of fluorescence has diminished but extends over a greater area. The solution also becomes viscous, indicating that the DNA is no longer packaged so tightly. The 30-fold variation in rate of sedimentation of the different chick nucleoids also suggests that the DNA of the erythrocyte nucleoid is packaged differently. Irradiation of the different chick nucleoids reduces their rate of sedimentation by varying degrees; their sedimentation rate may be converted to that of hen erythrocyte nucleoids quite simply by irradiation. The irradiation removes constraints still present in the nucleoids obtained from embryonic red cells so that they become unconstrained like the nucleoids of the hen erythrocyte. Variations in sedimentation rate in gradients containing ethidium also indicate that the less constrained nucleoids are derived from the more mature red blood cells. It seems unlikely that the kinds of variation in sedimentation behaviour outlined above could result from anything other than differences in conformation and constraint of the DNA.

The DNA of the immature nucleoids remains constrained and supercoiled by some mechanism remaining operative in the presence of the detergent and high salt concen-

tration present in the lysis mixture. But in hen erythrocyte nuclei this mechanism is absent, so that in the lysis mixture the DNA disperses. The maturation of the erythroblast to erythrocyte would then be marked by a progressive loss of the constraint either by nicking of the DNA or by the loss of some specific constraining mechanism.

It has been suggested that DNA might contain 2 kinds of information: one kind which is stored in the primary base sequence, and a second kind contained in its superstructure, the superstructure controlling the transcriptional activity of the DNA. Superstructures of active and inactive genes might therefore be different. It has also been argued that superstructural information might be heritable (Cook, 1973, 1974). An essential corollary of these suggestions is that DNA should be constrained, so that defined superstructures can be maintained within genes. The evidence presented here suggests that the DNA of growing cells is constrained, whereas that of inactive nuclei is not. It remains to be seen whether inactive genes in active nuclei are unconstrained and to what extent structural differences are the cause or the effect of differentiation.

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REFERENCES

BAUER, W. & VINOGRAD, J. (1968). The interaction of closed circular DNA with intercalative dyes. I. The superhelix density of SV40 in the presence and absence of dye. *J. molec. Biol.* **33**, 141–171.

BAUER, W. & VINOGRAD, J. (1971). The use of intercalative dyes in the study of closed circular DNA. *Prog. molec. subcell. Biol.* **2**, 181–215.

BOLUND, L., RINGERTZ, N. R. & HARRIS, H. (1969). Changes in the cytochemical properties of erythrocyte nuclei reactivated by cell fusion. *J. Cell Sci.* **4**, 71–87.

COOK, P. R. (1973). Hypothesis on differentiation and the inheritance of gene superstructure. *Nature, Lond.* **245**, 23–25.

COOK, P. R. (1974). On the inheritance of differentiated traits. *Biol. Rev.* **49**, 51–84.

COOK, P. R. (1975). Linkage of the loci for glucose-6-phosphate dehydrogenase and for inosinic acid pyrophosphorylase to the X chromosome of the field-vole, *Microtus agrestis*. *J. Cell Sci.* **17**, 95–112.

COOK, P. R. & BRAZELL, I. A. (1975). Supercoils in human DNA. *J. Cell Sci.* **19**, 261–279.

COOK, P. R., BRAZELL, I. A. & JOST, E. (1976). Characterization of nuclear structures containing superhelical DNA. *J. Cell Sci.* **22**, 303–324.

DOLFINI, S. (1971). Karyotype polymorphism in a cell population of *Drosophila melanogaster* cultured *in vitro*. *Chromosoma* **33**, 196–208.

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99**, 167–199.

ECHALIER, G. & OHANESSIAN, A. (1970). *In vitro* culture of *Drosophila melanogaster* embryonic cells. *In Vitro* **6**, 162–172.

GARON, C. F., BERRY, K. W. & ROSE, J. A. (1975). Arrangement of sequences in the inverted terminal repetition of adenovirus 18 DNA. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3039–3043.

HARRIS, H., WATKINS, J. F., FORD, C. E. & SCHOEFL, G. I. (1966). Artificial heterokaryons of animal cells from different species. *J. Cell Sci.* **1**, 1–30.

PUDNEY, M., VARMA, M. G. R. & LEAKE, C. J. (1973). Establishment of a cell-line (XTC-2) from the South African Clawed Toad, *Xenopus laevis*. *Experientia* **29**, 466–467.

ROMANOFF, A. L. (1960). *The Avian Embryo*. New York: Macmillan.

THOMAS, C. A. (1967). The rule of the ring. *J. cell. Physiol.* **70**, Suppl., 1, 1–12.

VINOGRAD, J., LEBOWITZ, J., RADLOFF, R., WATSON, R. & LAIPIS, P. (1965). The twisted circular form of polyoma viral DNA. *Proc. natn. Acad. Sci. U.S.A.* **53**, 1104–1111.

WORCEL, A. & BURGI, E. (1972). On the structure of the folded chromosome of *Escherichia coli*. *J. molec. Biol.* **71**, 127–147.

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