PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Nucleosome Structure

P. Oudet, J. E. Germond, M. Bellard, C. Spadafora and P. Chambon

Phil. Trans. R. Soc. Lond. B 1978 **283**, 241-258
doi: 10.1098/rstb.1978.0021

Phil. Trans. R. Soc. Lond. B. **283**, 241–258 (1978) [241]
Printed in Great Britain

Nucleosome structure

By P. Oudet, J. E. Germond, M. Bellard, C. Spadafora and P. Chambon
Institut de Chimie Biologique, Laboratoire de Biologie Moléculaire des Eucaryotes du C.N.R.S. et U. 44 de l'INSERM, Faculté de Médecine, 67085 Strasbourg, France

[Plates 1–4]

Electron microscopic and biochemical results are presented supporting the following conclusions:

(1) Two molecules of each histone H2A, H2B, H3 and H4 are necessary and sufficient to form a nucleosome with a diameter of 12.5 ± 1 nm and containing about 200 base pairs of DNA.

(2) H3 plus H4 alone can compact 129 ± 8 DNA base pairs into a sub-nucleosomal particle with a diameter of 8 ± 1 nm. In such a particle the DNA duplex is under a constraint equivalent to negative superhelicity.

(3) Chromatin should be viewed as a dynamic structure, oscillating between a compact structure (the nucleosome) and more open structures, depending on the environmental conditions.

1. Introduction

Supporting the model proposed by Kornberg (1974), electron microscopy and biochemical studies have established that the bulk of the chromatin DNA is compacted into repeating structural units, termed nucleosomes (for references, see Kornberg 1977). Each nucleosome was initially thought to contain about 200 base pairs of DNA associated with a histone octamer core consisting of two molecules each of histones H2A, H2B, H3 and H4. In addition an association of one H1 histone molecule with the nucleosome was suggested (Kornberg 1974) and supported by direct evidence (Varshavsky, Bakayev & Georgiev 1976). It should be pointed out that it has not been rigorously demonstrated that all nucleosomes have an identical histone core composition, although results from reconstitution experiments (Oudet, Gross-Bellard & Chambon 1975), immunological studies (Bustin, Goldblatt & Sperling 1976; Simpson & Bustin 1976), chemical cross-linking (Thomas & Kornberg 1975*a*, *b*), and careful measurements of the relative amounts of the histones in various cells (Olins, Carlson, Wright & Olins 1976), are all in agreement with such a histone octamer core composition. Nuclease digestion studies have shown that the most frequent value for the nucleosome DNA content is about 200 base pairs, but striking variations have been recently found according to the origin of the chromatin (Compton *et al.* 1976*a*, *b*; Spadafora, Bellard, Compton & Chambon 1976; Morris 1976*a*, *b*; Noll 1976; Thomas & Furber 1976; Thomas & Thompson 1977; Lohr *et al.* 1977*a*, *b*; Johnson *et al.* 1976). In contrast, a particle termed 'nucleosome core' (Sollner-Webb & Felsenfeld 1975; Axel, Melchior, Sollner-Webb & Felsenfeld 1974; Shaw *et al.* 1976; Simpson & Whitlock 1976; Noll & Kornberg 1977), produced by further nuclease degradation of nucleosomes from all cell types so far analysed, is invariant in its DNA content (about 140 base pairs). All the histones of the nucleosome are present in the nucleosome core particle except H1 (Shaw *et al.* 1976; Simpson & Whitlock 1976; Noll & Kornberg 1977). These observations prompted us to analyse further whether all four histones are actually necessary to

reconstitute a nucleosome. The results presented here demonstrate that in fact equimolar amounts of all four calf thymus histones H2A, H2B, H3 and H4 are necessary and sufficient to reconstitute nucleosomes containing about 200 base pairs of DNA. However, we also found that H3 and H4 alone can compact about 130 base pairs of DNA into a subnucleosomal particle, in agreement with some recent evidence suggesting that H3 plus H4 alone can organize DNA segments of almost the length of the nucleosome core DNA (Boseley *et al.* 1976; Camerini-Otero, Sollner-Webb & Felsenfeld 1976; Sollner-Webb, Camerini-Otero & Felsenfeld 1976).

Implicit in the original model of Kornberg (Kornberg 1974; Thomas & Kornberg 1975) was the possibility that a nucleosome whose histone core was constituted of two each of the four histones could open up into two separate half nucleosomes provided the nucleosome would have a dyad axis of symmetry. Several different models have been put forward describing how DNA could be organized by the histones in the nucleosome (Varshavsky & Georgiev 1975; Li 1975; Van Holde, Sahasrabuddha & Shaw 1974; Hyde & Walker 1975; Baldwin, Boseley, Bradbury & Ibel 1975; Pardon *et al.* 1975). More recently two models (Weintraub, Worcel & Alberts 1976; Richards *et al.* 1977) of nucleosome structure based on two separate half nucleosomes have been proposed. Since hydrophobic interactions are responsible for histone–histone associations (Li 1975; D'Anna & Isenberg 1974*a*, *b*; Moss *et al.* 1976*a*, *b*; Roark *et al.* 1976), one of the predictions of a two half nucleosome model would be the formation of half nucleosomes under conditions decreasing hydrophobic interactions. We present here electron microscopic evidence suggesting that under appropriate conditions a nucleosome could open up into two separate half nucleosomes.

2. Materials and methods

Individual highly purified calf thymus histones and the H3–H4 histone pair were prepared and their concentration was estimated as described elsewhere (Oudet *et al.* 1977). Nuclei from CV1 cells infected with Simian virus 40 (SV40) were isolated as previously reported and further purified by centrifugation (15 min, 1000 *g* 4 °C) through a 0.3 M sucrose cushion containing 10 mM Tris–HCl, pH 7.5, 60 mM NaCl and 40 mM KCl. The nuclear pellet was gently resuspended at about 50 μg DNA/ml in the same buffer without sucrose. All other materials were as already described (Oudet *et al.* 1975; Germond *et al.* 1975, 1976; Bellard, Oudet, Germond & Chambon 1976; Hossenlopp, Wells & Chambon 1975).

Samples for electron microscopy were diluted in 10 mM Tris–HCl, pH 7.5, and processed as previously described (Oudet *et al.* 1975), except that the material was stained with 0.25% uranyl formate (in water). All measurements were performed as already reported (Oudet *et al.* 1975) or with the help of a Digistrand (Matra, France) connected to a 980 A computer (Texas Instruments).

Histones and DNA were associated at 20 °C by stepwise dilution in polypropylene conical tubes (Eppendorf, Germany). Two methods were used. In method A, DNA (200–300 μg/ml) and the histones were mixed, as indicated in the text and the legends, in buffer A (20 mM Tris–HCl, pH 8.0, 0.2 mM EDTA, 5 mM dithiothreitol, 0.1 mM paramethylsulphonyl fluoride (PMSF)) containing 2 M NaCl. After 1 h the association was carried out by stepwise dilution with buffer A to 1.6 M NaCl (15 min), 1.2 M NaCl (15 min), 0.9 M NaCl (1 h), 0.8 M NaCl (1 h), 0.7 M NaCl (1 h), 0.5 M NaCl (15 min). Samples of the material were then either diluted for electron microscopy or diluted to 200 M NaCl and prepared for gel electrophoresis (slab

gels 13 cm long, 26 h at 70 V) in order to separate the DNA molecules according to the number of superhelical turns as previously reported (Germond *et al.* 1975, 1976). In method B, the DNA only was brought to 2 M NaCl in buffer A. Histones and buffer A were then added sequentially to reduce the salt concentration to 1.2 M NaCl. Adding the histones decreased the NaCl concentration to about 1.5 M and care was taken to thoroughly mix the histones with the DNA before adding buffer A. After 30 min at 1.2 M NaCl, the association was pursued and the samples were processed as described above for method A.

All other methods were as previously reported (Oudet *et al.* 1975; Germond *et al.* 1975, 1976; Bellard *et al.* 1976) or as described in the text.

3. Results

(*a*) *Equimolar amounts of the four histones H2A, H2B, H3 and H4 are necessary and sufficient to reconstitute nucleosomes*

A nucleosome can be defined as a bead-like structure of about 12.5 nm in diameter in which a DNA duplex segment of about 200 base pairs is compacted under a constraint equivalent to about one negative superhelical turn (Oudet *et al.* 1975; Germond *et al.* 1975; Keller 1975; Shure & Vinograd 1976). Therefore, using electron microscopy, the formation of nucleosomes on a DNA molecule of given length can be monitored by counting the number of beads with a diameter of 12.5 ± 1.0 nm (Oudet *et al.* 1975) and by showing, by measurements of the apparent reduction in length of the DNA molecule, that these beads contain on the average about 200 base pairs of DNA (Oudet *et al.* 1975; Bellard *et al.* 1976). In addition, when the nucleosomes are formed on a covalently closed DNA, it is possible, by using gel electrophoresis under appropriate conditions, to correlate the formation of the nucleosomes with the number of superhelical turns present in the covalently closed DNA (Germond *et al.* 1975, 1976). We have used these two methods to demonstrate that all four calf thymus histones are necessary to form a nucleosome.

(i) *Electron microscopic analysis*

Nucleoprotein complexes were formed by associating SV40 DNA form I (superhelical closed circular form) and the purified calf thymus histones as described in §2 and in the legend to figure 1. In a typical experiment, SV40 DNA form I was mixed with three out of the four H2A, H2B, H3 and H4 histones at a ratio of 1.8 molecules of each histone per 200 base pairs of DNA. The fourth histone was then added at either 0.6, 1.2, 1.8 or 2.4 molecules per 200 base pairs of DNA as indicated in figure 1. (In this paper all histone:DNA ratios are expressed as histone molecules per 200 base pairs of DNA.) After the end of the reconstitution period, aliquots were taken, mounted on grids and examined by electron microscopy. For each point the number of beads with a diameter of 12.5 ± 1.0 nm was counted and plotted against the fourth histone:DNA ratio (figure 1, panels *a–d*). In addition, in each case the amount of DNA compacted in the beads was determined at the fourth histone:DNA ratio of 1.8 by measuring the contour length of the reconstituted nucleoprotein complex. In all cases we have found an average value of 200 ± 20 base pairs per bead. These determinations, which are in good agreement with our previous estimations (Oudet *et al.* 1975; Germond *et al.* 1975) of the DNA length folded in a nucleosome reconstituted with calf thymus histones, indicate that the 12.5 nm beads which were counted were actually nucleosomes.

The results shown in figure 1 demonstrate clearly that all four histones are required in order

to reconstitute a nucleosome. In all four cases, there was an almost linear increase in the number of nucleosomes as the fourth histone was increased until a plateau value was reached when the fourth histone to DNA ratio was identical to that of the three other histones. Therefore a nucleosome contains equimolar amounts of the four histones.

Although no 12.5 nm beads were observed when one of the four histones was missing, the DNA did not look naked. An irregular thickening of the DNA molecule was observed in the

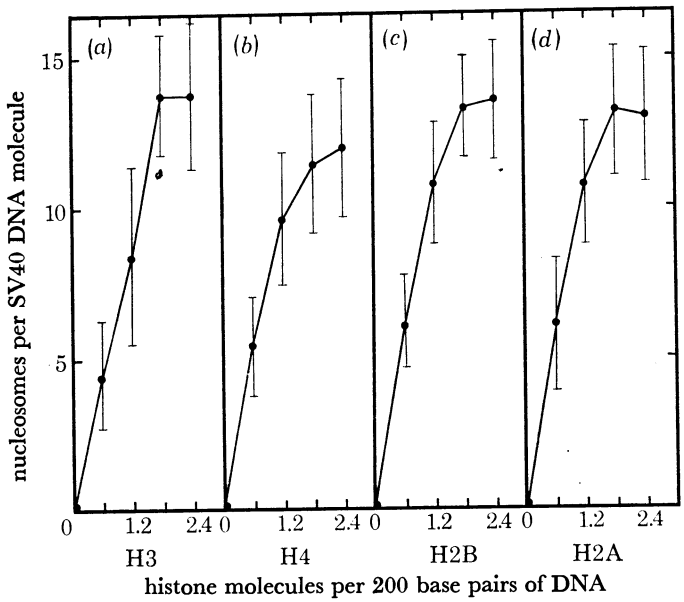


FIGURE 1. Formation of nucleosomes as a function of the addition of the fourth histone. SV40 DNA form I was associated (method A) to three out of the four calf histones H2A, H2B, H3 and H4 and increasing amounts of the fourth histone as indicated in the figure. The three histones at a ratio of 1.8 molecules each to 200 base pairs of DNA were as follows: (*a*) H2A, H2B and H4; (*b*) H2A, H2B and H3; (*c*) H2A, H3 and H4; (*d*) H2B, H3 and H4. The number of nucleosomes per DNA molecule was determined by electron microscopy after dilution to 3.5 mM NaCl. For each point, 30 molecules at least were counted and the standard deviation was calculated (vertical bars).

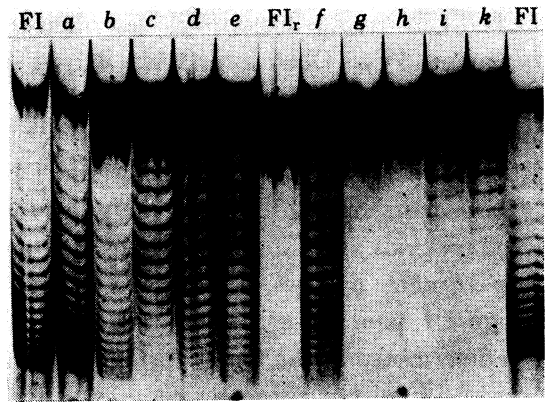


FIGURE 2. Gel electrophoresis of DNA from u.e.-treated complexes formed *in vitro* (method A) from SV40 DNA form I or form I_r and the four calf thymus histones or three out of the four histones. Complexes were reconstituted at a histone:DNA ratio of 2 molecules of each histone to 200 base pairs of DNA as follows: (*a*)–(*e*), complexes reconstituted with DNA form I; (*f*)–(*k*), complexes reconstituted with DNA form I_r; (*a*) and (*f*), all four histones were present; (*b*) and (*g*), H3 was omitted; (*c*) and (*h*), H4 was omitted; (*d*) and (*i*), H2A was omitted; (*e*) and (*k*), H2B was omitted. FI and FI_r, control untreated DNA form I and form I_r, respectively.

absence of histones H3 or H4, whereas thickening of the DNA as well as bead-like with variable diameters, smaller than that of the nucleosome, were observed in the absence of H2A or H2B.

(ii) *Gel electrophoretic analysis*

In order to analyse whether all four histones are required to impose a constraint on the DNA duplex, we have determined the number of superhelical turns which remained in the DNA molecule after treatment of the reconstituted nucleoprotein complexes with an untwisting activity (u.e.) (Germond *et al.* 1975). Nucleoprotein complexes were reconstituted from SV40 DNA form I or form I_r (relaxed covalently closed circular form) in the presence of all four histones or of three out of the four histones. After u.e. treatment of the complexes and deproteinization, the DNA was analysed by gel electrophoresis (figure 2). As previously shown (Germond *et al.* 1975; Keller 1975) relaxed SV40 DNA molecules (f.I_r) have the slowest migration rate in this gel system. Molecules which contain superhelical turns are separated according to the number of superhelical turns present, neighbouring bands corresponding to molecules differing by one turn. In agreement with our previous results (Germond *et al.* 1975),

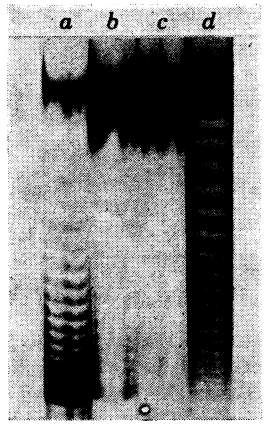


FIGURE 3. Gel electrophoresis of DNA from u.e.-treated complexes formed *in vitro* (method B) from SV40 DNA form I and the arginine-rich histones H3 and H4. (*a*) Control untreated DNA form I; (*b*) H3 alone at a ratio of 4 molecules per 200 base pairs of DNA; (*c*) H4 alone at a ratio of 4 molecules per 200 base pairs of DNA; (*d*) H3 and H4 at a ratio of 4 molecules each per 200 base pairs of DNA.

there was a good correlation between the average number of nucleosomes per complex and the average number of superhelical turns per DNA molecule, when all four histones were present (figure 2*a* and *f*, the number of nucleosomes being 18 ± 4 and 13 ± 4 on the nucleoprotein complexes reconstituted with DNA form I and DNA form I_r, respectively). Omitting H3 (figure 2*b* and *g*) or H4 (figure 2*c* and *h*) resulted in a very low number of superhelical turns per DNA molecule after u.e. treatment, whereas molecules with superhelical turns were visible when H2A (figure 2*d* and *i*) or H2B (figure 2*e* and *k*) were missing. Although it was surprising that in the two latter cases the average number of superhelical turns per molecule was much higher when the complexes were made with SV40 form I than with form I_r (compare figure 2*d* and *i*, and *e* and *k* – see below and Discussion for a possible explanation of this discrepancy), these observations suggested that H3 and H4 alone could fold the DNA, resulting in a constraint revealed by the presence of superhelical turns in the deproteinized DNA after u.e. treatment of the reconstituted complexes. Figure 3 shows very clearly that it is indeed the

H3–H4 pair which is responsible for the appearance of a constraint in the DNA of complexes reconstituted with these histones, since no superhelical molecules were obtained for complexes reconstituted with H3 or H4 alone (figure 3*b* and *c*), whereas most of the DNA molecules obtained from H3–H4 nucleoprotein complexes were supercoiled (figure 3*d*).

(*b*) *A subnucleosomal particle can be formed with histones H3 and H4 alone*

The results so far described prompted us to study in detail the structure of H3–H4 reconstituted nucleoprotein complexes. As will be shown below, electron microscopic studies and gel electrophoretic analysis of the constraint imposed on the DNA have led us to the conclusion that H3 and H4 alone can compact about 130 base pairs of DNA in a bead-like particle with a diameter of 8.0 ± 1.0 nm in which the DNA is under a constraint equivalent to negative superhelicity.

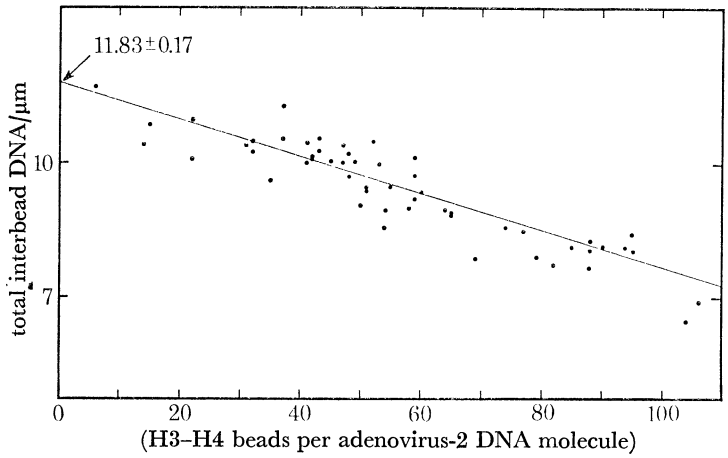


FIGURE 6. Determination of the DNA length associated with H3–H4 beads. The H3–H4 histone pair and adenovirus-2 DNA were associated (method B) at different histone:DNA ratios as described in the legend to figure 5. The length of the total interbead DNA was plotted as a function of the number of H3–H4 beads present on the adenovirus-2 DNA molecules. The method of least squares was used to determine the best fitting line.

(i) *Electron microscopic studies reveal the presence of H3–H4 beads containing about 130 base pairs*

SV40 DNA molecules form I (figure 4*c*, plate 1), form I_r (figure 4*d*) and form III (linear form) (figure 4*e*) were associated with H3 and H4 histones and examined by electron microscopy after spreading in a low ionic strength buffer as described in the legend to figure 4. In all these cases, bead-like structures were observed, connected by filaments whose diameters appeared identical to that of naked DNA. These beaded particles are clearly smaller (average diameter 8.0 ± 1.0 nm) and less compact than the nucleosomes (diameter 12.5 ± 1.0 nm) which are shown for comparison in figure 4*g*. These smaller particles were not made visible in the presence of H3 or H4 alone (not shown) and were not electron microscopic artefacts formed on the grids during spreading, since identical results were obtained after fixation of the nucleoprotein complexes in solution with 1% formaldehyde (not shown).

It is apparent from examination of figure 4 that the formation of H3–H4 particles was accompanied by a compaction of the DNA, since the contour length of the H3–H4 complexes appears shorter than that of pure SV40 DNA, (figure 4*a*). No DNA compaction was observed with H3 or H4 alone (not shown). Since SV40 DNA is too short to allow accurate determination

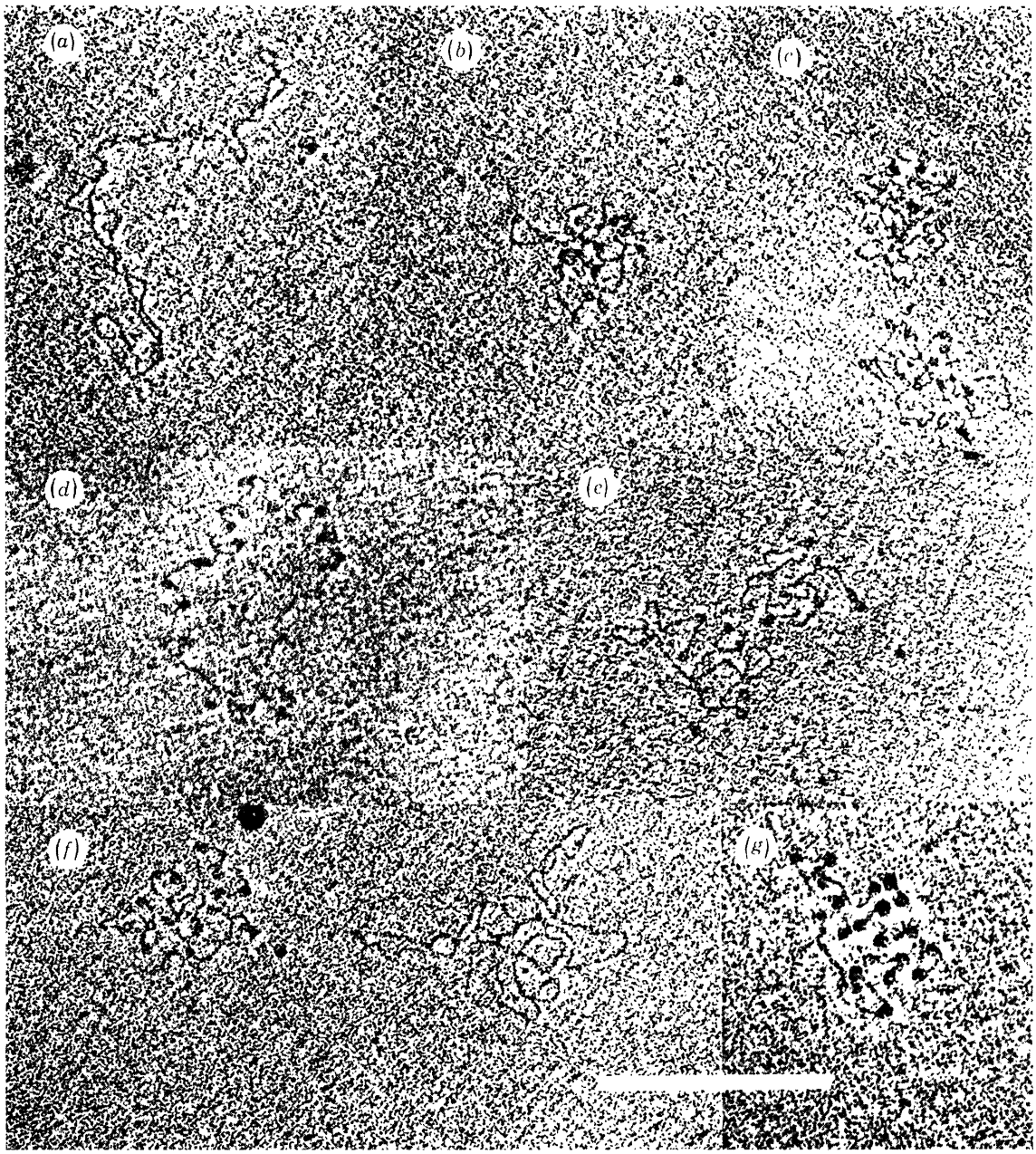


FIGURE 4. Association of SV40 DNA with the calf thymus H3–H4 histone pair. All associations were performed according to method B. (*a*) SV40 DNA form I alone examined in 50 mM NaCl; (*b*) and (*c*), DNA form I was associated with the H3–H4 pair (4 molecules of each histone per 200 base pairs of DNA) and examined after dilution to 50 mM NaCl (*b*) or 2 mM NaCl (*c*); (*d*) and (*e*), DNA form I_r and form III, respectively, were associated with the H3–H4 pair (4 molecules of each histone per 200 base pairs of DNA) and examined after dilution to 2 mM NaCl; (*f*) a mixture of identical amounts of DNA form I and form I_r was associated with the H3–H4 pair (2.8 molecules of each histone per 200 base pairs of DNA) and examined after dilution to 2 mM NaCl; (*g*) SV40 DNA form I was associated (method A) with the four calf thymus histones H2A, H2B, H3 and H4 at a ratio of 2 molecules of each histone per 200 base pairs of DNA and examined after dilution to 2 mM NaCl. The bar indicates 0.25 μm.

(*Facing p.* 246)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

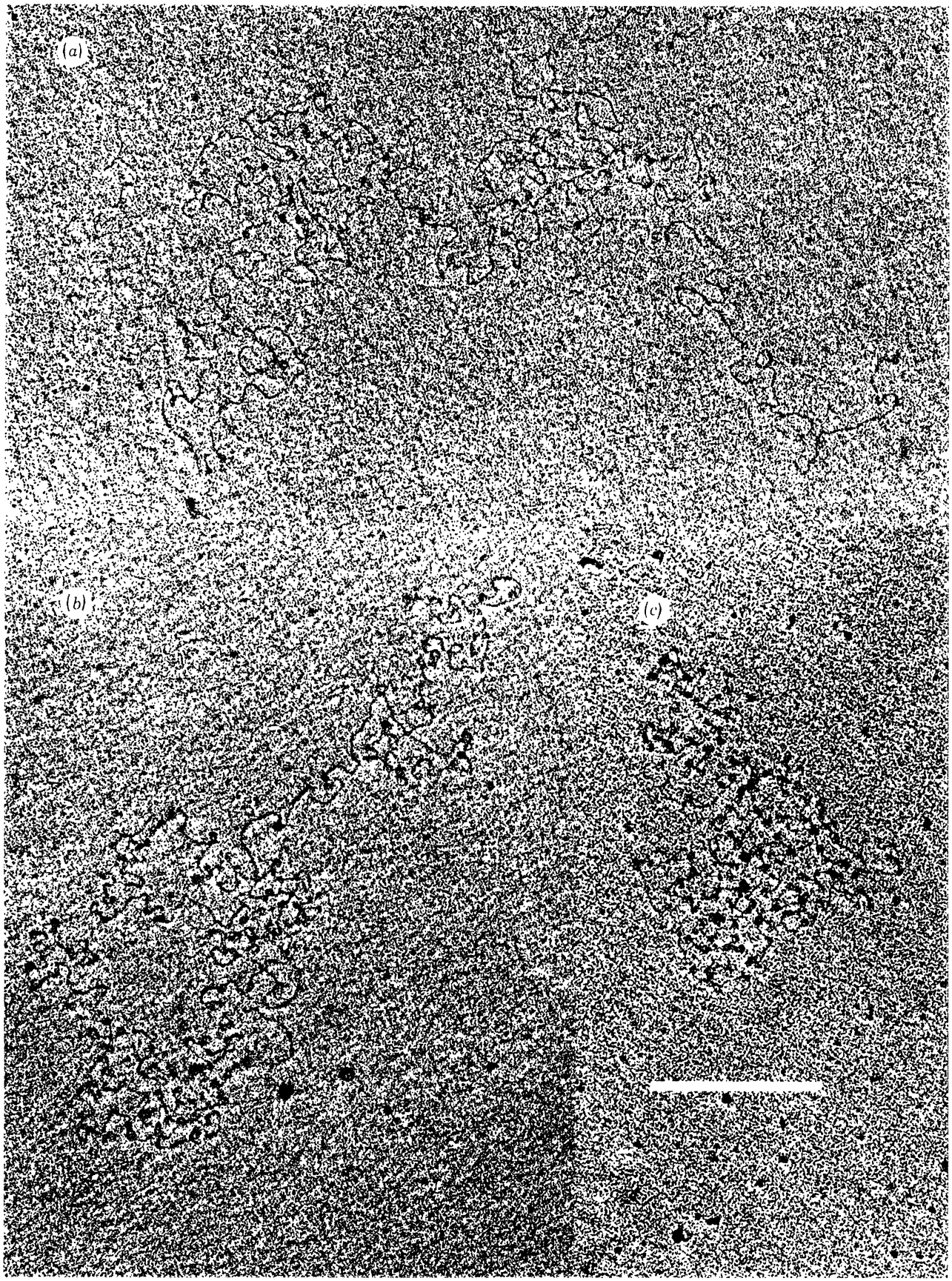


FIGURE 5. Association of adenovirus-2 DNA with the calf thymus H3–H4 histone pair. Adenovirus-2 DNA and the H3–H4 pair were associated (method B) at ratios of 2 molecules (*a*), 3.2 molecules (*b*) and 4 molecules (*c*) of each histone per 200 base pairs of DNA. Samples were examined after dilution to 2 mM NaCl. The bar indicates 0.25 μm.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

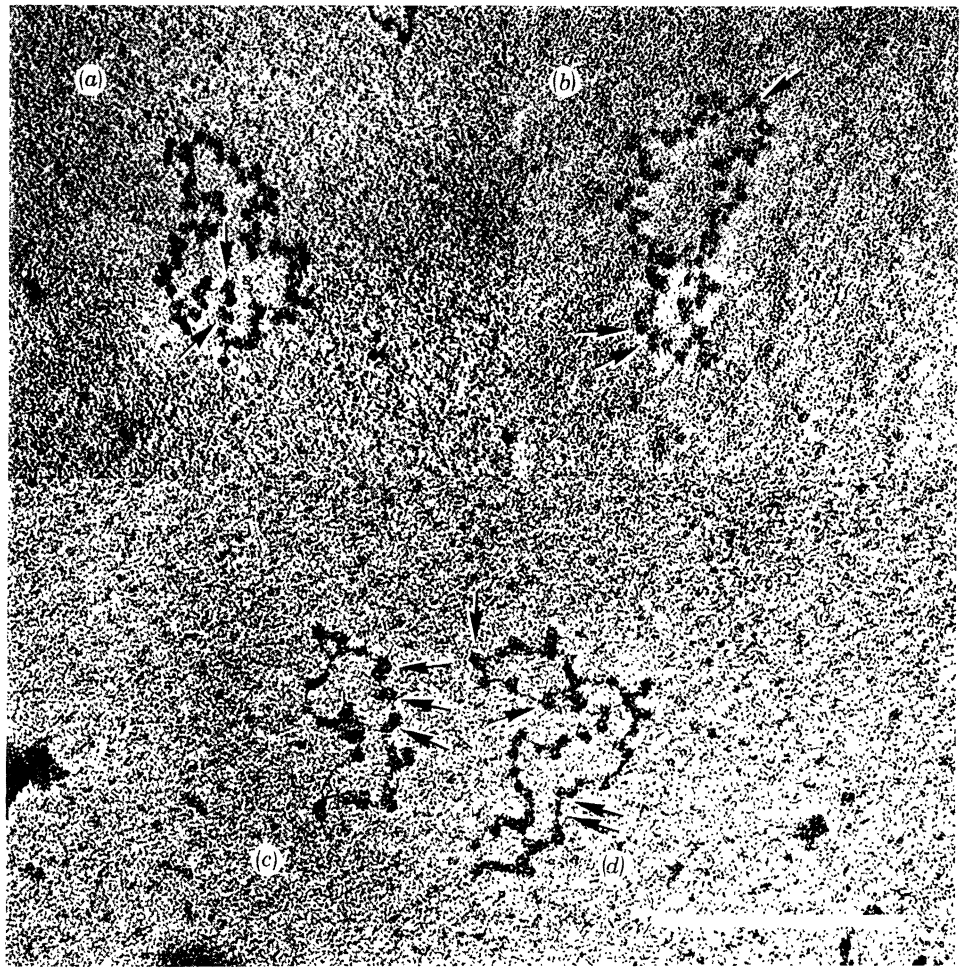


FIGURE 9. SV40 minichromosomes after incubation of nuclei from CV1 infected cells in a low ionic strength buffer (see text). Contour lengths of the circular structures were 0.64, 0.65 and 0.83 μm, for minichromosomes *a*, *b* and *d*, respectively. Minichromosome *c* was not measured due to the difficulty of tracing its contour. The bar represents 0.25 μm.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

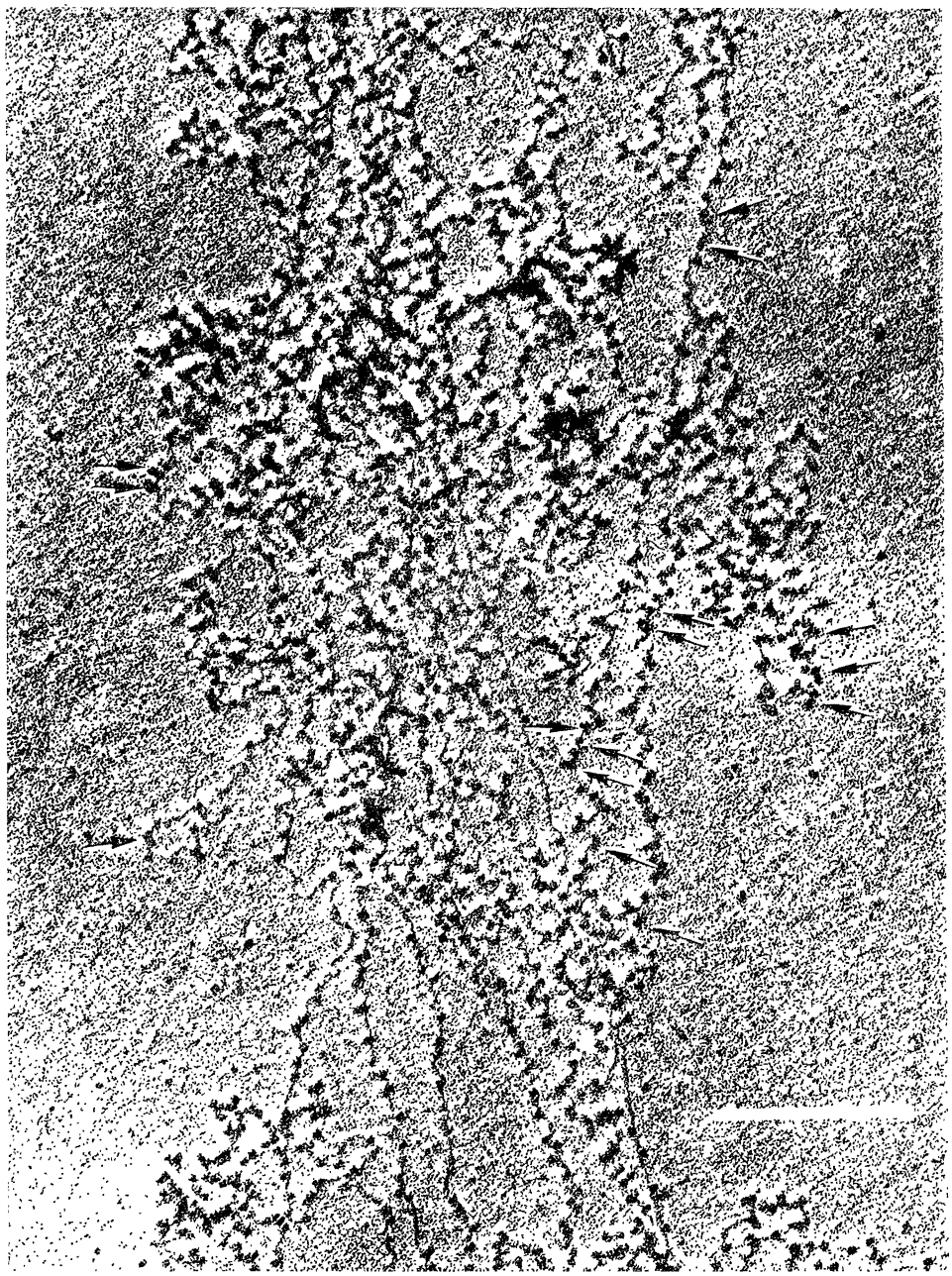


FIGURE 10. Cellular chromatin from CV1 infected cells after incubation of purified nuclei in a low ionic strength buffer (see text). The bar represents 0.25 μm.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

of the contour length reduction as a function of the number of H3–H4 beads, H3–H4 nucleoprotein complexes were reconstituted with adenovirus-2 DNA. As shown in figure 5, plate 2, similar H3–H4 beads were obtained. At increasing histone:DNA ratio, the number of beads clearly increased, while the contour length of the nucleoprotein complex decreased. A statistical analysis of the length of the total interbead DNA as a function of the number of H3–H4 beads indicates a very good correlation ($r = 0.91$) between the number of beads and the length of naked DNA. Extrapolation to zero H3–H4 bead gives a value of 11.8 ± 0.2 μm (35000 base pairs) for the adenovirus-2 DNA molecule in very good agreement with a previous measurement of the length of deproteinized adenovirus-2 DNA molecule (Oudet *et al.* 1975). The slope of the best fitting line (figure 6) indicates that an H3–H4 bead contains 129 ± 8 DNA base pairs, assuming that the mass per unit length for DNA measured by the Dubochet's method is 1.96×10^6/μm (Oudet *et al.* 1975).

TABLE 1. H3–H4 'SUBNUCLEOSOMAL PARTICLES' ARE FORMED PREFERENTIALLY ON SUPERHELICAL DNA

SV40 DNA form in the association mixture	number of 'beads' per SV40 DNA molecule		
	DNA form I	DNA form I_r	DNA form III
DNA FI + FI_r	12.8 ± 2.0	3.2 ± 1.7	—
DNA FI + FIII	12.4 ± 1.5	—	4.4 ± 1.4

The H3–H4 histone pair and the various SV40 DNA forms (in a 1:1 ratio) were associated (method B) at a histone:DNA ratio of 2.4 molecules of each histone to 200 base pairs of DNA. The number of H3–H4 beads per DNA molecule was counted by electron microscopy after dilution to 2 mM NaCl. In each case more than 30 DNA molecules were examined. The numbers are mean ± standard deviation.

(ii) *Electron microscopic and gel electrophoresis studies indicate that the constraint imposed on the DNA duplex in H3–H4 beads is equivalent to negative superhelicity*

Electron microscopic comparison of SV40 DNA form I molecules and H3–H4 SV40 nucleoprotein complexes adsorbed onto the charged grids at 50 mM NaCl (in order to make superhelicity visible (Germond *et al.* 1975)), shows that the superhelix density of the complex (figure 4*b*) is not higher than that of the deproteinized DNA (figure 4*a*). Since the contour length of the complex is shorter than that of pure DNA, this observation suggests that the free energy contained in the negative superhelical turns of SV40 DNA form I is used during the formation of H3–H4 beads (Vinograd, Lebowitz & Watson 1968; Davidson 1972). This possibility is further supported by the results presented in table 1 which demonstrate that H3–H4 beads are formed more readily with SV40 DNA form I than with its allomorphic forms I_r and III which have a lower free energy. Figure 4, panel *f*, illustrates the preferential formation of H3–H4 beads on DNA form I, when H3–H4 histones were associated with a mixture of DNA form I and form I_r. These results, together with those already presented in figure 3, indicate that the constraint imposed on the DNA in H3–H4 beads is equivalent to negative superhelicity (Vinograd *et al.* 1968).

In order to estimate the magnitude of this constraint, SV40 DNA form I and form I_r were associated with increasing amounts of H3–H4 histones. The number of H3–H4 beads per SV40 DNA molecule increased with increasing histone to DNA ratios (table 2). However, for unknown reasons (see §4), the yield of H3–H4 beads was unexpectedly low, particularly at the lower H3–H4 to DNA ratios, since a 100% yield would give about nineteen H3–H4

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

beads per SV40 DNA molecule at a ratio of 1.6 molecules of each histone per 200 base pairs of DNA. The constraint of the DNA in the H3–H4 beads was directly estimated at each histone:DNA ratio by treating the histone–DNA complexes with u.e. in order to relieve any extra-bead superhelicity. After deproteinization the DNA was analysed for superhelicity by gel electrophoresis. There was clearly an increase in the average number of superhelical turns per DNA molecule when the H3–H4 to DNA ratios were increased in the association

TABLE 2. FORMATION OF H3–H4 'SUBNUCLEOSOMAL PARTICLES' AS A FUNCTION OF HISTONE:DNA RATIO

H3–H4/DNA (molecules of each histone per 200 base pairs	number of 'beads' per SV40 DNA molecule	
	DNA form I	DNA form I_r
1.6	4.5 ± 1.7	4.0 ± 1.0
2.4	6.6 ± 1.1	6.3 ± 1.2
3.2	14.1 ± 1.9	9.8 ± 1.6
4.0	16.4 ± 1.6	12.2 ± 1.2

SV40 DNA form I or form I_r and the H3–H4 histone pair were associated according to method B at different ratios as indicated in the table. The number of H3–H4 beads per DNA molecule was determined by electron microscopy after dilution to 2 mM NaCl. In each case more than 30 DNA molecules were examined. The numbers are mean ± standard deviation.

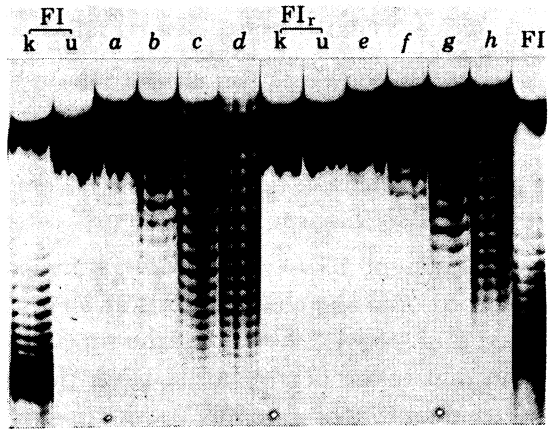


FIGURE 7. Gel electrophoresis of DNA from u.e.-treated complexes formed *in vitro* with SV40 DNA form I (*a–d*) or form I_r (*e–h*) and the H3–H4 histone pair as described in the legend to table 2. Ratios of histone:DNA expressed as molecules of each histone for 200 base pairs of DNA were as follows: 1.6 (*a* and *e*), 2.4 (*b* and *f*), 3.2 (*c* and *g*) and 4 (*d* and *h*). FI and FI_r are control DNA form I and form I_r, untreated (k) or treated (u) with u.e.

mixture (figure 7). The increase roughly paralleled the increase in the number of H3–H4 beads (table 2) whether the DNA present in the association mixture was DNA form I (figure 7*a–d*) or DNA form I_r (figure 7*e–h*). However, in both cases and for a given histone:DNA ratio, the average number of superhelical turns in the DNA molecules after u.e. treatment and deproteinization appears to be slightly lower than the average number of H3–H4 beads per complex as determined by electron microscopy (table 2).

Since the constraint imposed on the DNA duplex in a nucleosome is equivalent to about 1.0 negative superhelical turn (Germond *et al.* 1975), it was interesting to study the effect of the addition of H2A or H2B during the association of H3–H4 histones to the DNA. As already mentioned (§3(*a*)(i)) the electron microscopic examination of complexes formed in the

presence of H3–H4 plus H2A or H2B did not reveal the presence of well defined structures which could correspond to intermediate assembling steps between the H3–H4 beads and the nucleosomes. The results shown in figure 8*a–e* indicate that there was a significant increase in the average number of superhelical turns per DNA molecule when H2A or H2B histones were added to an association mixture containing SV40 DNA form I and H3–H4 histones (compare, for example, slots *a*, *c* and *e*). However, in the presence of SV40 DNA form I_r in

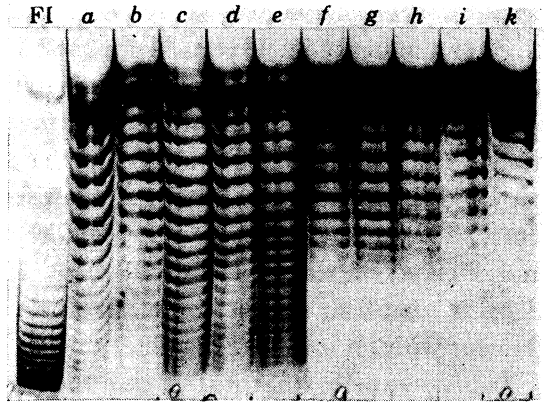


FIGURE 8. Gel electrophoresis of DNA from u.e.-treated complexes formed *in vitro* (method B) with SV40 DNA form I (*a–e*) or form I_r (*f–k*) and the H3–H4 histone pair in the absence (*a*, *f*) or in the presence of H2A (*b*, *c*, *g*, *h*) or H2B (*d*, *e*, *i*, *k*). The H3–H4:DNA ratio was 3.2 of each histone to 200 base pairs of DNA. Ratios of H2A or H2B histone expressed as molecules per 200 base pairs of DNA were 2 (*b*, *d*, *g* and *i*) or 4 (*c*, *e*, *h* and *k*). FI, control DNA form I.

the association mixture (figure 8*f–k*), the addition of histone H2A and H2B to H3–H4 histones resulted in a decrease in the average number of superhelical turns per DNA molecule (compare, for example, slots *f*, *h* and *k*). These contradictory results are best explained by assuming that the addition of histone H2A or H2B prevents u.e. from relieving the inter-bead superhelicity, which in the case of DNA form I results in an artefactual increase in the number of superhelical turns, whereas it leads to an artefactually small number of superhelical turns in the case of DNA form I_r. (Stabilization of pre-existing superhelical turns has been reported by Bina-Stein & Singer (1977) in the case of histone H1 addition and we have observed a similar effect (unpublished result) by mixing directly in 200 mM NaCl SV40 DNA and various histone combinations.)

(*c*) *Electron microscopy supports the view that a nucleosome may be constituted of two half nucleosomes*

A well defined chromatin, the SV40 minichromosome which consists of 20–24 nucleosomes (for references, see Bellard *et al.* 1976) was chosen in order to attempt the electron microscopic characterization of half nucleosomes. Since low ionic strength and low temperature are known to decrease hydrophobic interactions, purified nuclei from infected CV1 cells (§2) were diluted at 0 °C in a 1 mM triethanolamine HCl buffer (pH 8.5) containing 0.2 mM EDTA (final DNA concentration 3 μg/ml). After a 15–30 min incubation period, the lysed nuclei were diluted tenfold in the same buffer. Aliquots were either directly adsorbed on charged grids or centrifuged onto charged grids through 1 mM borate buffer (pH 8.5) (Miller & Bakken 1972). When the material was adsorbed on the charged grids without prior incubation at

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

0 °C, typical SV40 minichromosomes (Bellard *et al.* 1976) containing 20–24 nucleosomes were visible (not shown). In contrast, after a 15–30 min incubation period at 0 °C, all of the SV40 minichromosomes were replaced by circular structures (figure 9*a* and *b*, plate 3) with a contour length similar to that of typical SV40 minichromosomes, but consisting of 40–50 beads with a diameter of 9.3 ± 1.0 nm smaller than that of nucleosomes (12.5 ± 1.0 nm). That these smaller beads were derived from nucleosomes was indicated by the presence of intermediate structures showing the progressive disappearance of the nucleosomes and the concomitant appearance of the smaller beads (not shown). The observation that the final number of smaller beads was approximately twice the original number of nucleosomes suggested to us that each nucleosome was split into two half nucleosomes upon incubation at low ionic strength. This possibility was further supported by the observation of half nucleosome pairs (arrows, figure 9*a* and *b*). These pairs were more clearly seen when the incubated material was centrifuged onto a grid and positively stained only (arrows, figure 9*c* and *d*). However, centrifugation appears to stretch the half-nucleosomal structure. The small beads were indeed more spaced in figure 9*c* and *d* than in figure 9*a* and *b*, and often connected by a DNA filament. It seems even that in some cases centrifugation could bring about an almost complete unravelling of the nucleosomal structure, resulting in a filament with an irregular diameter thicker than that of naked DNA (figure 9*d*, double arrow). It is noteworthy that in this case the contour length of the minichromosome was significantly increased. If, after a 20 min incubation period at low ionic strength, the salt concentration was increased to 20 mM NaCl immediately before adsorption on the grids, the original nucleosomal structure was recovered (not shown). It seems therefore that the opening of the nucleosomes at low ionic strength may involve only the disruption of bonds which are easily reformed when the ionic strength is increased.

Two lines of evidence indicate that the conversion of nucleosomes to half nucleosomes and its reversion by increasing the salt concentration were not artefacts arising on the grids, but were in fact taking place in solution. The same half nucleosomal structures were obtained when the material incubated at low ionic strength was fixed with 1 % formaldehyde (pH 7.5, 5 min, 20 °C) before its adsorption on the charged grids. Moreover, when the material incubated at low ionic strength was fixed under the same conditions before the addition of 20 mM NaCl, we did not observe any reversion from half nucleosomal to nucleosomal structure.

The conversion of nucleosomes to half nucleosomes was not restricted to the viral chromatin, but was also observed for the cellular chromatin of the infected cells. Figure 10, plate 4, shows the cellular chromatin which was incubated at 0 °C for 30 min in the triethanolamine buffer. Most of the material consists of closely spaced beads with a diameter (9.5 ± 1.0 nm) identical to that of half nucleosomes of SV40 minichromosomes. It should be stressed that adsorption of the material on the grids without incubation at 0 °C resulted in a chromatin structure identical to that previously described for rat liver chromatin (Oudet *et al.* 1975), consisting of chains of nucleosomes with a diameter of 12.5 ± 1.0 nm. Pairs of half nucleosomes very similar to those of the SV40 chromatin were very frequently observed in unstretched segments of the chromatin fibre after incubation at low ionic strength (figure 10, arrows). On the contrary, on stretched fibres, not only half nucleosome pairs were absent, but the remaining half nucleosomes were irregularly spaced along a filament with variable diameter thicker than that of naked DNA. These observations suggest that a transition from nucleosomes to half nucleosomes and eventually to a completely extended structure could also take place in cellular chromatin under low ionic strength conditions.

4. Discussion

(*a*) *Equimolar amounts of all four histones H2A, H2B, H3 and H4 are required to form a nucleosome*

The existence of the nucleosome, as the repetitive structural unit responsible for the basic compaction of the DNA in eukaryotic chromatin, is now well established from morphological and biochemical studies (for references, see §1). It has been proposed (Kornberg 1974) that nucleosomes are homogeneous in histone content, every nucleosome containing 2 each of the four histones H2A, H2B, H3 and H4 (the histone octamer core) plus one H1. As mentioned in the Introduction this assumption has been supported by many lines of evidence and there is no indication that the bulk of the nucleosomes are heterogeneous in histone content. However, it has not been unequivocally demonstrated that every nucleosome contains the four histones H2A, H2B, H3 and H4.

It is clear from our present reconstitution studies, where DNA has been associated with all possible combinations of highly purified individual histones, that all four calf thymus histones H2A, H2B, H3 and H4 are necessary to assemble a nucleosome. It is important to stress that we have used three independent criteria to characterize the formation of a nucleosome, namely its morphology (a bead-like structure of 12.5 ± 1.0 nm), its DNA content (about 200 base pairs) and the constraint imposed on the compacted DNA duplex (equivalent to about one negative superhelical turn per bead). Our results are in agreement with, and extend those, of Sollner-Webb *et al.* (1976) and Camerini-Otero *et al.* (1976) who have shown that nuclease digests of reconstitutes containing all four histones H2A, H2B, H3 and H4 are indistinguishable from those of sheared chromatin. In addition, our results lead to the conclusion that not only are the four histones necessary to assemble a nucleosome, but also that every nucleosome contains equimolar amounts of the four histones.

Assuming that two molecules of each histone are required to assemble a nucleosome containing 200 base pairs of DNA, about 20 nucleosomes should be formed per SV40 DNA molecule (about 4700 base pairs; see Bellard *et al.* 1976) at a ratio of 1.8 molecules of each histone to 200 base pairs of DNA. Since the yield of nucleosome assembling is certainly not 100%, the formation of 14 or 15 nucleosomes (figure 1) at this histone:DNA ratio indicates that 2 molecules of each histone are most probably required to assemble a nucleosome. Clearly our results fully support the assumption that the histone core of every nucleosome comprises two each of the four histones H2A, H2B, H3 and H4.

It should, however, be kept in mind that this conclusion is drawn from *in vitro* reconstitution experiments and that different histone/DNA assembling could occur *in vitro* with histones of different origin or *in vivo* under different environmental conditions. On the other hand, such a possibility appears rather unlikely in view of other results, such as those of careful measurements of the relative amounts of the four histones in chromatin from various sources (Olins *et al.* 1976).

(*b*) *An H3–H4 subnucleosomal particle*

While our studies clearly demonstrate that all four histones H2A, H2B, H3 and H4 are required to form a nucleosome, it is also quite clear from our results that the arginine-rich histones, H3 and H4, together play a unique rôle in creating a well defined subnucleosomal nucleoprotein structure. It is very unlikely that the bead-like particles visible in H3–H4/DNA

reconstitutes are electron microscopic artefacts occurring on the grids, since they were also seen after fixation of the reconstitutes with formaldehyde. In addition similar particles were made visible, as isolated particles or short chains of beads, after mild micrococcal nuclease digestion (unpublished result). Moreover, there is a strong correlation between the number of particles which can be made visible and the magnitude of the constraint imposed on the DNA in a H3–H4 nucleoprotein complex. One would indeed expect that some contraint of the DNA duplex would be required in order to compact 130 base pairs of DNA duplex in a structure with a diameter of only 8.0 nm.

In addition to its different histone composition, the H3–H4 subnucleosomal particle is clearly different from the nucleosome in several respects. When viewed under identical conditions by electron microscopy, it appears less dense than the nucleosome and its diameter is significantly smaller (8.0 ± 1.0 nm compared with 12.5 ± 1.0 nm for the nucleosome). In view of the dehydrating conditions of electron microscopy, it is very likely that this dimension does not accurately reflect the hydrated dimension of the particle. It is worth mentioning, however, that there was no significant variation in the diameter of the H3–H4 particle whether the reconstitute was fixed with formaldehyde before the adsorption on the grid or positively stained with uranyl formate only without metal-shadowing. The length of the DNA segment which is associated to a H3–H4 particle was determined from the apparent length reduction of the adenovirus-2 DNA molecule as a function of the number of H3–H4 particles, in exactly the same way as that previously used to determine the size of the DNA segment associated to a nucleosome (Oudet *et al.* 1975). The difference is highly significant: 129 ± 8 base pairs were associated to an H3–H4 particle, whereas 193 ± 6 base pairs were previously found to be folded in a nucleosome reconstituted with the four calf thymus histones. Confirmation of the length of the DNA segment associated to a H3–H4 subnucleosomal particle was given by nuclease digestion studies. In agreement with the results of Camerini-Otero *et al.* (1976), we have found (unpublished results) that micrococcal nuclease digestion of H3–H4 reconstitutes yields discrete DNA fragments. At early times of the digestion, discrete DNA fragments of 130–140 base pairs were clearly visible after electrophoresis on polyacrylamide gels, whereas they disappeared at later times of the digestion to be replaced by smaller fragments in the range of 40–70 base pairs. In view of the excellent correlation between the length of the DNA segment contained in an H3–H4 particle as determined by electron microscopy, and the maximum length of the discrete DNA fragments obtained by micrococcal nuclease digestion, we conclude that the H3–H4 subnucleosomal particle is the structure responsible not only for the compaction of the DNA, but also for the DNA digest pattern obtained with micrococcal nuclease.

Both H3 and H4 histones are required to generate the 8.0 nm subnucleosomal particles. Other results (unpublished) indicate that the H3–H4 subnucleosomal particles arise from a unique H3/H4 complex with a 1:1 histone stoichiometry, since for a given amount of histone H3, the number of H3–H4 particles increased in proportion to the amount of H4 added, until a plateau value was reached for an equimolar amount of the two histones. Beyond this point some aggregation occurred which was presumably due to histone H4 self-aggregation (Edwards & Shooter 1969; Sperling & Bustin 1974). The existence of an H3–H4 tetramer has been demonstrated (Kornberg & Thomas 1974; Roark, Geoghegan & Keller 1974) in histone solutions prepared by the salt extraction procedure of van der Westhuyzen & von Holt (1971), and H3–H4 tetramers have been formed in the presence of salt from separate solutions of H3 and H4 (D'Anna & Isenberg 1974*c*). Since the formation of H3–H4/DNA complexes takes

place under conditions favouring the formation of H3–H4 tetramers and since equimolar amounts of the two histones are required to form an H3–H4 subnucleosomal particle, it appears very likely that each H3–H4 particle results from the interaction of one H3–H4 tetramer with 130 base pairs of DNA. In this respect it is relevant to mention that H3–H4 subnucleosomal particles were formed with the same efficiency from H3–H4 tetramers prepared according to van der Westhuyzen & von Holt (1971) or from H3 and H4 histones purified under acidic conditions and which were mixed under salt conditions favouring the formation of tetramers (unpublished).

Provided the histones were added together, the formation of the H3–H4 subnucleosomal particles does not appear to depend critically on the reconstitution method, since essentially the same results were obtained whether the histones and the DNA were mixed in 2 M NaCl (method A, §2) or in 1.5 M NaCl followed by rapid dilution to 1.2 M NaCl (method B). Our results are therefore in agreement with those of Sollner-Webb *et al.* (1976) who did not find the critical dependence on the reconstitution method for H3–H4/DNA complexes reported by Boseley *et al.* (1976). On the other hand, mixing H3 and H4 with the DNA in 250 mM NaCl did not result in any discrete subnucleosomal structure indicating that the histone–DNA association must be carried out in a high ionic strength environment to allow the specific interactions to occur. It is very likely that the H3–H4 subnucleosomal particle is formed at approximately 1.2 M NaCl, since the same yield of H3–H4 particles was obtained when the ionic strength of the association mixture was progressively decreased as described in §2 or abruptly decreased from 1.1 M to 0.2 M NaCl. Abrupt dilutions above 1.4 M NaCl did not result in any H3–H4 subnucleosomal particles (unpublished results). This formation of H3–H4 particles at approximately 1.2 M NaCl is interesting in two respects: first, because it has previously been shown that 1.2–1.4 M NaCl is precisely the range of salt concentration at which histones H3 and H4 dissociate reversibly and cooperatively as an equimolar complex from calf thymus chromatin DNA (Burton, Hyde & Walker 1975; secondly, because we have previously reported that the histone core of the nucleosome, which comprises all four histones H2A, H2B, H3 and H4, dissociate reversibly from the DNA at 0.8–0.9 M NaCl (Germond *et al.* 1976). It appears therefore that the affinity of histones H3 and H4 for the DNA could be lowered by their association to histones H2A and H2B. Further studies are in progress to get a better insight in this problem which is obviously related to the assembling of a nucleosome.

As pointed out in §3, the yield of H3–H4 beads is low compared with the yield of nucleosome reconstitution under similar association conditions. The method of preparation of the histones cannot be invoked to explain this observation since the same yield was obtained with H3–H4 histones purified as tetramers or as individual histones (see above). In keeping with the observation of Boseley *et al.* (1976) the inclusion of urea in the association buffer (Rubin & Moudrianakis 1975; Camerini-Otero *et al.* 1976; Sollner-Webb *et al.* 1976) to prevent a possible histone self-aggregation, did not result in any improvement, and in fact lowered the yield (unpublished observation). Obviously this failure does not preclude other more successful modifications of the reconstitution method being found by a systematic study of the parameters which are involved in the formation of the H3–H4 beads. Alternatively, it is possible that the low yield of H3–H4 beads, particularly at the lower H3–H4:DNA ratios (table 2) is related to the relatively low concentration of histones in the association mixture, since it has been reported by Burton *et al.* (1975) that the binding of H3 and H4 to DNA is highly cooperative in the presence of 1.25 M NaCl. Such a cooperativity could be related to an H3–H4 dimer–

tetramer equilibrium (Roark *et al.* 1974), although such an equilibrium has been questioned (D'Anna & Isenberg 1974*c*). In any case, it is interesting to note that Sollner-Webb *et al.* (1976) have also obtained better H3–H4 reconstitutions (as probed with DNase I) at high H3–H4:DNA ratio (total protein:DNA mass ratio of 1).

The finding that about the same DNA length is folded in the H3–H4 subnucleosomal particle and the 'nucleosome core' (see Introduction) with almost the same conformational constraint, obviously suggests that histones H3 and H4 alone, presumably in the form of a tetramer, define the length of the basic fold of the nucleosome core DNA. Such a basic subnucleosomal H3–H4 structure was one of the main proposals of the Kornberg's chromatin model (Kornberg 1974) and its possible existence was recently supported by X-ray diffraction (Boseley *et al.* 1976) and nuclease digestion (Camerini-Otero *et al.* 1976; Sollner-Webb *et al.* 1976) studies, which have shown that the histone pair H3–H4 is essential for the formation of a nucleoprotein reconstitute with some chromatin-like features. The recent finding (Simpson 1976) that H3 and H4 are associated with the ends of the nucleosome core DNA is also in agreement with our results and such a basic subnucleosomal structure.

The possibility of forming a structure which presents some similarities with the nucleosome core with the arginine-rich histones raises two questions: what could be the rôle of histones H2A and H2B in the folding of the DNA in the nucleosome core and in the nucleosome, and could the H3–H4 subnucleosomal particle be an intermediate step in the assembling of the nucleosome?

It appears at the present time that H2A and H2B could play a double rôle; first, they could interact with the H3–H4 subnucleosomal particle helping to stabilize the DNA fold in the nucleosome, and secondly they could bind to the so-called 'linker' DNA (Shaw *et al.* 1976; Kornberg 1977) to yield the full nucleosome. The importance of H2A and H2B in the stabilization of the DNA fold in the nucleosome core is supported by several observations. First, Camerini-Otero *et al.* (1976) and Sollner-Webb *et al.* (1976) have shown that, while the nuclease digest pattern of the DNA associated to H3 and H4 alone presents some chromatin-like features, the addition of H2A and H2B is actually required to form a structure which is recognized by all nuclease probes as that of the nucleosome core. Secondly, we have observed by electron microscopy that the H3–H4 subnucleosomal particle is less dense than the nucleosome or the nucleosome core. Thirdly, Boseley *et al.* (1976) have reported that a complex of histones H3 + H4 with DNA gives low angle X-ray diffraction maxima similar to those found for chromatin, although weaker. Evidence that H2A and H2B are involved in the binding of the 'linker' DNA are of two sorts. First we have shown (Oudet *et al.* 1975; Bellard *et al.* 1976) by electron microscopy and nuclease digestion that both 'core' and 'linker' DNA are contained in nucleosomes devoid of histone H1. Secondly, Camerini-Otero *et al.* (1976) and Sollner-Webb *et al.* (1976) have shown that micrococcal nuclease and DNase I produce DNA fragments (up to 200 base pairs) larger than the nucleosome core from chromatin lacking the lysine-rich histones. In addition, the DNase I digest band intensity in the region of 140 and 190 base pairs (nucleosome core and full nucleosome, respectively) was the same for native chromatin, chromatin stripped of the lysine-rich histone and chromatin reconstituted from H2A, H2B, H3 and H4. Clearly such a rôle of histones H2A and H2B in binding to the linker DNA could contribute to explain the observed variations in the lengths of the linker DNA, in view of the variations in structure of H2A and H2B, as opposed to the highly conserved structure of H3 and H4 (Compton *et al.* 1976*a*; Spadafora *et al.* 1976; see also Kornberg 1977).

It is not clear whether the H3–H4 subnucleosomal particle could be an intermediate step in the assembling of the nucleosome, since up to now we have failed to assemble first an H3–H4 subnucleosomal particle and then to obtain a nucleosome by adding the H2A–H2B pair at 1.1 M NaCl during the reconstitution procedure (unpublished results). This observation could reflect our present inability to find the right association conditions for converting an H3–H4 subnucleosomal particle to a nucleosome or indicate that specific interactions between all four histones (in the form of an octamer?) should take place (in the presence of the DNA? – see Kornberg (1977)) to allow the formation of a nucleosome. It is clear, however, that H2A and H2B should be added as a pair, since the addition of H2A or H2B alone to H3 and H4 does not result in the assembling of a structure which would be intermediate between an H3–H4 subnucleosomal particle and a nucleosome. We have indeed not seen any well-defined structure when the DNA was associated to H2A, H3 and H4 or to H2B, H3 and H4. In this respect, it is interesting to note that both Felsenfeld and his collaborators (Camerini-Otero *et al.* 1976; Sollner-Webb *et al.* 1976) and Boseley *et al.* (1976) have found that the addition of H2A or H2B to H3 and H4 were detrimental to the formation of a chromatin-like structure. These detrimental effects of H2A or H2B addition are very probably due to interactions of H2A with H3 and H2B with H4 which could weaken the H3–H4 interactions (D'Anna & Isenberg 1974*b*, *c*).

In summary, our results provide strong support for the initial proposal of Kornberg (1974) that the tetramer $(H3)_2(H4)_2$ by defining the core of the repeat unit has a fundamental rôle in chromatin structure. We further conclude that histones H2A and H2B are essential in stabilizing the basic H3–H4 subnucleosomal structure and in binding to the linker DNA to yield a full nucleosome.

(*c*) *Half nucleosomes*

As pointed out in the Introduction, the finding of a pair of each type of histones in the nucleosome has suggested that a nucleosome could be made up of two symmetrical halves. Very recently Altenburger, Hörz & Zachau (1976) have provided some evidence that a nucleosome could have a symmetrical arrangement by showing that the DNA in chromatin is cleaved by DNase II at 100 base-pair intervals under certain conditions. Our present results obtained with SV40 minichromosomes suggest very strongly that nucleosomes can split in half. In addition, our results show that the nucleosome–half nucleosome equilibrium is markedly dependent upon the ionic strength as would be expected if a strong electrostatic repulsion were required to disrupt the hydrophobic bonds which hold the histone octamer core together. It should be stressed, however, that up to now we have not been able to obtain a clearcut transition from nucleosomes to half nucleosomes by incubating purified SV40 minichromosomes (Bellard *et al.* 1976) at very low ionic strength, which suggests that some nuclear component could be important, in addition to low ionic strength, to promote the half nucleosome conversion.

That most of the DNA which was initially compacted in nucleosomes is still compacted in half nucleosomes is indicated by the fact that the contour length of an SV40 minichromosome containing 40–50 half nucleosome beads was only slightly longer (about 650 nm, figure 9*a*, *b*) than that of an SV40 minichromosome consisting of 20–21 nucleosomes (about 500 nm), while naked SV40 DNA is about 1600 nm (see Bellard *et al.* 1976). This conclusion was further supported by the determination of the DNA length contained in one half nucleosome. This was achieved, as previously for the nucleosomes (Bellard *et al.* 1976), by measuring the total

length of apparently naked DNA and the number of half nucleosomes present on unstretched SV40 minichromosomes containing only 36–40 half nucleosomes. It was found (unpublished result) that, on the average, each half nucleosome contains 96 base pairs, a value which corresponds to almost half of the DNA length contained in an SV40 nucleosome. Of course, this conclusion rests on the assumption that one is actually dealing with symmetrical half nucleosomes (see below).

There is no doubt that the half nucleosome beads correspond to a new type of chromatin particle. The dimension of the half nucleosome is indeed significantly different from those of the nucleosome and the H3–H4 subnucleosomal particle when viewed under identical electron microscopy conditions (the diameter of the half nucleosome was not significantly modified when it was positively stained only with uranyl formate without metal-shadowing). Furthermore, all of the half nucleosomes appear under the electron microscope to be almost as compact as nucleosomes and in any case much more compact than the H3–H4 subnucleosomal particles. The homogeneous and compact appearance of the half nucleosome beads provides a strong indication that they actually correspond to symmetrical halves of nucleosomes and not, for instance, to H3–H4 subnucleosomal particles on one hand and to H2A–H2B particles, yet undiscovered, on the other. We would therefore favour the view that the half nucleosome beads as they are revealed by electron microscopy correspond to the 'symmetrically paired half nucleosomes' which have been postulated in the models proposed by Weintraub *et al.* (1976) and by Richards *et al.* (1977).

It is clear from our results that not only the SV40 minichromosome but also the cellular chromatin could be converted to a half nucleosomal structure at very low ionic strength. The cellular and the SV40 half nucleosomes are very probably identical, since they have the same diameter, the same compactness and they can be seen side by side on the same grid. This simultaneous conversion of the two types of chromatin to a half nucleosomal structure suggests that histone acetylation does not play an important rôle in the nucleosome to half nucleosome transition, since it has been shown that histones of the viral chromatin are acetylated to a much greater extent than their cellular counterparts (Schaffhausen & Benjamin 1976, and personal communication).

The finding that cellular chromatin can be made visible as chains of nucleosomes with a diameter of 12.5 ± 1.0 nm or as flexible chains of half nucleosomes with a diameter of 9.3 ± 1.0 nm raises the question of the relation between nucleosomes, half nucleosomes and ν-bodies (Olins & Olins 1974). There is indeed a discrepancy between the diameter of the chromatin repeat unit (ν-body) as determined by Olins & Olins (1974) and by Woodcock, Safer & Stanchfield (1976) (about 6–8 nm) and the diameter of the chromatin repeat unit (nucleosome) as determined by Oudet *et al.* (1975), Franke *et al.* (1976) and Laird, Wilkinson, Foe & Chooi (1976) (about 11–13 nm). This difference cannot be ascribed only to different electron microscopic staining and viewing methods, since it remained even when identical staining procedures were used. It is striking that the smaller diameter of the chromatin repeat unit was observed only when chromatin was swollen in distilled water before centrifugation onto the grid (Olins & Olins 1974; Woodcock *et al.* 1976). It is therefore possible that the different results which have been reported for the dimension of the chromatin subunit could be, at least in part, explained by assuming that the ν-bodies are in fact half nucleosomes. In this respect it is interesting to mention that Tsanev & Petrov (1976) have observed in rat liver chromatin large-size particles (about 12 nm) and small-size particles (about 8 nm) in various proportions depending on the

conditions of preparation of the chromatin sample. It is possible that their small-size particles correspond to half nucleosomes.

Finally, the fact that under certain conditions, both nucleosomes and half nucleosomes could disappear leaving a filament thicker than naked DNA is interesting, since it does suggest that chromatin could be viewed as a dynamic structure where the DNA could be either tightly compacted in nucleosomes, or more accessible in half nucleosomes or easily accessible in the completely extended structure. It remains to be seen to which extent such reversible transitions could take place *in vivo* under different environmental conditions and play a rôle in chromatin function, i.e. transcription and replication of the genome.

We thank Dr F. Gannon for helpful discussions. The excellent technical assistance of Mrs E. Sittler, Miss D. Mertz and Mr G. Dretzen, and the help of Mrs B. Chambon in the preparation of the manuscript, are gratefully acknowledged.

This work was supported by grants from the C.N.R.S. (A.T.P. Différenciation – Contract 2117; A.T.P. Internationale – Contract 2054), by the I.N.S.E.R.M. (Contract CRL 76.5.099.1) and of the F.R.M.F. C. Spadafora is a recipient of an E.M.B.O. long term fellowship.

References (Oudet *et al.*)

Altenburger, W., Hörz, W. & Zachau, H. 1976 *Nature* **264**, 517–522.
Axel, R., Melchior, W., Sollner-Webb, B. & Felsenfeld, G. 1974 *Proc. natn. Acad. Sci. U.S.A.* **71**, 4101–4105.
Baldwin, J. P., Boseley, P. G., Bradbury, M. & Ibel, K. 1975 *Nature* **253**, 245–249.
Bellard, M., Oudet, P., Germond, J. E. & Chambon, P. 1976 *Eur. J. Biochem.* **70**, 543–553.
Bina-Stein, M. & Singer, M. F. 1977 *Nucl. Acids Res.* **4**, 117–127.
Boseley, P. G., Bradbury, M., Butler-Browne, G. S., Carpenter, B. G. & Stephens, R. M. 1976 *Eur. J. Biochem.* **62**, 21–31.
Burton, D. R., Hyde, J. E. & Walker, I. O. 1975 *FEBS Lett.* **55**, 77–80.
Bustin, M., Goldblatt, D. & Sperling, R. 1976 *Cell* **7**, 297–304.
Camerini-Otero, R. D., Sollner-Webb, B. & Felsenfeld, G. 1976 *Cell* **8**, 333–347.
Compton, J. L., Bellard, M. & Chambon, P. 1976*a* *Proc. natn. Acad. Sci. U.S.A.* **73**, 4382–4386.
Compton, J. L., Hancock, R., Oudet, P. & Chambon, P. 1976*b* *Eur. J. Biochem.* **70**, 555–568.
D'Anna, J. A. & Isenberg, I. 1974*a* *Biochemistry* **13**, 2098–2104.
D'Anna, J. A. & Isenberg, I. 1974*b* *Biochemistry* **13**, 4992–4997.
D'Anna, J. A. & Isenberg, I. 1974*c* *Biochem. biophys. Res. Commun.* **61**, 343–347.
Davidson, N. 1972 *J. molec. Biol.* **66**, 307–309.
Edwards, P. A. & Shooter, K. V. 1969 *Biochem. J.* **114**, 227–236.
Franke, W. W., Scheer, U., Trendelenburg, M. F., Spring, H. & Zentgraf, H. 1976 *Cytobiologie* **13**, 401–434.
Germond, J. E., Hirt, B., Oudet, P., Gross-Bellard, M. & Chambon, P. 1975 *Proc. natn. Acad. Sci. U.S.A.* **72**, 1843–1847.
Germond, J. E., Bellard, M., Oudet, P. & Chambon, P. 1976 *Nucl. Acids Res.* **3**, 3173–3192.
Hossenlopp, P., Wells, D. & Chambon, P. 1975 *Eur. J. Biochem.* **58**, 237–251.
Hyde, J. E. & Walker, I. D. 1975 *Nucl. Acids. Res.* **2**, 405–421.
Johnson, E. M., Littau, V. C., Allfrey, V. G., Bradbury, M. & Matthews, H. R. 1976 *Nucl. Acids Res.* **3**, 3313–3329.
Keller, W. 1975 *Proc. natn. Acad. Sci. U.S.A.* **72**, 4876–4880.
Kornberg, R. D. 1974 *Science, N.Y.* **184**, 868–871.
Kornberg, R. D. 1977 *A. Rev. Biochem.* **46**. (In the press.)
Kornberg, R. D. & Thomas, J. D. 1974 *Science, N.Y.* **184**, 865–868.
Laird, C. D., Wilkinson, L. E., Foe, V. E. & Chooi, W. Y. 1976 *Chromosoma* **58**, 169–192.
Li, H. J. 1975 *Nucl. Acids Res.* **2**, 1275–1289.
Lohr, D., Corden, J., Tatchell, K., Kovacic, R. T. & Van Holde, K. E. 1977*a* *Proc. natn. Acad. Sci. U.S.A.* **74**, 79–83.
Lohr, D., Kovacic, R. T. & Van Holde, K. E. 1977*b* *Biochemistry* **16**, 463–471.
Miller, O. L. & Bakken, A. H. 1972 *Acta endocrinol.* Suppl. **168**, 155–177.
Morris, N. R. 1976*a* *Cell* **8**, 357–363.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Morris, N. R. 1976*b* *Cell* **9**, 627–632.
Moss, R., Cary, B. D., Crane-Robinson, C. & Bradbury, M. 1976*a* *Biochemistry* **15**, 2261–2267.
Moss, R., Cary, B. D., Abercrombie, C., Crane-Robinson, C. & Bradbury, M. 1976*b* *Eur. J. Biochem.* **71**, 337–350.
Noll, M. 1976 *Cell* **8**, 349–355.
Noll, M. & Kornberg, R. D. 1977 *J. molec. Biol.* **109**, 393–404.
Olins, A. D., Carlson, R. D., Wright, E. B. & Olins, D. E. 1976 *Nucl. Acids Res.* **3**, 3271–3291.
Olins, A. D. & Olins, D. E. 1974 *Science, N.Y.* **183**, 330–332.
Oudet, P., Germond, J. E., Bellard, M., Sures, I., Gallwitz, D. & Chambon, P. 1977 submitted for publication.
Oudet, P., Gross-Bellard, M. & Chambon, P. 1975 *Cell* **4**, 281–300.
Pardon, J. F., Worcester, D. L., Wooley, J. C., Tatchell, K., Van Holde, K. E. & Richards, B. 1976 *Nucl. Acids Res.* **2**, 2163–2176.
Richards, B., Pardon, J., Lilley, D., Cotter, R. & Wooley, J. 1977 *Cell Biol. internat. Rep.* **1**, 107–116.
Roark, D. E., Geoghegan, T. E. & Keller, G. H. 1974 *Biochem. biophys. Res. Commun.* **59**, 542–547.
Roark, D. E., Geoghegan, T. E., Keller, G. H., Matter, K. V. & Eagle, R. L. 1976 *Biochemistry* **15**, 3019–3025.
Rubin, R. L. & Moudrianakis, E. N. 1975 *Biochemistry* **14**, 1718–1726.
Schaffhausen, B. S. & Benjamin, T. L. 1976 *Proc. natn. Acad. Sci. U.S.A.* **73**, 1092–1096.
Shaw, B. R., Herman, T. M., Kovacic, R. T., Beaudreau, G. S. & Van Holde, K. E. 1976 *Proc. natn. Acad. Sci. U.S.A.* **73**, 505–509.
Shure, M. & Vinograd, J. 1976 *Cell* **8**, 215–226.
Simpson, R. T. 1976 *Proc. natn. Acad. Sci. U.S.A.* **73**, 4400–4404.
Simpson, R. T. & Bustin, M. 1976 *Biochemistry* **15**, 4305–4312.
Simpson, R. T. & Whitlock, J. P. 1976 *Nucl. Acids Res.* **3**, 117–127.
Sollner-Webb, B., Camerini-Otero, R. D. & Felsenfeld, G. 1976 *Cell* **9**, 179–193.
Sollner-Webb, B. & Felsenfeld, G. 1975 *Biochemistry* **14**, 2915–2920.
Spadafora, C., Bellard, M., Compton, J. L. & Chambon, P. 1976 *FEBS Lett.* **69**, 281–285.
Sperling, R. & Bustin. M. 1974 *Proc. natn. Acad. Sci. U.S.A.* **71**, 4625–4629.
Thomas, J. O. & Furber, V. 1976 *FEBS Lett.* **66**, 274–280.
Thomas, J. O. & Kornberg, R. D. 1975*a* *Proc. natn. Acad. Sci. U.S.A.* **72**, 2626–2630.
Thomas, J. O. & Kornberg, R. D. 1975*b* *FEBS Lett.* **58**, 353–358.
Thomas, J. O. & Thompson, R. J. 1977 *Cell.* (In the press.)
Tsanev, R. & Petrov, P. 1976 *J. microsc. Biol. Cell* **27**, 11–18.
Van der Westhuyzen, D. R. & Von Holt, C. 1971 *FEBS Lett.* **14**, 333–337.
Van Holde, K. E., Sahasrabuddha, C. G. & Shaw, B. 1974 *Nucl. Acids Res.* **1**, 1579–1586.
Varshavsky, A. J., Bakayev, V. V. & Georgiev, G. P. 1976 *Nucl. Acids Res.* **3**, 477–492.
Varshavsky, A. J. & Georgiev, G. P. 1975 *Mol. Biol. Rep.* **2**, 255–262.
Vinograd, J., Lebowitz, J. & Watson, R. 1968 *J. molec. Biol.* **33**, 173–197.
Weintraub, H., Worcel, A. & Alberts, B. 1976 *Cell* **9**, 409–417.
Woodcock, C. L. F., Safer, J. P. & Stanchfield, J. E. 1976 *Expl Cell Res.* **97**, 101–110.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

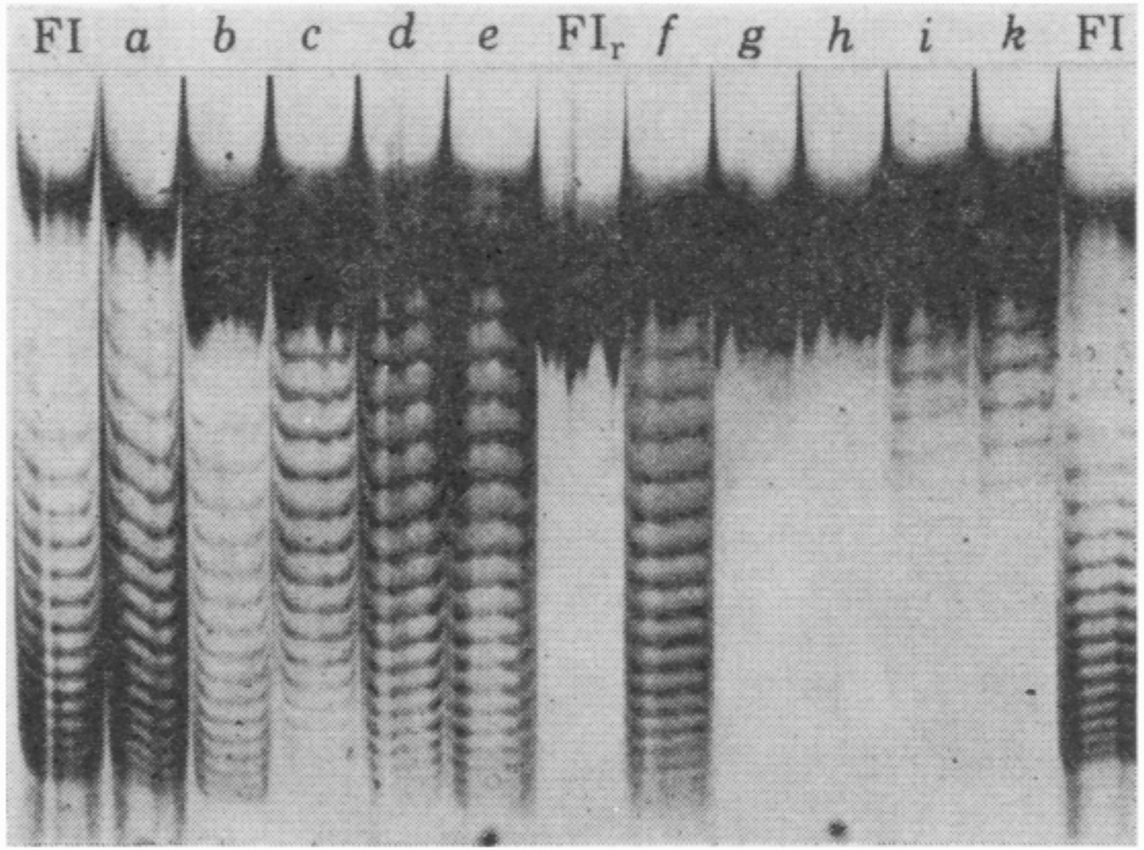


FIGURE 2. Gel electrophoresis of DNA from u.e.-treated complexes formed *in vitro* (method A) from SV40 DNA form I or form I_r and the four calf thymus histones or three out of the four histones. Complexes were reconstituted at a histone:DNA ratio of 2 molecules of each histone to 200 base pairs of DNA as follows: (*a*)–(*e*), complexes reconstituted with DNA form I; (*f*)–(*k*), complexes reconstituted with DNA form I_r; (*a*) and (*f*), all four histones were present; (*b*) and (*g*), H3 was omitted; (*c*) and (*h*), H4 was omitted; (*d*) and (*i*), H2A was omitted; (*e*) and (*k*), H2B was omitted. FI and FI_r, control untreated DNA form I and form I_r, respectively.

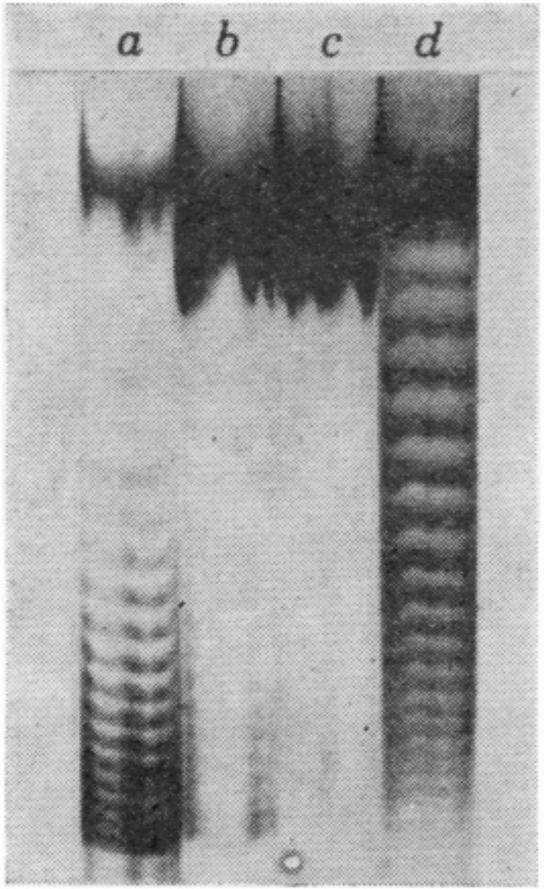


FIGURE 3. Gel electrophoresis of DNA from u.e.-treated complexes formed *in vitro* (method B) from SV40 DNA form I and the arginine-rich histones H3 and H4. (*a*) Control untreated DNA form I; (*b*) H3 alone at a ratio of 4 molecules per 200 base pairs of DNA; (*c*) H4 alone at a ratio of 4 molecules per 200 base pairs of DNA; (*d*) H3 and H4 at a ratio of 4 molecules each per 200 base pairs of DNA.

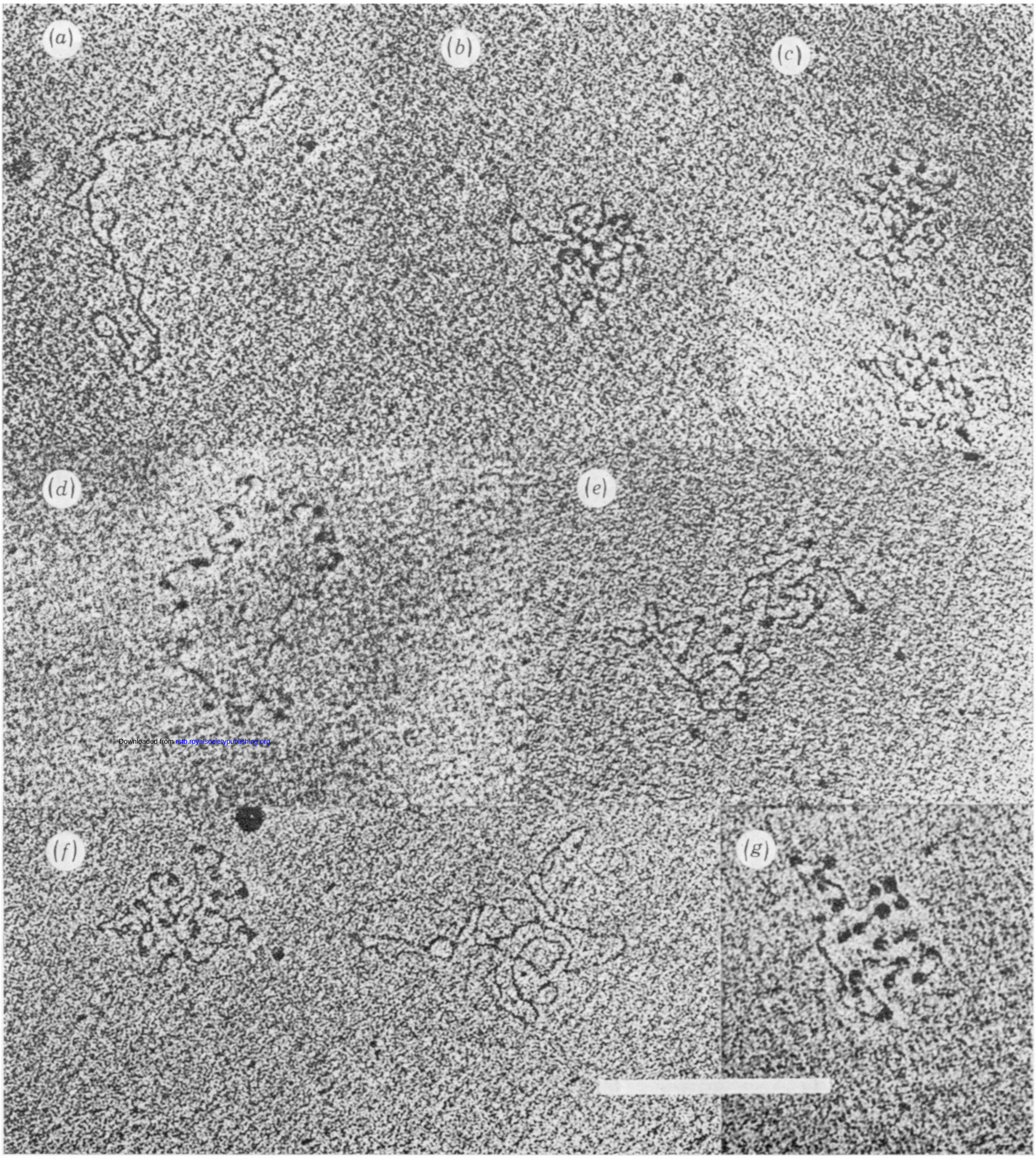


FIGURE 4. Association of SV40 DNA with the calf thymus H3–H4 histone pair. All associations were performed according to method B. (*a*) SV40 DNA form I alone examined in 50 mM NaCl; (*b*) and (*c*), DNA form I was associated with the H3–H4 pair (4 molecules of each histone per 200 base pairs of DNA) and examined after dilution to 50 mM NaCl (*b*) or 2 mM NaCl (*c*); (*d*) and (*e*), DNA form I_r and form III, respectively, were associated with the H3–H4 pair (4 molecules of each histone per 200 base pairs of DNA) and examined after dilution to 2 mM NaCl; (*f*) a mixture of identical amounts of DNA form I and form I_r was associated with the H3–H4 pair (2.8 molecules of each histone per 200 base pairs of DNA) and examined after dilution to 2 mM NaCl; (*g*) SV40 DNA form I was associated (method A) with the four calf thymus histones H2A, H2B, H3 and H4 at a ratio of 2 molecules of each histone per 200 base pairs of DNA and examined after dilution to 2 mM NaCl. The bar indicates 0.25 μm.

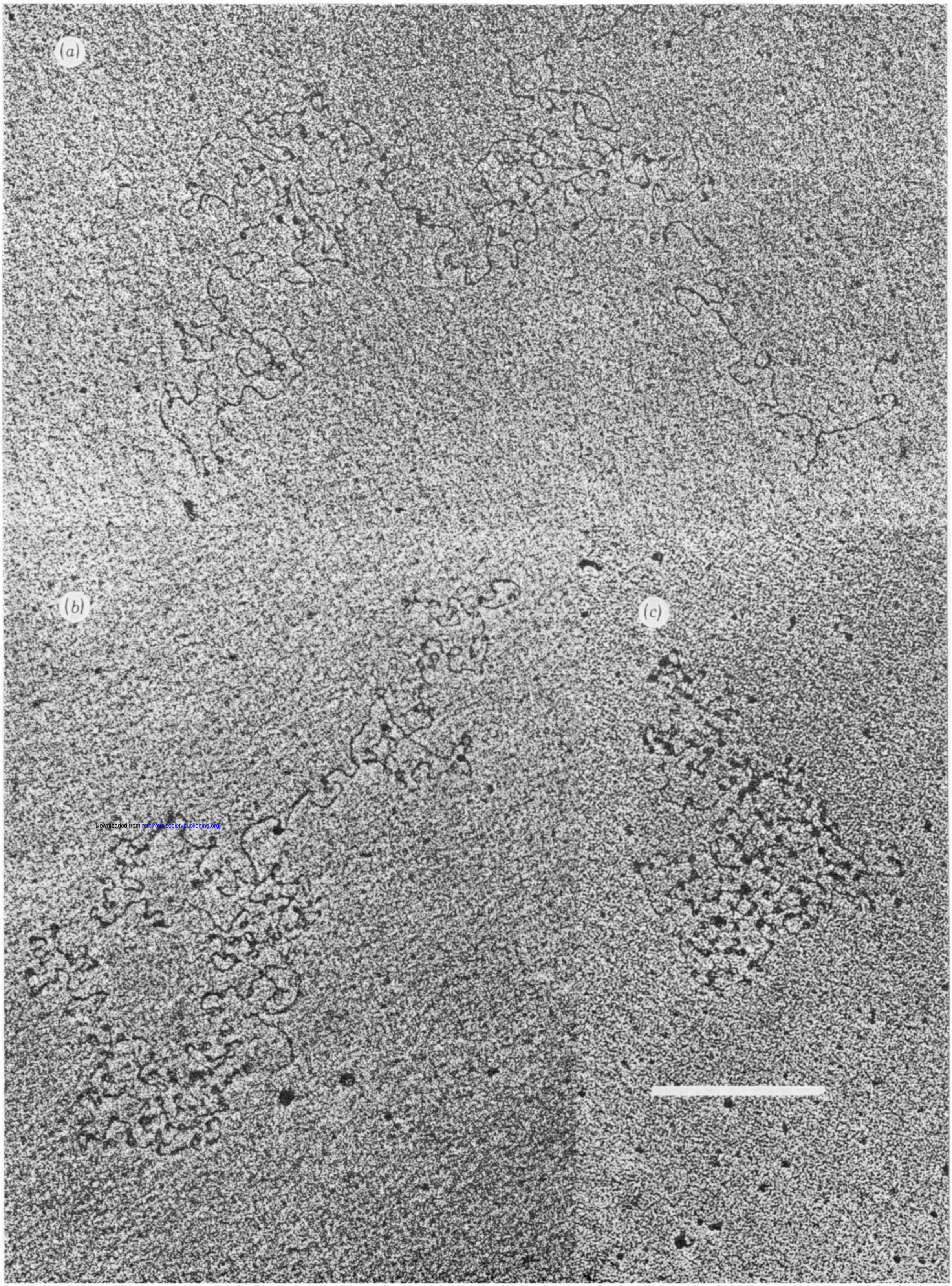


FIGURE 5. Association of adenovirus-2 DNA with the calf thymus H3–H4 histone pair. Adenovirus-2 DNA and the H3–H4 pair were associated (method B) at ratios of 2 molecules (*a*), 3.2 molecules (*b*) and 4 molecules (*c*) of each histone per 200 base pairs of DNA. Samples were examined after dilution to 2 mM NaCl. The bar indicates 0.25 μm.

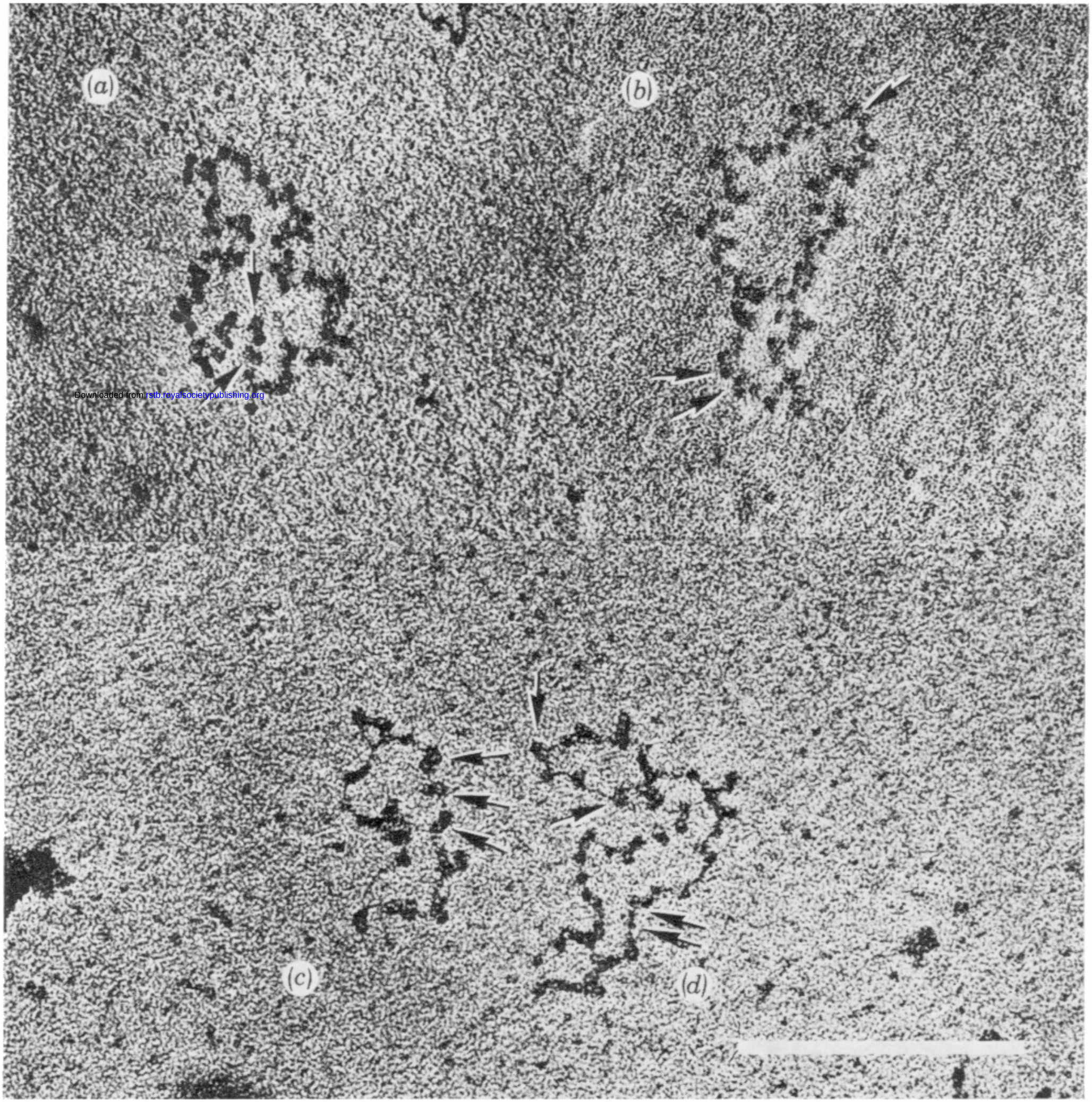
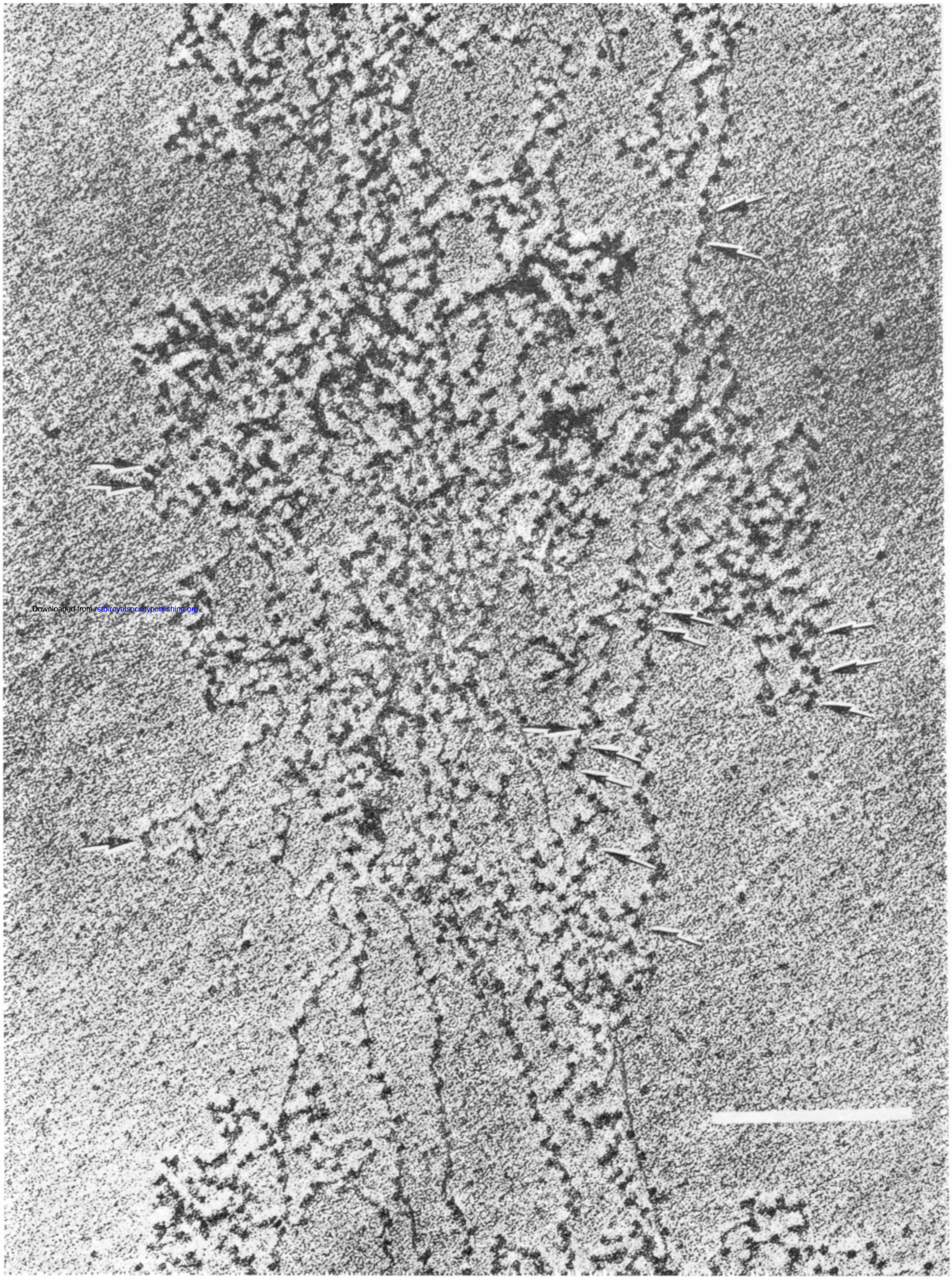


FIGURE 9. SV40 minichromosomes after incubation of nuclei from CV1 infected cells in a low ionic strength buffer (see text). Contour lengths of the circular structures were 0.64, 0.65 and 0.83 μm, for minichromosomes *a*, *b* and *d*, respectively. Minichromosome *c* was not measured due to the difficulty of tracing its contour. The bar represents 0.25 μm.



PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

FIGURE 10. Cellular chromatin from CV1 infected cells after incubation of purified nuclei in a low ionic strength buffer (see text). The bar represents 0.25 μm.

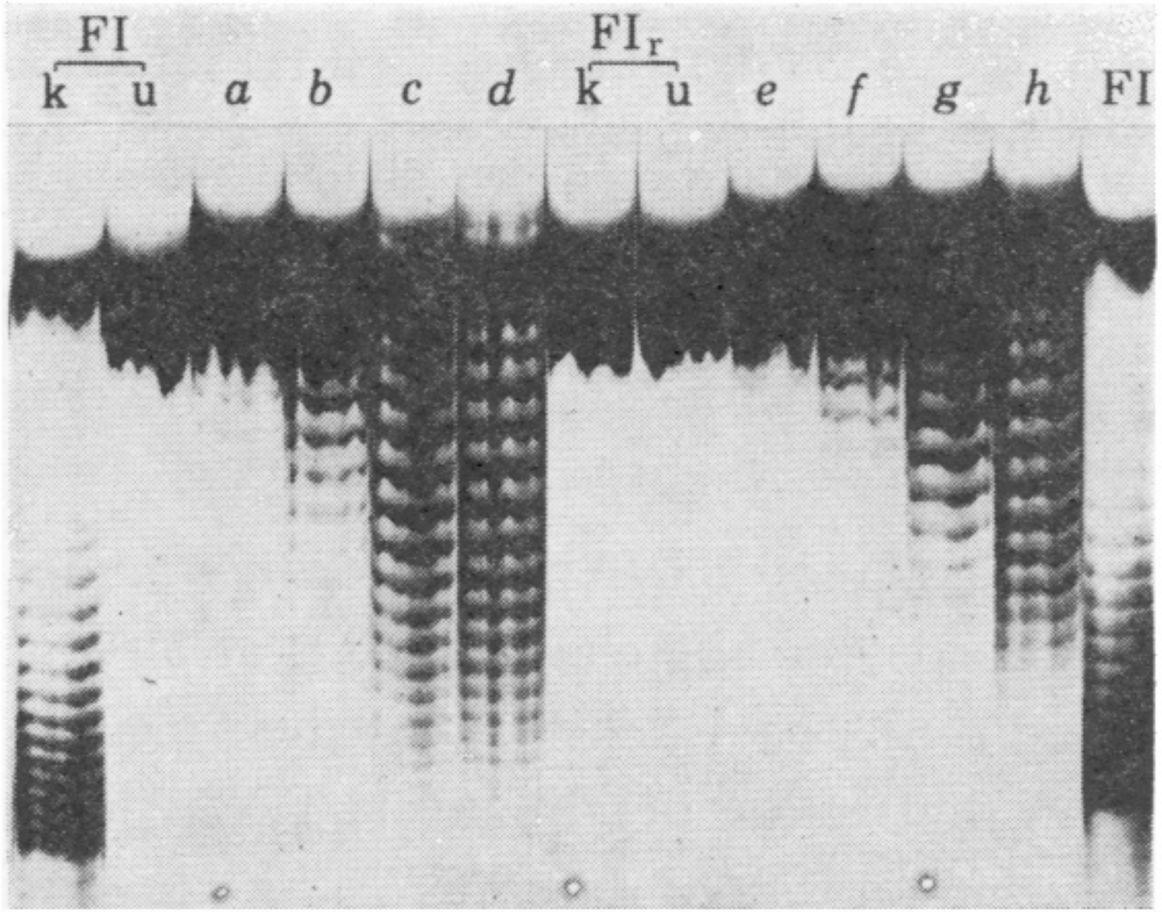


FIGURE 7. Gel electrophoresis of DNA from u.e.-treated complexes formed *in vitro* with SV40 DNA form I (*a–d*) or form I_r (*e–h*) and the H3–H4 histone pair as described in the legend to table 2. Ratios of histone:DNA expressed as molecules of each histone for 200 base pairs of DNA were as follows: 1.6 (*a* and *e*), 2.4 (*b* and *f*), 3.2 (*c* and *g*) and 4 (*d* and *h*). FI and FI_r are control DNA form I and form I_r, untreated (k) or treated (u) with u.e.

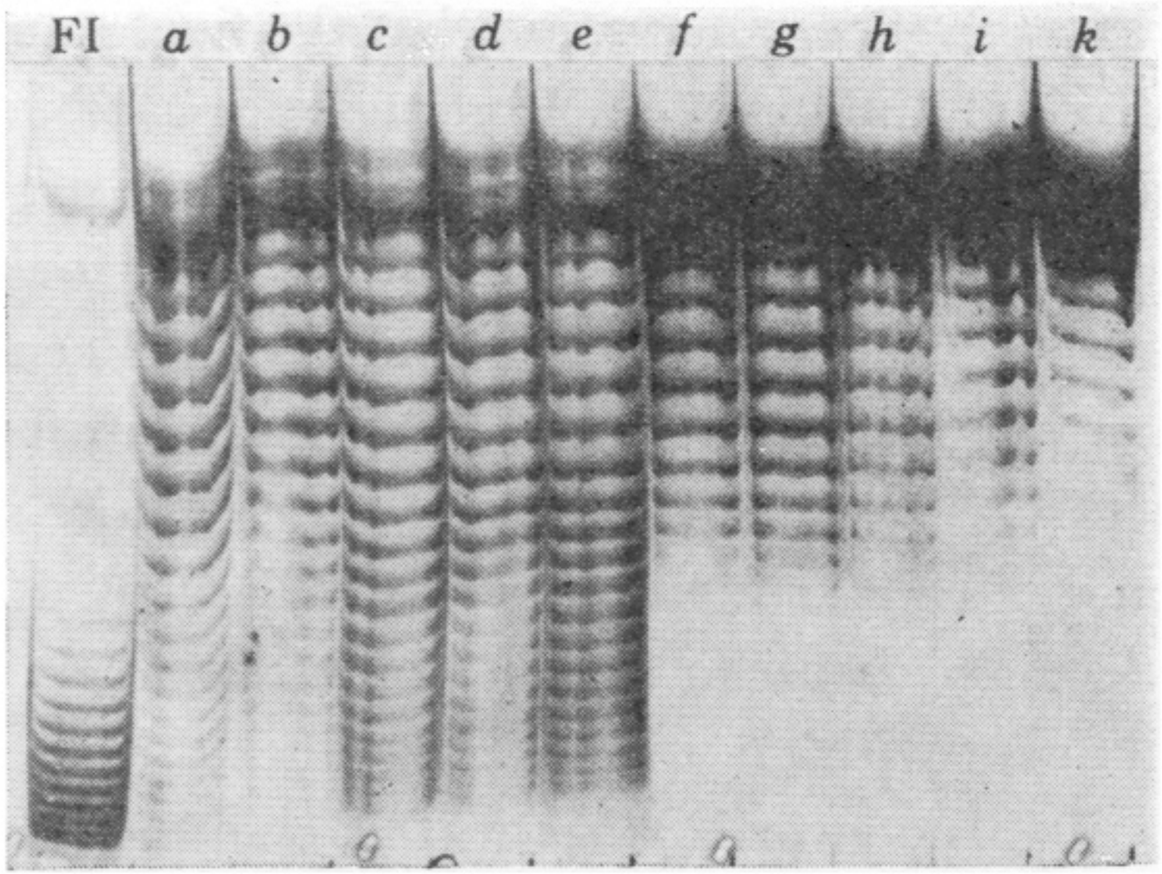


FIGURE 8. Gel electrophoresis of DNA from u.e.-treated complexes formed *in vitro* (method B) with SV40 DNA form I (*a*–*e*) or form I_r (*f*–*k*) and the H3–H4 histone pair in the absence (*a*, *f*) or in the presence of H2A (*b*, *c*, *g*, *h*) or H2B (*d*, *e*, *i*, *k*). The H3–H4:DNA ratio was 3.2 of each histone to 200 base pairs of DNA. Ratios of H2A or H2B histone expressed as molecules per 200 base pairs of DNA were 2 (*b*, *d*, *g* and *i*) or 4 (*c*, *e*, *h* and *k*). FI, control DNA form I.