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# Chromatin core particle unfolding induced by tryptic cleavage of histones

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ABSTRACT

Chromatin 'core particles' have been digested with trypsin to varying extents. The resulting particles are homogeneous by the criterion of ultracentrifuge boundary analysis. Sedimentation coefficients are lowered as cleavages are introduced into the histones, showing that an unfolding of the core particle occurs. This unfolding is further characterised by a lower melting temperature together with a premelting phase, higher molar ellipticity in the circular dichroism spectra at 280 nm and increased kinetics of digestion by both micrococcal nuclease and DNase I. Differences are also observed in the products of nuclease digestion. The most consistent interpretation of the data involves an unfolding process whereby free rods of DNA are released to extend from a nucleoprotein core.

## INTRODUCTION

The structural integrity of chromatin is dependent upon both histone-DNA and histone-histone interactions. The current view of nucleosome structure is that of an octameric core of histones (1) which is enclosed by DNA (2). Neutron scattering studies performed in our laboratories have suggested that the histone core has an approximately flat cylindrical shape, with linked partial nucleoprotein annuli at the top and bottom (3). In this structure, two heterologous histone tetramers involve intimate inter-histone interactions (4,5,6) whilst histone-DNA interaction is accomplished via salt linkages between DNA phosphate groups and basic histone residues of both the protein surface and the N-terminal 'tails'. The N-termini of the histones are all rich in basic amino acids, and early NMR studies (7,8) have indicated that these regions show a low propensity for structure formation when isolated in solution. They have been shown to be released as random-coil structures on removal of protein-DNA interactions (5,9), and to be of enhanced accessibility to some proteases (4,10,11).

The ability to introduce cleavages into the histone 'tails' is a useful structural perturbation, from which conclusions concerning the intact structure may be drawn. Sahasrabuddhe and Van Holde (12) showed that

trypsin digestion of PS particles (13) resulted in a lowering of the sedimentation coefficient, consistent with an 'opening' of the structure. Earlier workers (14,15,16) had shown that chromatin exhibits increased viscosity and lowering of melting temperature upon protease digestion. Whilst these studies suggest that cleavage of histone 'tails' is accompanied by a loosening of DNA folding, it is of interest to study the effects upon purified monomer chromatin subunits (140 base pair 'core particles') prepared by the methods of low shear (17,18), and to correlate changes with histone cleavage visualised using high resolution polyacrylamide gel electrophoresis (19). This is the aim of this paper.

## EXPERIMENTAL

### Materials

Staphylococcal nuclease (E.C.3.1.4.7.) and Deoxyribonuclease I (E.C.3.1.4.5.) were obtained from Worthington Biochemicals. Trypsin (E.C.3.4.21.4.) and tosyl-L-lysine chloroketone (TLCK) were obtained from Sigma Chemical Company.

### Preparation of Core Particles

Chicken erythrocyte nuclei, isolated by the methods of Shaw *et al.* (17) were digested with staphylococcal nuclease for 10 min. After addition of EDTA to 10mM to terminate the nuclease action the nuclei were lysed and applied to a Bio-Gel A-5m column (17). Fractions from the peak containing 140 base pair core particles were pooled and dialysed against 10mM Tris, 0.7mM EDTA pH 8.0. The core particles gave bands of 140 and 160 base pairs on gel electrophoresis, and sediment with a single boundary at 11.2 $\pm$ 0.1 S.

### Preparation of Core Protein

The method is based upon previously published procedures (4,20). Chicken erythrocyte nuclei, isolated as above (17) were lysed in water. After washing the resulting chromatin in 0.6 and 0.65M NaCl it was pelleted at 30,000 rpm for 40 min. The gel was then made 2.0M in NaCl and 10mM in [N-cyclohexyl-amino] ethane sulphonic acid (CHES) and the pH quickly adjusted to 9.0. After 24 hr this was centrifuged at 42,000 rpm for 10 hr and the supernatant taken as core protein solution. This has been characterised elsewhere (4,5,6,21).

### Trypsin Digestions

Trypsin digestions of core particles and core protein solutions in their respective buffers were performed using trypsin concentrations of 0.005 to 25 μg/ml at 4°C for 16 hr. Reactions were terminated by addition

of TLCK to final concentrations of 40-200 μg/ml, followed by extensive dialysis to remove small fragments and excess TLCK.

Electrophoresis of Proteins

After lyophilization, 10 μg quantities of histone or nucleohistone samples were dissolved in buffer containing 2% SDS, and directly applied to a 25 x 25 cm polyacrylamide gel slab, following the method of Laemmli (19). The running gel was 15% acrylamide-0.4% bisacrylamide, pH 8.8, and the stacking gel was 6% acrylamide-0.16% bisacrylamide, pH 6.8. The running buffer contained 50mM Tris, 0.77M glycine, 0.25% SDS, pH 8.3, and electrophoresis was performed at 90v for ~ 15 hr. Gel slabs were stained in 0.05% Coomassie Brilliant Blue R250 and destained in 10% acetic acid-10% methanol.

Sedimentation Velocity

Sedimentation velocity experiments were performed on a Beckman model E ultracentrifuge, equipped with UV scanner optics operating at 265 nm. Rotor speeds of 48,000 and 52,000 rpm were used, with temperatures of around 4°C. Sedimentation coefficients were determined from the slopes of ln r (r = distance of boundary from rotor centre) against time plots, using linear regression analysis. These were adjusted to $S_{20,w}$ values using standard viscosity and temperature corrections.

To assess sample homogeneity, G(S) functions were calculated according to the method of Van Holde and Weischet (22). G(S) is found by extrapolating the apparent sedimentation coefficient at various positions along the sedimentation boundary to infinite time. This extrapolation method gives a distribution of sedimentation coefficient independent of diffusion, since boundary spreading due to heterogeneity and diffusion proceed by different powers in time. A complete theoretical explanation will be presented in Van Holde and Weischet (22).

Melting Profiles

All samples were dialysed exhaustively against $2.5 \times 10^{-4}$M EDTA, degassed by passage of helium and overlayed with Dow Corning 200 silicone fluid. Melting curves were obtained in a Beckman Acta III spectrophotometer using a heating rate of 0.25°C/min. The dialysis buffer was used as blank. Temperatures and absorbances were recorded directly on punch tape, later used as input for a computer program which calculated percentage hypochromicity against temperature after correcting for thermal expansion. First derivative plots were calculated by fitting 20 contiguous point sets to a least squares linear regression, plotting the resulting slopes as a

function of temperature.

Circular Dichroism Spectra

CD spectra in the range 250-320 nm were recorded with a Durrum-Jasco CD-SP recorder, calibrated against camphor sulphonic acid, and were smoothed using a flat bed digitiser and computer program.

Staphylococcal Nuclease Digestion of Core Particles

Core particles and trypsinised core particles in 10mM Tris, 0.75mM EDTA pH 7.2 were made 1.0mM in calcium and digested with 100 units/ml staphylococcal nuclease. 0.5 $A_{260}$ aliquots were removed at various digestion times and the reaction terminated by addition of EDTA to 10mM and cooling on ice. DNA was extracted (23) and dissolved in one tenth electrophoresis buffer pH 7.8. DNA samples were electrophoresed on 6% polyacrylamide gel slabs according to Loening (24). After 9 hr at 80v the gels were stained in ethidium bromide solution and photographed using Polaroid Type 55 film. The gels were also stained in Toluidine blue solution and scanned using an Ortec densitometer.

Deoxyribonuclease I Digestions

Particles in 10mM Tris, 0.7mM EDTA pH 7.2 were made 5mM in magnesium anddigested with 100 units/ml DNase I. Aliquots were removed after various times of digestion and the DNA extracted (23), and electrophoresed on 8% polyacrylamide-urea gels (25). Gels were run for 6-7 hr at 150v followed by staining in ethidium bromide and photography as above. Negatives were scanned using an Ortec densitometer.

RESULTS

Polyacrylamide Gel Electrophoresis

Figure 1 shows the results of trypsin digestion of core particles at 4$^{o}$C, using increasing concentrations of enzyme. It is clear that, as in the case of chromatin (10,26), the histones are not equally accessible. H3 is most readily attacked, which may be related to its proximity to the DNA 'ends' (27), closely followed by H2A. H2B and, to a lesser extent, H4, appears to be rather more resistant. The appearance of the band which runs between H2A and H4 correlates well with the disappearance of H3, and therefore, as noted previously (4), is likely to be a cleavage product of H3. This product is itself cleaved at higher enzyme concentrations, at which point further cleavages must also be induced in the other histones, since more than four bands may be seen on the gel.

An interesting comparison may be made between these results and those

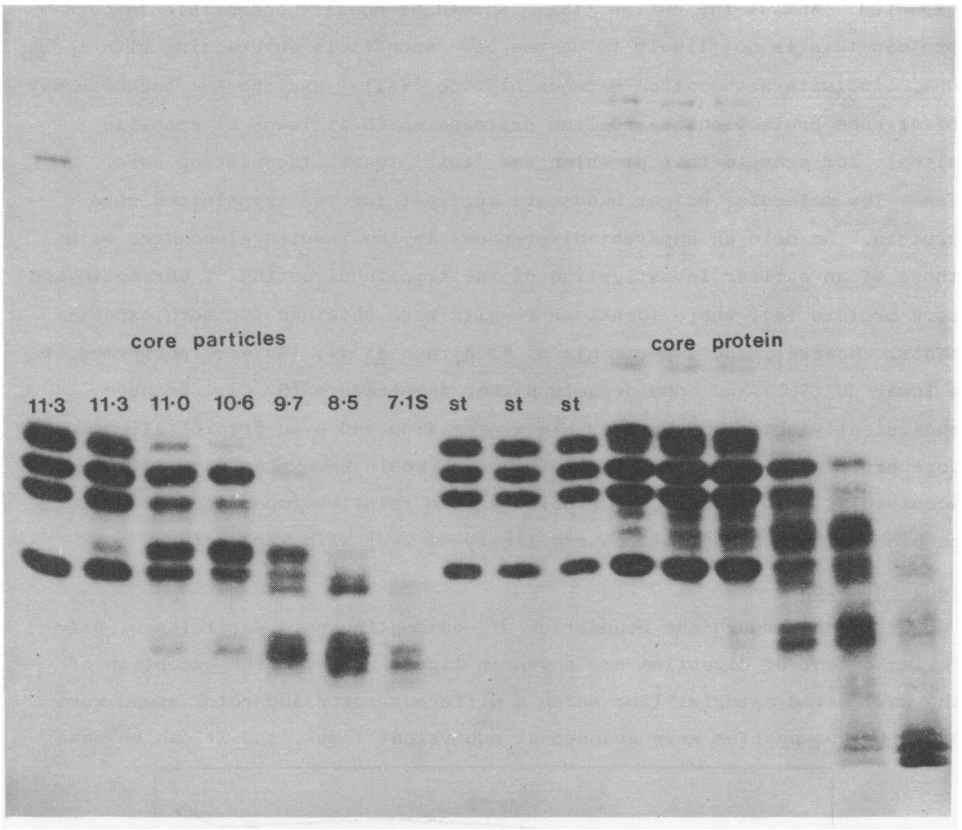


Figure 1: SDS polyacrylamide gel electrophoresis of tryptic digests of core particles and core protein. Slots 1 to 7, core particles treated with trypsin at enzyme concentrations 0, 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml, slots 8 to 10, acid extracted histone standards, slots 11-16, trypsin digested core protein using enzyme concentrations 0, 0.01, 0.05, 0.1, 0.5 and 1.0 μg/ml. Intact histones run in the order H3 (slowest), H2B, H2A and H4 (fastest). Sedimentation coefficients of trypsin digested core particles are indicated.

of digestion of isolated core protein (4,5,6,20) in 2M NaCl. Digestions were performed at the same temperature and for the same time as those on the core particles. The relative accessibilities of the four histones, and the products formed, are similar in the two cases, suggesting that the histone tails are not greatly protected by the DNA. Nevertheless, certain differences are apparent. In particular the region between the positions for H2A and H4 indicates that whilst H3 cleavage in core particles produces a single resistant fragment, in core protein two fragments are produced, one of which is rather broad. These results are consistent with a lowered specificity for cleavage site in isolated core protein, as might be

expected. Whilst the entire 'tail' should be equally accessible in core protein this is not likely to be the case when it is interacting with the DNA. Intimate association between histone 'tails' and the DNA backbone may offer some protection towards the protease which is lower at specific sites; for example that at which the 'tail' leaves the histone core. Fewer low molecular weight bands are apparent for the trypsinised core protein. We note an apparent discrepancy in the results given here with those of an earlier investigation of the trypsin digestion of chromatin and core protein (4), where identical results were obtained for both experiments. However, the experiments of Weintraub et al. (4) were performed at a lower pH (7.1) and considerably higher temperature (37°C). We have consistently observed considerable aggregation and even precipitation when core protein is incubated at $> 25^{\circ}C$, which would be expected to influence accessibility to trypsin. Furthermore, the relative importance of hydrophobic and ionic interactions are likely to vary with temperature.

Sedimentation

UV scans through the boundaries of sedimenting core particles of differing extent of digestion are shown in figure 2. With the exception of the undigested material (for which a different rotor and rotor speed were used) the boundaries were scanned at equivalent times, and it can be seen

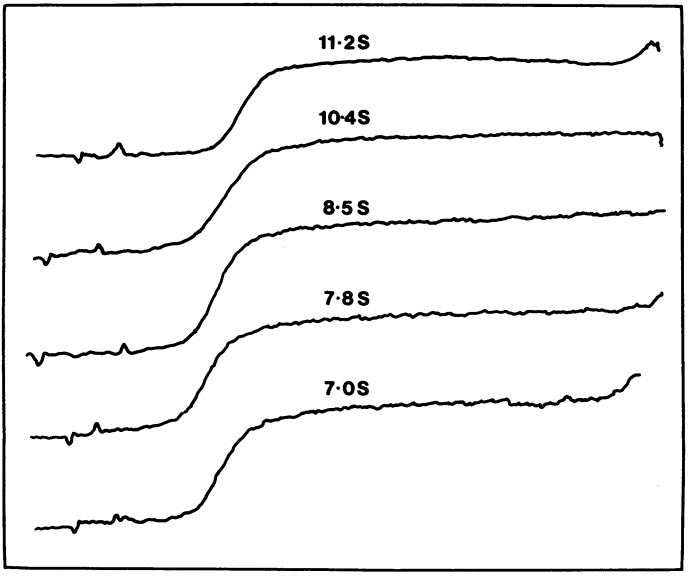


Figure 2: UV scans through sedimentation boundaries of trypsin digested core particles at equivalent times (with the exception of the intact core particles, for which a different rotor and rotor speed was used).

that they are of approximately equal width. This suggests that we have produced a reasonably homogeneous population of digested particles for each enzyme concentration. This is supported by the graphs of ln r (r = boundary position) against time, shown in figure 3. In each instance a good straight line is evident, without curvature and with little scatter of points.

A better indication of homogeneity can be obtained by analysing the sedimentation boundaries. The widths of the boundaries result from diffusion as well as sample heterogeneity. The diffusion contribution has been removed using the method of Van Holde and Weischet (22), leaving an integral distribution of sedimentation coefficient G(S). The quantity G(S) is defined as the weight fraction of the sample having a sedimentation coefficient less than a particular value. Figure 4 is a plot of G(S) for the trypsin digested samples. For a homogeneous sample G(S) will be a step function, changing from zero to one at the sedimentation coefficient of the sample. Undigested core particles are nearly homogeneous by this criterion. The trypsin digested samples are characterised by broader G(S) functions, but are still nearly homogeneous. Thus whilst it seems unlikely that our digestion conditions produce a completely uniform solution of cleaved particles, it does appear that the digests are quite homogeneous, and are most probably composed of mixtures of particles at very similar stages of digestion.

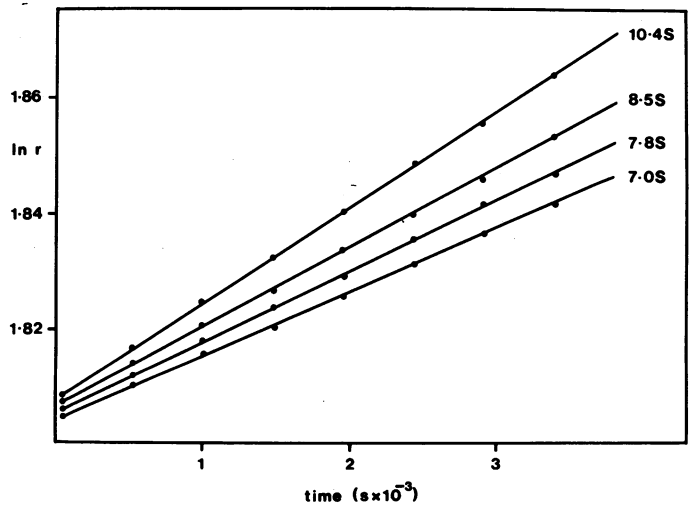


Figure 3: Plots of ln r (r = distance of boundary from rotor centre) against time (sec) for sedimenting trypsin digested core particles.

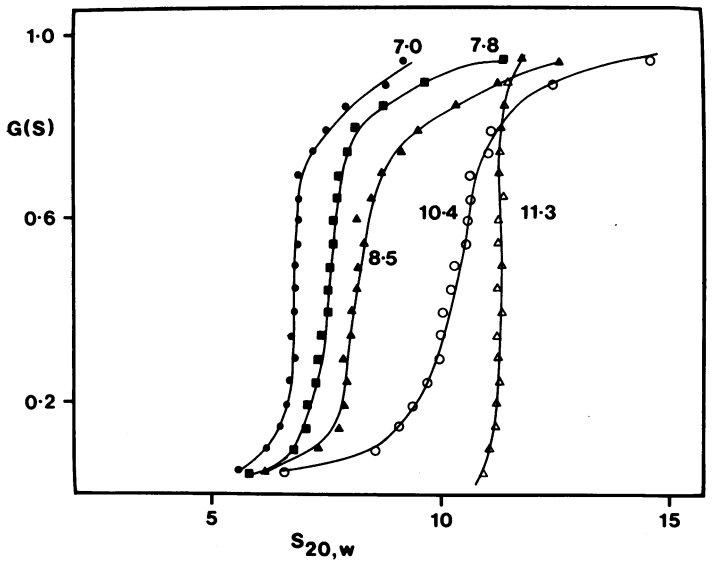


Figure 4: Plots of G(S) against $S_{20,w}$ for trypsin digested core particles.

The sedimentation coefficients calculated from these and similar data are shown in these figures, and also in figure 1. Thus we are able to correlate histone cleavage with changes in hydrodynamic behaviour.

Almost total cleavage of H3 to its first product produces only a very small lowering of $S_{20,w}$. We therefore think that the 'shortened' H3 can continue to constrain the DNA in a manner similar to its uncleaved parent. As further histone cleavage proceeds, a greater lowering of $S_{20,w}$ becomes apparent. At 10.6S all the histones except H2B have been cleaved, but the first product of H3 cleavage is still intact. Loss of intact H2B results in a further reduction to 9.7S; at this stage almost no parent histones remain. Thus the structure has unfolded to a considerable extent by cleavage of the 'tails'. It is likely that this process exposes further potential cleavage sites, thereby producing the further lowering of $S_{20,w}$ and the multiplicity of bands seen on the gel. Very extensive digestion reduces the $S_{20,w}$ beyond 7S, where loss of protein from the complex must be considerable.

Melting Profiles

The melting profiles of 10.4S and 7.7S trypsinised core particles are shown in figure 5, and those of undigested core particles and 140 base pair DNA are included for comparison. The derivative plot of these data, also

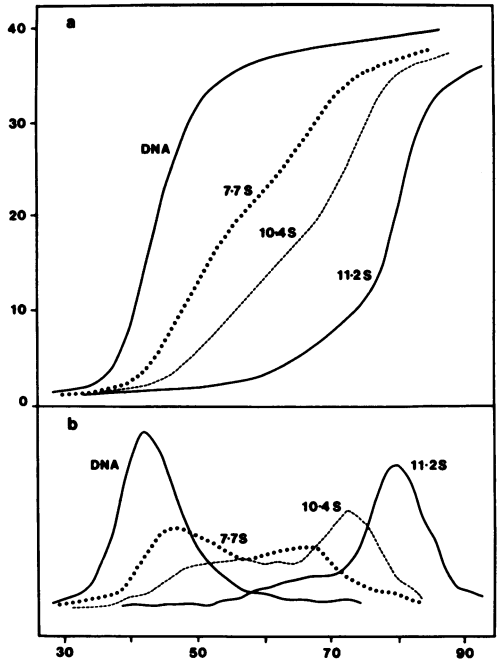


Figure 5: (a) Melting profiles of intact and trypsin digested core particles and 140 base pair DNA. (b) The same data shown in first derivative mode.

shown in figure 5, clearly indicates that the melting of partially digested particles is biphasic, being composed of higher and lower melting components. The relative proportions of these depends on the extent of digestion (this being confirmed by the melting behaviour of products of intermediate sedimentation coefficient, not shown). Thus it appears that histone cleavage lowers the overall $T_m$ and exposes two types of DNA, with melting properties similar to nucleoprotein and free DNA respectively. Earlier studies of digestion of chromatin (14,16) apparently failed to detect a similar 'pre-melting' behaviour.

Circular Dichroism

The CD spectra of core particles, 140 base pair DNA and trypsin digested core particles, in the region 260 to 300 nm, are shown in figure 6. Both chromatin (28,29) and isolated chromatin subunits (13,30) have characteristically low ellipticity in this region, when compared with free DNA, reflecting some conformational difference in DNA structure. Figure 6 shows that with increased extent of digestion, the CD spectra become more like

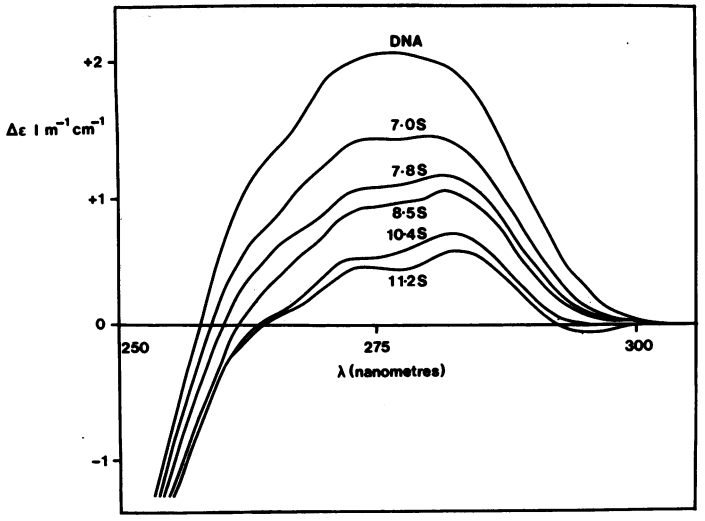


Figure 6: Circular dichroism spectra of intact and trypsin digested core particles and 140 base pair DNA.

that of DNA. The spectra look very like weighted summations of those of free DNA and core particles, thus strengthening our belief that the lowering of $S_{20,w}$ is accompanied by formation of two types of DNA with properties similar to those of the above. Of course, we cannot exclude the possibility that all the DNA is continuously converted via a series of conformations with intermediate spectra on the basis of these spectra alone. This is, however, very unlikely when the other data of this paper are taken into account.

Staphylococcal Nuclease Digestion

140 base pair core particles are subject to further digestion by staphylococcal nuclease, both as isolated core particles (18) and in chromatin (10,31). Weintraub and Van Lente (10) have further shown that the 'sub-monomer' peaks produced by further digestion of chromatin are different if the chromatin has been subjected to a prior trypsin digestion.

The most striking aspect of the staphylococcal nuclease digestion of trypsinised core particles, shown in figure 7, is the faster kinetics. Under identical conditions of digestion, sub-monomer bands are produced at much shorter times than for untrypsinised particles, suggesting a considerably enhanced accessibility of the DNA to the nuclease. This is very apparent even for the 10.4S particle, where we know that H2B and the first product from H3 are still uncleaved. Gel scans for the staphylococcal

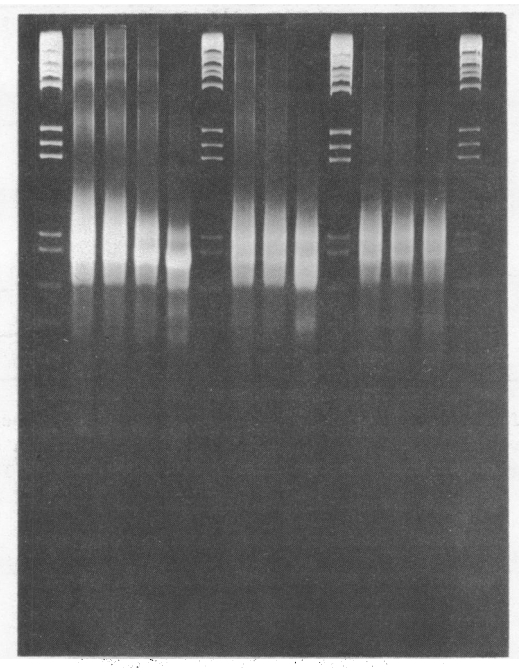

Figure 7: Polyacrylamide gel electrophoresis of staphylococcal nuclease digests of intact and trypsin digested core particles, together with PM2 Hae III restriction digests. Slots 2 to 5, 11.2S core particles digested for 0, 30s, 5 and 30m; slots 7 to 9, 10.4S particles digested for 10, 30s and 5m; slots 11 to 13, 7.7S particles digested for 10, 20 and 60s. PM2 Hae III fragments visible are assigned to 94 (fastest), 114, 141 and 155 base pairs (see also figure 8).

nuclease digestion of 11.2S and 10.4S particles, shown in figure 8, show that the patterns of sub-monomer bands produced are different. The 135-140 base pair peak is considerably reduced in the 10.4S particle, whereas there is a greater intensity around 120 base pairs. The bands corresponding to around 90 and 100 base pairs appear to be replaced by one of intermediate mobility after extended digestion. Thus the unfolding resulting from trypsin digestion of the histones apparently alters the specificity for cleavage by the nuclease, as well as allowing the digestion to proceed more rapidly.

DNase I Digestion

DNase I digestion patterns from intact core particles and 10.6S and 8.0S trypsin digested particles are shown in figure 9. As with the staphylococcal nuclease digestion, the reaction kinetics are considerably faster for the trypsinised particles, again suggesting an enhancement in accessi-

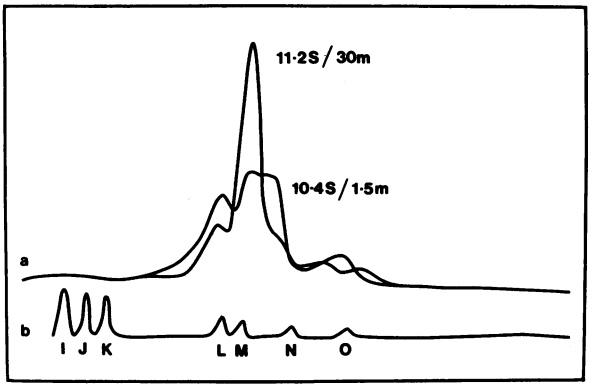


Figure 8: Scans of gels from electrophoresis of staphylococcal nuclease digests of core particles (30m digestion) and 10.4S trypsin digested core particles (1.5m digestion). Also shown is a scan of a Hae III restriction digest of PM2 DNA; O (94), N (114), M (141), L (155) and K (265 base pairs).

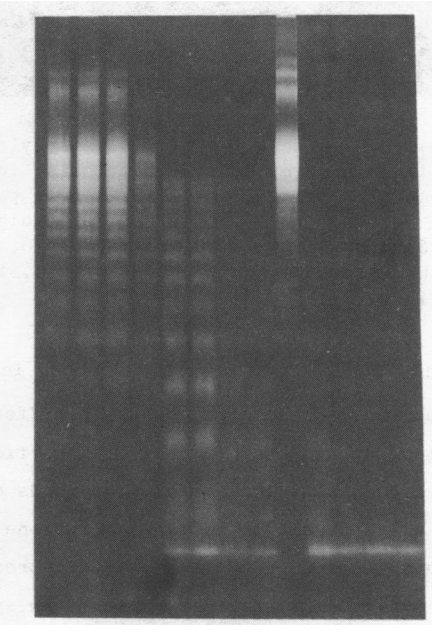

Figure 9: Polyacrylamide gel electrophoresis of DNase I digests of intact and trypsin digested core particles. Slots 1 to 4, 11.2S core particles digested for 30, 45s, 1 and 5m; slots 5 to 8, 10.6S particles digested for 30s, 1, 1.5 and 5m; slots 9 to 13, 8.0S particles digested for 0, 30s, 1, 1.5 and 5m.

bility. This rate difference may also be seen from a comparison of the scans from the negatives of similar gel photographs, shown in figure 10.

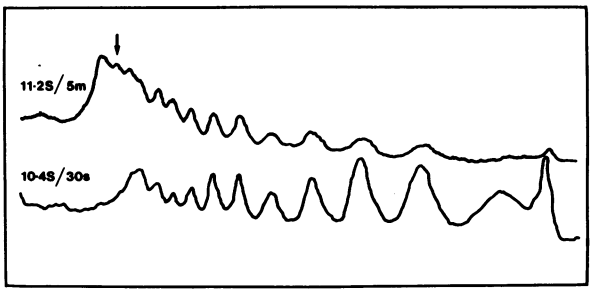


Figure 10: Scans of gels from electrophoresis of DNase I digests of core particles (5m digestion) and 10.4S trypsin digested particles (30s digestion). The arrow indicates the position of 140 base DNA.

It is evident from these data that all the particles give rise to typical DNase I 10 base ladders (32). However, the trypsinised particles appear not to give the higher bands, even at the very shortest digestion times. The number of higher bands which are absent increases with the lowering of $S_{20,w}$. These results are consistent with two possibilities. Firstly, the kinetics of digestion may be so increased for the trypsinised particles that the larger fragments have already been further cleaved at the earliest times. Alternatively, the structural feature of nucleosomal DNA which is responsible for the typical DNase I digestion pattern may be restricted to shorter DNA lengths for the particles of reduced $S_{20,w}$. The latter explanation is in better agreement with the $T_m$ and CD behaviour. Despite the absence of the higher bands, it is of great importance that the bands still seen from the trypsinised particles retain their 10 base spacing, in phase with those from the undigested core particles. Furthermore, the background between bands is not noticeably higher for these particles. Thus even though considerable unfolding of the DNA must have occurred, the structure resulting in the cleavage to the 10 base fragments is in part preserved. That this feature is partially retained even when the sedimentation coefficient has been lowered to 8.0S (where bands up to 50 bases may be distinguished) strengthens the general view that the 10 base fragments result from a feature related in some way to a fundamental property of the DNA itself, probably the 10 base pitch of DNA. This, in turn, further supports the contention that nucleosomal DNA is in the B form.

## DISCUSSION

Histone cleavage within core particles by trypsin results in marked effects upon sedimentation velocity, CD spectra, melting behaviour and the

kinetics and products of digestion by nucleases. Despite the interior positioning of the histone core (2), the initial products of proteolysis, as well as the order of histone attack, are similar in the intact core particle and with the DNA removed. Thus whilst the bulk of the protein is internal, the N-terminal regions must be close to the exterior of the particle and therefore accessible to the enzyme. $^{13}C$ NMR spectra of core protein in 2M NaCl (3,5,33) indicate that these regions are left without a defined structure once the DNA has been removed.

Cleavage of all four histone tails reduces the sedimentation coefficient of the resulting particle to ~ 9.7S. Using lower trypsin concentrations, such that H2B remains intact, a particle of ~ 10.5S is produced, which must, therefore, be closer to the native folded structure. Thus uncleaved H2B is capable of holding the structure together to a considerable extent.

At first sight it might be expected that the cleavage of the histone tails would produce a greater degree of unfolding. However the tails are likely to have two functions. The first is to exert a mechanical restraint on the DNA. The second is a mechanism whereby more efficient charge neutralisation of phosphate groups may be achieved. Under the conditions of our experiments, the N-terminal fragments should remain bound to the DNA, thus preserving the latter function. We have previously observed (33) that considerable protein-surface-DNA interaction must also occur. The retention of such contacts together with the continued charge neutralisation must be responsible for maintaining the integrity of the 9.7S particle.

Having unfolded to this extent, it is likely that further sites are available for protease attack, and so further unfolding will occur, as the results indicate. At this stage, the actual histone core structure will be perturbed, with concomitant loss of structure.

The melting profiles, circular dichroism spectra and DNase I digestion patterns may all best be explained on the basis of structural unfolding whereby part of the DNA retains the properties of the intact core particle, whilst the remaining portion behaves much more like free DNA.

H3 is more susceptible to proteolysis than the other histones, and has been shown (27) to be near to the DNA termini. Thus we think it likely that the particle unfolds by release of rods of DNA from their ends, whilst retaining a central core where DNA is folded in a manner very similar to that of the intact nucleosome.

Thus the released rods would provide an increased frictional drag, thereby lowering the sedimentation coefficient of the particle. They are

also likely to behave similarly to free DNA, hence melting at a lower $T_m$ than the folded DNA, and providing increased CD ellipticity at 280 nm. That increased digestion produces a further lowering of $S_{20,w}$, increased premelting and larger ellipticity, strongly suggests that the unfolding continues by the release of longer rods of DNA from the ends. Even after extended digestion to around 8S particles, however, a proportion of the DNA must still be very nucleosomal in its folding, since the 10 base fragments and some higher multiples are still produced by DNase I cleavage. The size of the highest bands visible puts a lower limit on the DNA length for the central folded section. That only lower multiples of 10 bases are seen for the trypsinised particles is also of some interest in the interpretation of the mechanism whereby these bands are generated from intact chromatin.

Possible modes of nucleosomal disassembly are of interest from another point of view. The observation that active genes have the subunit structure typical of bulk chromatin (34,35,36) has led to the suggestion that transcription does not require the release of histones from the DNA, but merely a partial disruption of the nucleosome (3,37,38). Thus histone-histone interactions may be altered to produce a 'linearisation' of the structure, where the major interactions retained are salt linkages between the histone 'tails' and the DNA phosphate groups. In this context the major function of the N-terminal 'tails' would be in maintaining the structure of the disrupted nucleosome, rather than its intact structure. Trypsin cleavage of the 'tails' may be considered as the reverse of this process, to the extent that the integrity of the 'tail'-DNA interactions have been partially lost. That the remaining histone core is partially successful in maintaining nucleosomal conformation adds weight to the idea that the real function of the 'tails' may be connected with the dynamics of nucleosome disassembly.

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