Mammalian Chromatin Substructure Studies with the Calcium–Magnesium Endonuclease and Two-Dimensional Polyacrylamide-Gel Electrophoresis

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The basic regularity of chromatin substructure that has been reported in rat liver chromatin (Hewish & Burgoyne, 1973*b*) was also detected in mouse chromatin. The regular series of DNA fragments produced by the action of Ca–Mg endonuclease on rat chromatin were studied further. The smallest single-stranded class has a molecular weight of approx. 45000–63000 and the smallest double-stranded class has a molecular weight of approx. 120000–150000. Studies of the substructure of the DNA fragments produced by the Ca–Mg endonuclease have shown that the regular series of double-stranded fragments have regular series of single-stranded fragments within them. It was concluded that the regular series of double-stranded fragments was probably a consequence of the regular series of single-stranded fragments. Digestion time-courses are presented for mouse and rat nuclear DNA.

The chromatin of mammalian cell nuclei consists of DNA complexed with basic and acidic proteins which have long been suspected to be involved in control of the expression of the genetic material. Previous studies have shown that the nuclear DNA is quite extensively covered by the nuclear proteins and much of the genome is thus inaccessible to the actions of various enzymes, such as deoxyribonuclease (Pederson, 1972; Billings & Bonner, 1972; Mirsky *et al.*, 1972), RNA polymerase (Bonner & Huang, 1963; Silverman & Mirsky, 1973; Paul & Gilmour, 1969) and DNA polymerase (Silverman & Mirsky, 1973; Wang, 1969). Removal of the chromatin proteins removes the inhibiting influence and allows the enzymes to act on the DNA (Billings & Bonner, 1972; Mirsky & Silverman, 1972; Silverman & Mirsky, 1973; Wang, 1969). There is, however, some argument about the degree to which the DNA is covered by the histones and the degree to which the covering proteins interfere with the approach of probe molecules (e.g. Clarke & Felsenfeld, 1971; Itzhaki, 1970).

Previous work from this laboratory (Hewish & Burgoyne, 1973*b*) has shown that as the DNA of isolated nuclei was digested *in situ* by an endogenous nuclear DNAase* (designated the Ca–Mg endonuclease), a series of discrete degradation products arose which could be separated by polyacrylamide-gel electrophoresis into a series of bands. The bands appeared to be the result of a non-random spacing of proteins on the DNA such that a non-random series

* Abbreviations: DNAase, deoxyribonuclease; RNAase, ribonuclease.

of sites susceptible to DNAase action was created. An analysis of the mobilities of the DNA bands in polyacrylamide gel by the method of Williamson (1970) indicated that the molecular weights of the bands were related as members of an arithmetic series.

The present paper has two main aims. First, we wish to demonstrate that the non-random breakdown of DNA is not a phenomenon completely limited to rat tissues. Secondly, we wish to examine the substructure of the DNA pieces produced by the action of Ca–Mg endonuclease on rat nucleoprotein DNA. The mouse was used as a technically suitable alternative animal and two-dimensional polyacrylamide-gel electrophoresis was used to examine the substructure of the DNA pieces produced in rat liver nuclei.

In all of our studies to date, chromatin has always been digested *in situ* as far as possible to minimize the possibility of reorganization of chromatin structure before the endonuclease attack on the DNA. Thus digestions have been carried out with intact nuclei.

Materials and Methods

Animals and cell stocks

Livers were obtained from Swiss Albino mice and from Hooded Wistar rats approx. 2 and 3–4 months old respectively.

Preparation of nuclei

Nuclear preparations were prepared by a modification of the procedure of Burgoyne *et al.* (1970). This

procedure used polyamines as stabilizing agents and chelating agents to remove bivalent metals. The following modifications, which gave more consistent yields of nuclei, were made. The buffered 2.4M-sucrose was replaced by 2.1M-sucrose in the same buffer, and the final wash and resuspension solution had 1mM-EDTA and 0.2mM-EGTA [ethanedioxybis-(ethylamine)tetra-acetic acid] in the buffered 0.34M-sucrose.

This nuclear preparation procedure gave rat and mouse liver nuclei that contained a Ca–Mg endonuclease which was capable of attacking the nuclear DNA *in situ* and fragmenting it (Hewish & Burgoyne, 1973*a,b*; Burgoyne *et al.*, 1970). Buffer A consisted of 60mM-KCl–15mM-NaCl–0.15mM-spermine–0.5mM-spermidine–15mM-2-mercaptoethanol–15mM-Tris–HCl, pH7.4.

Preparation of DNA from digests

A number of different phenol extraction procedures were used successfully. Long RNAase digestions were avoided, where possible, as the handling procedures during digestion and subsequent re-purification of the DNA tended to lessen the clarity of the electrophoretic patterns and there was usually very little RNA in the DNA preparations. DNA solutions that were too dilute for electrophoresis were concentrated and three different concentration procedures were used without giving noticeably different results. The procedures were dialysis against polyethylene glycol, film evaporation and alcohol precipitation.

Polyacrylamide gels

Three different polyacrylamide-gel systems were used in these studies. All used the same standard electrophoresis buffer solution in the gel and in the reservoirs. The buffer was 0.04M-Tris–0.02M-sodium acetate–2mM-EDTA adjusted to pH7.8 with acetic acid.

Gel system A. This consists of 2.5% (w/v) acrylamide and is the Loening (1967) system.

Gel system B. This is the same as system A, but with 7M-urea.

Gel system C. This consists of 7.5% (w/v) acrylamide, 0.2% *NN'*-methylenebisacrylamide, 0.05% *NNN'N*-tetramethylethylenediamine and 7M-urea.

Procedure for two-dimensional electrophoresis

This procedure depended on the observation that 7M-urea did not denature the native rat or mouse DNA, but did inhibit renaturation and cross-linking after heat denaturation (see the Results section).

Two gels were prepared.

(*a*) The primary gel was 2.5% (w/v) acrylamide, 7M-urea (system B). Primary gels were set in silicone-treated glass tubes (length 8cm, cross-sectional area 0.30cm²).

(*b*) The secondary gel was a slab of 7.5% (w/v) polyacrylamide and 7M-urea (system C). It contained 40ml of gel and was polymerized and run in a vertical Perspex holder. The dimensions of the gel were: approx. length 10.0cm; width 5.9cm; thickness 0.7cm. The slab holder should have approx. 5cm length of space above the main slab to hold the load plus a polyacrylamide plug. The polyacrylamide plug was a 2cm-long piece of 7.5% (w/v) polyacrylamide–7M-urea with the same cross-section shape as the main slab.

All gels were thoroughly pre-run before use with buffered 7M-urea solution in place of the load. Pre-running conditions were 15h at 1.5mA for the primary gel and 8h at 50mA for the secondary gel.

An average load contained approx. 100–250μg of DNA in 0.05ml of standard electrophoresis buffer and was made denser with a little sucrose (5–10%, w/v). A load was placed on top of a primary gel after removal of the buffered 7M-urea and current passed until the most mobile band of the DNA had travelled approx. 5.6cm (i.e. a little shorter distance than the width of the secondary gel). For our samples this took approx. 110 minutes at 5mA/tube.

The distance of travel of the fastest band was checked by running parallel gels containing one-tenth of the DNA that was loaded on the main gel.

After the primary gel had run, it was removed from its tube and placed in a test tube containing enough buffered 7M-urea to immerse it completely. The covered tube was then placed in a boiling-water bath for 15min and then cooled in ice. During this treatment the DNA was denatured *in situ*. Large bubbles occasionally appeared within the primary gel during the heating phase, but they were almost completely re-sorbed during the cooling phase and did not seem to interfere with the results.

The heat-treated primary gel was trimmed to approx. 5.8cm length so that it laid evenly along the top of the secondary gel.

The primary gel was held firmly in place by a plug of 7.5% (w/v) polyacrylamide–7M-urea (system C). The plugs were made conveniently by cutting approx. 2cm lengths out of a blank secondary gel. Plugs were cut from thoroughly pre-run, or thoroughly washed, blank gels. The plugs had small vertical grooves cut to allow the buffered 7M-urea to escape easily as the plug was pressed down evenly on to the primary gel.

The whole assembly was finally topped up with buffered 7M-urea and a voltage applied. For our samples a convenient combination of current and time was 19h at 32.5mA. Electrophoresis was carried out at 0–4°C.

Staining and observation

Ethidium bromide staining was used as described previously (Hewish & Burgoyne, 1973b) except the acetic acid was replaced by 1 mM-EDTA, sodium salt, pH 7.4. Thus the gels were stained in 20 μg of ethidium bromide/ml in 1 mM-EDTA, pH 7.4, and de-stained in 1 mM-EDTA, pH 7.4. Staining was usually for 24 h, although the bands could usually be observed after 30 min. Destaining took approx. 48 h.

The DNA bands gave an orange fluorescence when observed under u.v. light, and excitation wavelengths of 340, 354 and 254 nm were used.

Measurement of DNA in bands

Small one-dimensional electrophoresis runs were stained and destained as above. The fluorescent bands were cut out and then further sliced into thinner discs of gel, each weighing 100–200 mg. Each piece was weighed, pressed between quartz plates 1 mm apart and the fluorescence measured directly in an Eppendorf fluorimeter. The procedure was standardized with similar pieces of gel that contained known amounts of DNA. The total DNA/slice could then be calculated and summed to give the total DNA/band. All values were expressed as percentages of the total DNA on the gel.

Sucrose gradient centrifugation

Alkaline sucrose gradients were prepared and used by the method of Olivera & Lehman (1967). Neutral gradients were made up identically in 0.15 M-NaCl–0.015 M-sodium citrate, pH 7.0. Centrifugation was at 50000 rev./min for 10 h at 4°C. On completion of centrifugation, the tubes were removed from the rotor and fractions (0.16 ml) were collected by puncturing the bottoms of the tubes. The E_{254} of the fractions was measured and then fractions were pooled in groups of three to obtain sufficient material for electrophoresis. The pooled fractions were dialysed against standard electrophoresis buffer, concentrated, and run on 2.5% (w/v) polyacrylamide gels (system A) as for nuclear DNA above.

Preliminary studies on single-dimensional electrophoresis

Native and denatured DNA from liver nuclei digests were run in gel systems containing 7 M-urea (systems B and C), and it was observed that although heat denaturation did cause an overall decrease in mobility, as expected from the work of Fisher & Dingman (1971), Dingman et al. (1972a,b) and Harley et al. (1973), there still remained a pattern of bands not unlike that given by native DNA. Native DNA was seen to remain largely and possibly entirely native in gels containing 7 M-urea because it retained a characteristically higher overall mobility when compared with DNA that had been heat-treated. Despite the differences in overall mobility between native and denatured DNA in gels the qualitative similarity between the patterns suggested, falsely, that each denatured band arose from the corresponding native band. However, this was shown to be untrue. A batch of native DNA was electrophoresed in the absence of urea (system A), and the second- and third-order bands cut out (before staining) were mashed, made 7 M with urea, and re-electrophoresed in a urea gel (system C). The obvious and expected result was not obtained. Instead of the second- and third-order native bands giving rise to one second- and one third-order denatured band respectively, the second-order native band gave rise to two denatured bands and the third-order native band gave rise to three denatured bands. Thus a full analysis of all native bands became necessary.

Results

Substructure of the rat DNA fragments: analysis of a two-dimensional electrophoretogram

Plate 1 shows a two-dimensional electrophoretogram of DNA from rat liver nuclei that was digested in situ by its Ca–Mg endonuclease. Electrophoresis of the native DNA bands was carried out in one dimension, then the DNA was denatured and electrophoresis carried out in the second dimension.

A number of deductions can be made from electrophoretograms of this type.

Deduction (a). Each size class of native DNA contained a corresponding size class of denatured DNA, plus all permitted size classes of denatured DNA which were smaller than itself. Thus the smallest, most mobile band of native DNA gave rise to only one size class of denatured DNA (co-ordinates A1 on Plate 1a). The next most mobile band of native DNA gave rise to two size classes of denatured DNA (co-ordinates A2 and B2 on Plate 1a); the third native band (in order of decreasing mobility, or increasing

Table 1. Denatured DNA size classes in the native DNA size classes

A summary of the results shown in Plate 1. X* marks the origin. There were higher-order bands than D4, but as the order rose they were increasingly difficult to resolve.

Size class of denatured DNA	Order of mobility of native band				
	1	2	3	4	X*
A	A	A	A	A	
B		B	B	B	
C			C	C	
D				D	
X*					

size) gave rise to three size classes of denatured DNA (co-ordinates A3, B3 and C3 on Plate 1*a*). Table 1 summarizes the relation of these size classes.

Clearly there is some sort of integral relation between the numbers and sizes of the denatured bands and the sizes of the native bands. It seems that a series of definite, permitted size classes of denatured DNA exists in the digested DNA and has resulted in the generation of definite size classes of native DNA.

This supports our initial suggestion (Hewish & Burgoyne, 1973*b*) that the native DNA bands were simply related to each other by all having molecular weights which were simple multiples of the molecular weight of the smallest. The results do not prove this unequivocally, however.

Deduction (*b*). The size classes of native and denatured DNA were not composed of populations of molecules of absolutely identical molecular weights, because some of the bands (e.g. A1 on Plate 1*a*) were diagonally sloped. This sloping indicated that the dispersal of the band in the primary gel was at least partially due to true heterogeneity of size within each DNA band and not merely to technical imperfections in the formation of the gel or loading of the material. However, these conclusions do not indicate whether the heterogeneity was due to the characteristics of the endonucleolysis process or was produced subsequently to this by other nucleases or shearing.

Deduction (*c*). Loose concatenates did not make up a significant proportion of the bands on these gels, i.e. the bands of denatured DNA were not just hydrogen-bonded concatenates of the smaller species. If such concatenates did make up a significant proportion of the DNA they would also have appeared above the bands A1, B2, C3 and C4 (Plate 1*a*). In fact the zone above these bands was virtually free of DNA. (See the appropriately marked zone on Plate 1*a*.) At very high loadings of DNA, faint streaks were seen in this region, and this probably indicated that very minor proportions of concatenates could be formed. However, even these may be only formed at relatively high concentrations of DNA.

Comparison of rat and mouse native-DNA fragments

Plate 2 shows that, in digests of mouse and rat nuclei, the observable bands of native DNA had very similar mobilities. Although the pattern of mobilities of the main size classes of DNA were extremely similar between rat and mouse it is possible that there may have been species differences in the relative distribution of DNA between the high-molecular-weight bands and the lower-molecular-weight bands. Fig. 1 shows the time-course of development of the two most mobile bands in both rat and mouse. In this particular experiment the mouse nuclei appeared to maintain more DNA in the high-molecular-weight bands than the rat nuclei. However, repeat experiments have

shown that the rate at which the patterns develop and thus the slopes of the lines shown in Fig. 1 show considerable variations between experiments. This may partly reflect a variable content of Ca–Mg endonuclease, as it is well known that this enzyme varies in activity, depending on the metabolic status of the cell (Hewish & Burgoyne, 1973*a*).

No degradations were followed for longer than 110 mins. Over this period the maximum degradation of rat DNA gave over 80% of the recovered DNA in the three fastest bands and the maximum degradation of mouse DNA ever observed gave 45% of the recovered DNA in the fastest three bands over 110 mins. After about 15 min of digestion nearly all of the DNA is small enough to enter the gel and can be found in some part of the band system. We have not studied the rate of production of acid-soluble DNA fragments during the Ca–Mg endonuclease digestion in nuclei, but there is one report that shows they are not a major product of the degradation process (Yamada *et al.*, 1973).
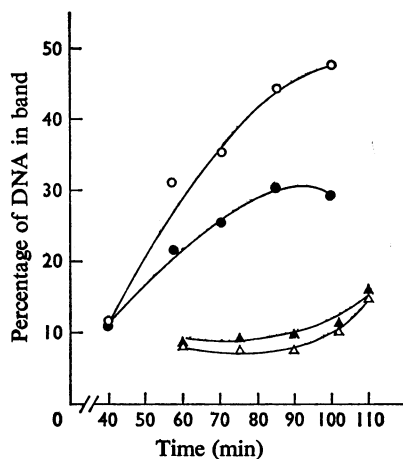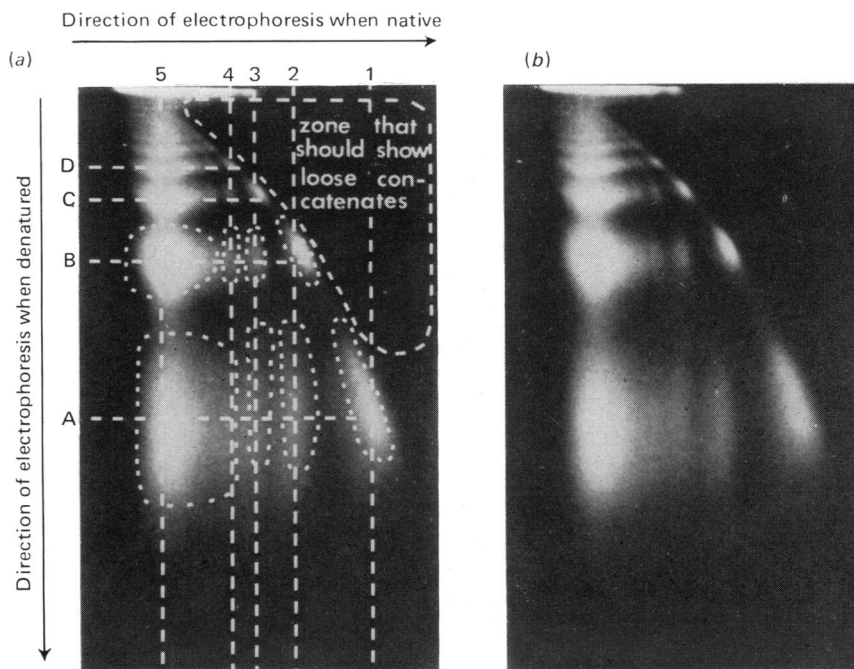

Fig. 1. *Comparison of digestion time-course between rat and mouse*

Nuclei were allowed to digest in the presence of Ca^{2+} and Mg^{2+} for periods ranging from 40 to 120 min. The DNA was then purified, exposed to RNAase A, and repurified by a phenol extraction to remove protein followed by a Sephadex G-50 step to remove RNAase-resistant oligoribonucleotides. The native DNA was then separated on 2.5% polyacrylamide gels (system A) and the two bands with highest mobilities were cut out for DNA determinations. Band 1 is the most mobile band and band 2 is the next band in order of decreasing mobility. The DNA content of each band is expressed as a percentage of the total DNA that has run into the gel, and very little DNA failed to penetrate the gel at the digestion times used in this experiment. Rat DNA: ○, band 1; ●, band 2. Mouse DNA: △, band 1; ▲, band 2.

Direction of electrophoresis when native

(a)

5   4 3   2   1

D

C

B

A

zone that should show loose concatenates

Direction of electrophoresis when denatured

(b)

EXPLANATION OF PLATE I

Two-dimensional electrophoresis of rat DNA fragments

Two prints (*a* and *b*) of the same electrophoretogram are shown. Print (*b*) is untouched and print (*a*) is identical with print (*b*) except that it has reference marks and notes superimposed on it. A sample of rat liver DNA was obtained by incubating a suspension of 1 ml of liver nuclei for 60 min at 37°C in 0.34 M-sucrose in buffer A, also containing 1 mM-EDTA, 0.2 mM-EGTA, 10 mM-$MgCl_2$, 1 mM-$CaCl_2$. The reaction was terminated by the addition of 5 ml of 100 mM-sodium EDTA, pH 7.4, and 4 ml of 4% (w/v) sodium dodecyl sulphate. Then 10 ml of 1.8 M-NaCl was added to the lysate which was next extracted with 10 ml of 78% (w/v) phenol. To the aqueous phase 2 vol. of ethanol was added to precipitate DNA and the precipitated DNA was washed by being redissolved in 1.5 ml of water and reprecipitated with 2 vol. of ethanol. The precipitate was finally dissolved in standard electrophoresis buffer. The load sample was 0.05 ml in volume and contained 250 μg of DNA. The DNA was electrophoresed, in its native state, along the upper, horizontal axis and then the bands were denatured and electrophoresed downwards. Technical details are given in the Materials and Methods section. The co-ordinates 1, 2, 3, 4 refer to the native bands on the upper horizontal axis in decreasing order of mobility. The co-ordinates A, B, C, D refer to classes of denatured DNA in order of decreasing mobility. Note that each native band contains a denatured DNA class, which is characteristic of it, plus all denatured classes smaller than the characteristic class. Note the absence of loose concatenates. Dotted lines have been placed around the zones A1, A2, and A3 and A4 → Ax, also around zones B2, B3, B4 and B5 → Bx, in order to mark them more clearly.
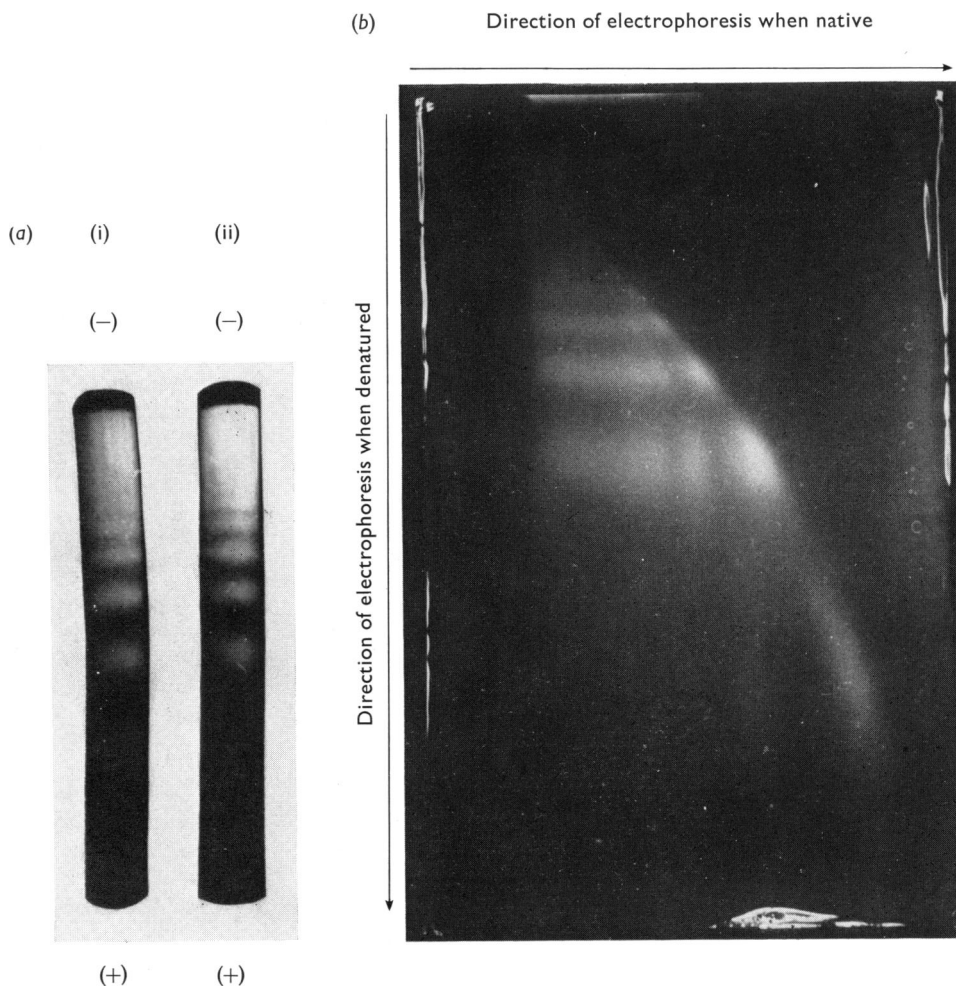
EXPLANATION OF PLATE 2

Comparison of mobilities of bands from rat and mouse DNA

(a) Nuclei from rat or mouse liver were autolysed in the presence of Ca^{2+} and Mg^{2+} and DNA was prepared from the nuclear digests as described for Plate 1. (i) Rat liver nuclei were incubated at 37°C for 60min and the DNA loaded on to the gel (load was 30µg of DNA). (ii) Mouse liver nuclei were incubated at 37°C for 60min and the DNA loaded on to the gel (load was 27.5µg of DNA). (b) Two-dimensional electrophoretogram of mouse DNA fragments. Mouse liver nuclei were digested and DNA fragments prepared as for Plate 1, and the electrophoresis procedure was as for Plate 1. The digestion time was 100min, and the load was 113µg of DNA.

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Table 2. *Molecular weight and sedimentation coefficient of DNA band 1 under neutral and alkaline conditions*

Digested nuclear DNA was purified by phenol extraction and centrifuged on neutral and alkaline sucrose gradients. Fractions from the gradient were analysed by polyacrylamide-gel electrophoresis to locate the various DNA species, and the approximate molecular weights of the first bands were calculated by extrapolating from the relationships of Burgi & Hershey (1963) and Studier (1965).

Gradient	$s^0_{20,w}$ (S)	Approximate mol.wt.
Neutral	5.3	150000–120000
Alkaline	3.8	65000– 45000

Molecular weight of the smallest classes of DNA molecules

Rat liver nuclei were isolated and allowed to autodigest their DNA in the presence of 1 mM-CaCl$_2$ and 10 mM-MgCl$_2$. The DNA fragments produced were purified by phenol extraction and run on neutral and alkaline sucrose gradients. Fractions from the gradients were subjected to polyacrylamide-gel electrophoresis to determine the position of the various DNA species in the gradient. The results of two such analyses are shown in Table 2. In neutral sucrose gradients the first band sedimented with a molecular weight of 120000–150000, calculated by the method of Burgi & Hershey (1963). In an alkaline gradient it sedimented with a molecular weight of 45000–63000, which is approximately half the former value, within the limits of error of the determinations.

Discussion

The results present in this paper are quite compatible with the earlier (Hewish & Burgoyne, 1973b) conclusion that the patterns of double-stranded DNA fragments represent a regular series of molecular weights N, $2N$, $3N$, $4N$ etc., where N is approx. 120000–150000. This explanation of the phenomenon still seems the most satisfactory. Essentially the regularity of the pattern of degradation of the nuclear DNA is thought to reflect a corresponding regularity in the pattern of protein bound to the DNA rather than being due to base recognition as with the restriction nucleases. The proteins probably restrict the action of the Ca–Mg endonuclease by simple steric hindrance.

The evidence from the two-dimensional electrophoresis indicates that the regular size classes of the native DNA are a secondary consequence of a regular pattern of single-strand breaks. Single-strand breaks are much more frequent than double-strand breaks, and double-strand breaks occur when two single strands occur near enough together.

The hypothesis postulated that protein bound to the DNA produced a regular pattern of steric hindrance. There is thought to be a very simple, repetitive, structure that has a simple average repeat distance corresponding to single-strand DNA pieces with molecular weights of approx. 60000–70000. However, this simple theory alone does not at present satisfactorily explain some features of the time-courses of degradation of higher-order bands to lower-order bands. We have suggested (Hewish & Burgoyne, 1973b) that a higher level of steric hindrance may exist and have postulated a class of nucleoproteins that, more or less randomly, sterically inhibit sites that the substructure proteins have left sterically susceptible.

The evidence for a higher level of steric hindrance is not nearly as strong as the evidence for the first level of steric hindrance. However, postulating two levels of steric hindrance does provide a simple and adequate explanation of the results and provides a basis for further investigation.

These studies leave a large number of important and interesting questions unanswered. One of the main questions is that posed by the various classes of repetitive DNA in the cells (e.g. mouse satellite DNA and ribosomal DNA). The electrophoresis and staining procedures only allow the bulk of the DNA to be observed and thus still leave open the question of whether the system of bands with integral relationships even contain these minor species of DNA.

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