STUDY OF CALF THYMUS DEOXYRIBONUCLEOPROTEINS BY MEANS OF GEL ELECTROPHORESIS.EFFECT OF IONIC COMPOSITION ON THE MODE OF CHROMATIN FRAGMENTATION.

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Summary.Calf thymus soluble deoxyribonucleoproteins (DNP) obtained by sonication of chromatins isolated both in a "physiological" salt medium and in a buffered water were resolved into four fractions on electrophoresis.The DNA extracted from DNP obtained in a salt medium occurs in fragments of discrete sizes while the sonicated chromatin gel obtained in a buffered water gives rise to a heterogeneous population of DNA fragments upon deproteinization.It is suggested that regularly spaced "weak" points exist in native chromatin and that the regularity is destroyed during isolation procedures involving the transfer of the nuclei into water.

In the cell nucleus chromatin exists in a rather complex medium /1/ which is strikingly different in ionic strength and composition from the media commonly used for its isolation /e.g.2,3/. In the present work we have investigated by means of gel electrophoresis the role of ionic conditions used to prepare soluble DNP on the mode of chromatin breakage by mechanical shear.

METHODS.Preparation of nuclei.Nuclei were prepared from calf thymus which was frozen in dry ice immediately after slaughter and stored at -70° C.The tissue was homogenized in a Waring blender in 0,1 M KCl-0,05 M NaCl-1 mM $MgCl_2$-1 mM $CaCl_2$-1 mM Tris-HCl,pH 6,5 (buffer A).The homogenate was passed through several layers of cheesecloth.After filtration the material was sedimented at 1000 g for 10 min.The nuclear pellet was washed three times by resuspension in buffer A by hand homogenization and centrifugation at 1000 g for 10 min,then it was washed another four times in 0,1 M KCl-0,05 M NaCl-1 mM $MgCl_2$-1 mM $CaCl_2$-20 mM Tris-HCl,pH 7,5 (buffer B or "salt" medium).

Preparation of "salt" DNP (DNP_S).The nuclear pellet from the last wash was resuspended in buffer B at $A_{425} \sim 100$.The disruption of nuclei and simultaneous fragmentation of chromatin were made with sonifiers at frequencies 20 and 36 kHz.The samples were sonicated for various lengths of time with several 1-min pulses. They were extensively cooled during the sonication procedure so that the temperature never exceeded 10° C.At each frequency constant volumes of the solutions were used.The sonicated samples were clarified by centrifugation at 10000 g for 10 min.The resulting supernatant is referred to as DNP_S.In some cases to obtain DNP_S the degradation of chromatin was accomplished by five passages at 1500 atm in a French pressure cell.

Preparation of "water" DNP (DNP_w). The gelatinous chromatin pellet was prepared from the nuclei by five washes in 1 mM Tris-HCl, pH 7,5. To obtain DNP_w the gel was then fragmented either by sonication or by passing through a French pressure cell and clarified as described above for DNP_s.

All procedures were carried out at 4° C. The DNP preparations were stored in an ice-bath and showed no significant variations in their electrophoretic properties for at least a week.

Preparation of DNA. DNA was isolated from the soluble DNP by phenol extraction.

Gel electrophoresis. The DNP_w samples were routinely electrophoresed in 0,5 % agarose gels. For electrophoresis of DNP_s composite gels containing 0,5 % agarose and up to 2 % acrylamide were also used. 0,1-0,2 ml of the DNP solution containing ~2 A_{260} units of the material were applied onto each gel. For electrophoretic analyses of DNA 3 % polyacrylamide-0,5 % agarose composite gels were used. For electrophoresis of DNP_w and DNA respective gels were prepared and run in 40 mM Tris-20 mM sodium acetate-2 mM Na_2EDTA, pH 8 and for electrophoresis of DNP_s-in buffer B, pH 8. Electrophoresis was carried out at 4 V/cm and 4° C. The details of the gel cooling were described previously /4/. When buffer B was used for electrophoresis it was necessary to check and to adjust its pH to the initial value periodically during the run. After electrophoresis two DNP gels run in parallel were stained one for DNA and the second for protein. The DNA component of DNP was stained using a 0,1 % solution of Methylene Blue (MB) in 7 % acetic acid and the protein component-in a 1 % solution of Amido Black 10 B in 7 % acetic acid. MB-stained gels were destained in water and AB-stained gels-in 7 % acetic acid.

RESULTS. Characterization of DNP_s. The yield of DNP_s comprised 1,5-20 % of the input material depending on the length of sonication time (30 sec-20 min). Electrophoresis in 0,5 % agarose gels permits the separation of DNP_s obtained at sufficient sonication times into four fractions (Fig.1a). Only the intermediately moving DNP_s components II and III can be stained both for DNA and for protein (band III stains for protein very faintly). The slowly moving band I can be stained only for protein while the fast moving band IV stains only with MB (Fig.1a, Table 1). At short sonication times (30 sec-5 min) band IV is absent from the electrophoretic patterns of DNP_s (Fig.1b). Here we used 0,5 % agarose gels only for the sake of uniformity, because this gel concentration is optimal for fractionation of DNP_w (see below). The separation between DNP_s fractions could be significantly increased by using more concentrated gels (Fig.1c). The same results were obtained when fragmentation of chromatin was performed using a French pressure cell.

Characterization of DNP_w. The yield of DNP_w comprised 90-95 % of the input material. When DNP_w obtained at sufficient sonication times (e.g. 2,5 min, 5 min) was electrophoresed in 0,5 % agarose gels the four fractions were observed (Fig.1d). The three slower

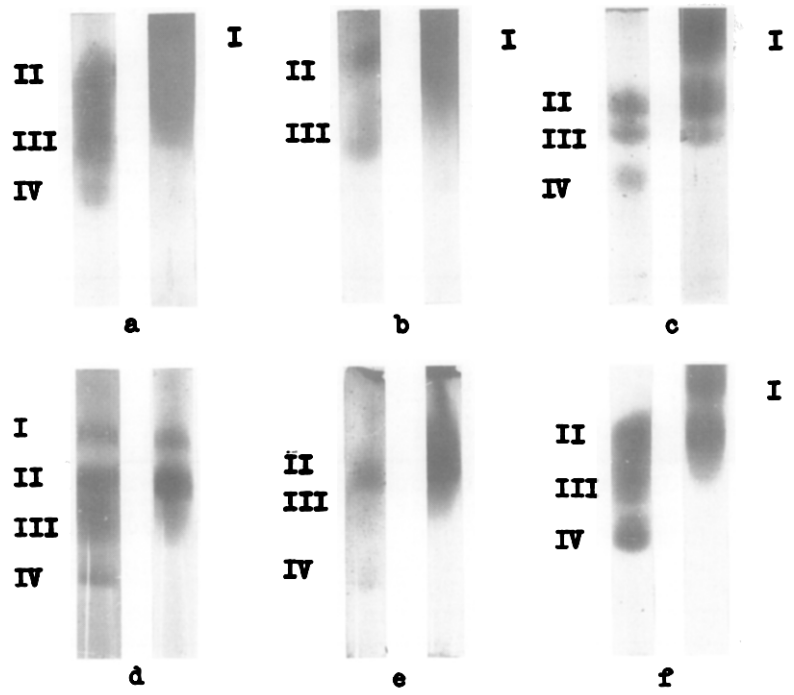

Fig.1 Gel electrophoretic fractionation of DNP.
In all cases except (c) DNP samples were obtained by sonication of respective chromatins at 36 kHz for the lengths of time indicated below: (a) DNP_s,10 min ; (b) DNP_s,30 sec ; (d) DNP_w,5 min ; (e) DNP_w,10 sec ; (f) $DNP_{w\rightarrow s}$,10 min. (c) DNP_s was obtained by five passages of the nuclei through a French pressure cell at 1500 atm.

Electrophoresis was performed as described in METHODS at 4 V/cm for 2 hr in 0,5 % agarose gels (a,b,d-f) and for 2,5 hr in 1,5 % polyacrylamide-0,5 % agarose gels (c).

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bands (I-III) stain both for DNA and for protein,while the fastest one (IV) cannot be visualized by staining for protein (Table 1) and,therefore,should be formed by a practically protein-free DNA.Chromatin gel produces band IV even at sonication times as short as 10 sec (Fig.1e).The fact that bands IV of $DNP_w^{10\ sec}$ and of $DNP_w^{5\ min}$ migrate with equal velocities in 0,5 % agarose gels arises out of the inability of the gel to resolve DNA in this size range.Electrophoresis of these two DNP_w samples in 3 % composite gels shows that the average size of DNA fragments which give rise to band IV of $DNP_w^{10\ sec}$ is longer than that of DNA fragments forming band IV of $DNP_w^{5\ min}$.At short sonication times (10,30 sec) a lot of material does not enter the 0,5 % agarose gel and band I is absent from the electrophoretic patterns of DNP_w (Fig.1e).

Table 1. Comparison of some properties of DNP samples isolated in different media.

Type of DNP	Electrophoretic fractions of DNP			Electrophoretic pattern produced by DNA extracted from DNP
	Fraction No.	Stain for protein	Stain for DNA	
DNP_s	I	+	−	Regular series of bands
	II	+	+	
	III	±	+	
	IV	−	+	
DNP_w	I	+	+	A broad band with no regularities
	II	+	+	
	III	+	+	
	IV	−	+	
$DNP_{w \rightarrow s}$	I	+	−	Same as for DNA of DNP_s but very smeared
	II	+	+	
	III	±	+	
	IV	−	+	

DNA isolated from DNP_s. DNAs prepared from DNP_s samples obtained at various sonication times were subjected to electrophoresis in 3 % composite gels. Typical DNA preparations gave a regular series of bands on electrophoresis (Fig.2a,b). They contained several relatively high mol.wt. components migrating in 3 % gel as narrow bands. The extensive sonication of chromatin in buffer B (15, 20 min) led to the disappearance of these bands (Fig.2c). Their mol.wts ranged from 1×10^6 to 5×10^6 daltons, as approximately measured by comparing their mobilities with those of DNAs of known sizes /4/. All DNA samples also contained three low mol.wt. components of discrete sizes independently of the duration of sonication of chromatin (30 sec-20 min). At long sonication times (15, 20 min) the pattern produced by DNA of DNP_s on electrophoresis became smeared (Fig.2c). The sizes of the three low mol.wt. DNA fragments roughly estimated by running the DNA samples on Sepharose 6B column and using the calibration curve of Prunell and Bernardi /5/ are about 3×10^5, 1×10^5 and $2\text{-}4 \times 10^4$ daltons, respe-

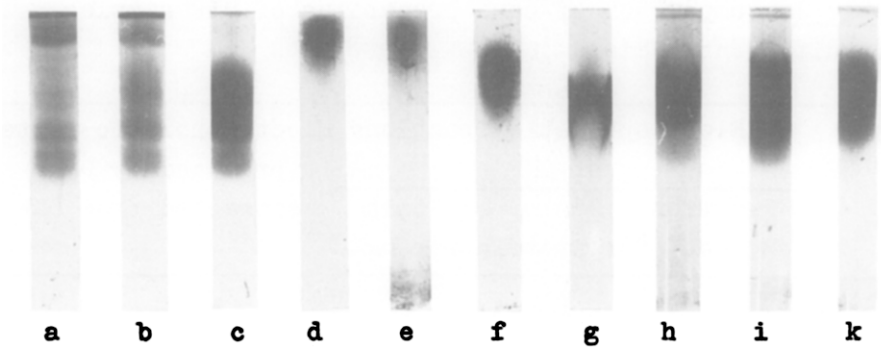

Fig.2 Electrophoretic comparison of DNAs isolated from various DNP samples.
DNAs were extracted from DNP samples obtained by sonication of chromatins at 36 kHz for the lengths of time indicated below : (a) DNP_S,30 sec ; (b) DNP_S,5 min ; (c) DNP_S,15 min ; (d) DNP_W, 10 sec ; (e) DNP_W,30 sec ; (f) DNP_W,2,5 min ; (g) DNP_W,5 min ; (h) $DNP_{W\to S}$,2,5 min ; (i) $DNP_{W\to S}$,5 min ; (k) $DNP_{W\to S}$,10 min. Electrophoresis was performed at 4 V/cm for 2 hr using 3 % polyacrylamide-0,5 % agarose composite gels.

ctively.At short sonication times in each of the three low mol. wt.DNA bands two subfractions could be observed (Fig.2a).

DNA isolated from DNP_W.DNAs extracted from DNP_W samples obtained at various times of sonication of the chromatin gel migrated over a broad region of the 3 % composite gel (Fig.2,d-g) exhibiting a random distribution with respect to chain length.The effect of prolonged sonication of the chromatin gel was a progressive shift of the broad band of its DNA to the lower mol.wt.region.No discrete bands could be detected even at very short sonication times (10 sec).

Transfer of the chromatin gel into a "salt" medium.Chromatin gel was precipitated by adding buffer B in a great excess.The precipitate was collected by centrifugation at 1000 g for 10 min and the pellet was washed several times with buffer B.The resulting chromatin was subjected to sonication for various lengths of time.The material remaining in supernatant after centrifugation at 10000 g for 10 min is referred to as $DNP_{W\to S}$.Under the same conditions of sonication the yield of $DNP_{W\to S}$ is similar to that of DNP_S.

Fig.1f shows that the typical $DNP_{W\to S}$ preparation produces a pattern similar to that produced by DNP_S on electrophoresis in 0,5 % agarose gels.Four bands are present,band I stains only with AB and band IV-only with MB.The differences between $DNP_{W\to S}$ and DNP_S preparations are revealed by comparing the size distributions produced by respective DNAs in 3 % composite gels.

DNA isolated from $DNP_{W \to S}$. Under conditions of sonication in which DNA of DNP_S gives fragments of discrete sizes on electrophoresis, DNA of $DNP_{W \to S}$ gives a very heterogeneous population of fragments (compare Fig.2, a-c and h-k). We have not found any regularities in the size distribution of the DNA of $DNP_{W \to S}$ with the exception of samples obtained at very short sonication times (30 sec, not shown). DNAs of $DNP_{W \to S}$ and DNP_W are similar in that they are both heterogeneous with respect to chain length. However, electrophoretic comparison of the fragmentation kinetics reveals an important difference. DNA isolated from $DNP_{W \to S}$ travels in the region of the three low mol.wt. bands produced by DNA fragments of DNP_S independently of the sonication time, while the broad band produced by DNA of DNP_W undergoes a progressive displacement toward lower mol.wts region of the gel upon increasing the time of sonication of chromatin (Fig.2, h-k and d-g, respectively). Hence, it should be concluded that the mode of fragmentation of the chromatin gel isolated in water and then transferred into a "salt" medium is somewhat intermediate between those of chromatins obtained in a "salt" medium and in water, respectively. These results are tabulated for comparative purposes (Table 1).

DISCUSSION. The first stage of our work was directed toward construction of such a medium for isolation and further investigation of chromatin which approaches the ionic conditions within the nuclei. According to /6/ the morphological appearance of chromatin in isolated nuclei is most close to that observed *in situ* when the concentration of each of the Mg^{2+} and Ca^{2+} divalent cations in the isolation medium is 0,9 mM. Basing upon these and another data /1,7,8/ we have chosen the following medium: 100 mM KCl-50 mM NaCl-1 mM $MgCl_2$-1 mM $CaCl_2$-20 mM Tris-HCl, pH 7,5 (buffer B). This buffer was used throughout all the steps of preparation and analysis of chromatin (tissue homogenization, washing of the nuclei, disruption of the nuclei and gel electrophoresis). The DNP isolated in buffer B was compared with that isolated conventionally in a buffered water.

The results obtained show that both DNP samples resolve into several fractions on electrophoresis. The material containing in the slowly moving band I of DNP_W shows an abrupt dependence of its electrophoretic mobility on the gel concentration. At acrylamide concentrations > 1 % in composite gels it does not enter the gel (not shown) while fraction I of DNP_S migrates in the gel

at acrylamide concentration 1,5 % (Fig.1c) and higher.This indicates that fraction I of DNP_w is composed of the more large and/or rigid particles /4/.Fig.1 shows that fraction I of DNP_s does not develope a blue colour upon staining with MB.Probably,the DNA in particles of DNP_s giving rise to this fraction is more tightly covered by protein and is packed in a more compact fashion than in those of DNP_w.At sufficient degrees of fragmentation of chromatin in buffer B it liberates the fragments forming band IV of DNP_s which does not stain for protein and,therefore,may be tentatively interpreted as being "naked" DNA.In DNP_w such fraction appears even after very short sonication times (e.g.10 sec,Fig.1e).

The most interesting observation is that DNA of DNP_s occurs in fragments of discrete sizes (Fig.2a,b) indicating that the chromatin isolated in buffer B breaks in a regular fashion upon sonication.In contrast,DNA extracted from DNP_w exhibited a broad size distribution which shifted to the lower mol.wts when the sonication time was increased (Fig.2,d-g).This indicates a random breakage of the chromatin gel.It should be stressed that the DNA in the "salt" chromatin is broken down to the short fragments much more easily than in the chromatin gel (compare Fig.2,a and d) or than pure DNA (not shown).One of the possible explanations is that in the points of the "salt" chromatin in which preferential breakage occurs the DNA structure is somehow modified.Examination of the electrophoretic pattern produced by DNA extracted from $DNP_{w \to s}$ leads to the conclusion that respective chromatin breaks in a fashion similar to that of chromatin isolated in a "physiological" medium but the precision of breakage is somehow distorted.The DNA fragments isolated from its sonication products demonstrate a slight heterogeneity in size which results in a high background in the interband regions so that the size distribution seems heterogeneous (Fig.2,h-k).The most probable interpretation of the discrete electrophoretic patterns produced by the DNA of DNP_s is that chromatin in our "salt" medium is packed in a way providing the existence of regularly spaced points for preferential breakage by sonication shear.A reasonable conclusion is that in the cell nucleus chromatin is also arranged in a regular fashion which can be essentially disturbed during the isolation procedure.In particular,this distortion can be ascribed to a redistribution of histones along DNA in chromatin /9/.

Several authors obtained different evidences of the regular

arrangement of native chromatin and the existence of the "weak" points in it /10,11/.

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