

## Studies on chromatin. II. Isolation and characterization of chromatin subunits

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### ABSTRACT

Earlier findings /1-10/ bearing on a subunit organization of chromatin were confirmed and in some points detailed. Besides this, a large-scale isolation of chromatin subunits, their protein composition, electron microscopic appearance and CsCl banding pattern are described. Although the purified chromatin subunit contains all five histones, the relative content of histone H<sup>1</sup> in it is two times lower than that in the original chromatin. It is shown that a mild digestion of chromatin with staphylococcal nuclease produces not only separate chromatin subunits and their "oligomers" but also deoxyribonucleoprotein particles which sediment more slowly than subunits. It appears that these particles and subunits are produced from different initial structures in the chromatin.

Finally, a crystallization of the purified chromatin subunit as a cetyltrimethyl ammonium salt is described.

### INTRODUCTION

Recent work /1-10/ has demonstrated the existence of a subunit structure in chromatin. Hewish and Burgoyne have reported /1/ that a large proportion of DNA in isolated rat liver nuclei is digested by an endogeneous nuclease to fragments which are integral multiples of a unit length. Further studies by Noll /4/, Felsenfeld /5/, Van Holde /6,7/ and other investigators /8,9/ which were based on the use of staphylococcal nuclease as a probe for chromatin substructure have shown that a mild nuclease treatment of rat liver nuclei results in the formation of a set of deoxyribonucleoprotein (DNP) particles which contain separate chromatin subunits (we shall call them "monosomes") together with various "oligomers" of this repeating DNP particle. Each monosome contains from 150 to 200 base pairs of DNA and approximately

equal by weight amount of histones/3,4/. Parallel electron microscopic studies of Olins and Olins /2,11/ have resulted in visualization of subunits in chromosomal fibers.

In the present paper we describe the results of nuclease digestion of a double-labelled chromatin, which confirm and in some points detail the previous findings/1-9/. Besides this, crystallization of the purified chromatin subunits (monosome), a large-scale isolation of monosomes, their protein composition, electron microscopic appearance and CsCl banding pattern are described. We report also the existence of DNP particles smaller than monosomes in a mild nuclease digest of the chromatin. Ancestor structures for these particles in the chromatin appear to be different from ancestor structures for monosomes.

#### MATERIALS AND METHODS

Preparation of labelled chromatin. Mice carrying Ehrlich ascites tumor cells were injected intraperitoneally with a mixture of  $Me-^3H$ /thymidine and  $L-^{14}C$ /lysine or a hydrolysate of *Chlorella*  $^{14}C$ -proteins /12,13,16/. Chromatin was prepared as described previously /12,15,16/. The procedure included isolation of nuclei, extraction of nuclei with 0.30 M NaCl, 2 mM  $MgCl_2$ , 5 mM triethanolamine (TEA)-HCl, pH 7.6 and additional purification of chromatin by centrifugation through 1.7 M sucrose, 5 mM TEA-HCl, pH 7.6 /16/. The specific radioactivity of  $^3H$ -DNA ranged from 10,000 to 25,000 cpm/ $\mu g$ ; the specific radioactivity of proteins - from 3,000 to 8,000 cpm/ $\mu g$ . Histones contained 60-70% of the total  $^{14}C$ -counts.  $^3H$ -DNA contained less than 0.5% of the total  $^{14}C$ -counts /15,16/.

Nuclease digestion of chromatin. Chromatin gel was gently suspended in 1 mM TEA-HCl, pH 7.6 to a final concentration of 1 mg of DNA per ml. Staphylococcal nuclease (Schwarz/Mann, 12,000 units/mg) was then added at 0°C to a final concentration of 2.5  $\mu g/ml$ . Immediately thereafter 50 mM  $CaCl_2$  was added to a final concentration of 1 mM followed by incubation at 37°C for a required time. The digestion was stopped by addition of 50 mM Na-EDTA, pH 7.6 to a final concentration of 2 mM followed by chilling in an ice

bath. The sample was centrifuged at 10,000 g for 10 min. Aliquots were taken from the initial suspension and the supernatant to determine the total and acid-soluble radioactivity. The supernatant was used immediately or after fixation with 1% HCHO at pH 7.0 /15,16/. Virtually no degradation of histones occurred during nuclease digestion of the chromatin and during subsequent handling of DNP samples (data not shown).

Analytical sucrose gradient centrifugation. A sample of unfixed or HCHO-fixed DNP (0.25 ml) was layered onto a linear 5-40% sucrose gradient which contained a 2.4 M sucrose shelf at the bottom. Centrifugation was carried out in the SW40 rotor (Beckman) at 39,000 rpm for 16 hr at 3°C. Fractions were collected directly onto glass fiber filters, washed with cold 7%  $\text{CCl}_3\text{COOH}$  and ethanol and thereafter counted with toluene-PPO-POPOP in the computerized SL-40 counter (Intertechnique) /15,16/. No significant differences could be observed between the patterns of fixed and unfixed DNP samples.

Preparative sucrose gradient centrifugation. A sample of unfixed DNP (60 ml) was layered onto 1,500 ml of a linear 10-40% sucrose gradient in the T115 zonal rotor (Beckman). Centrifugation was carried out at 33,000 rpm for 40 hr at 3°C. Appropriate fractions were pooled and used for the next experimental stage.

Gel chromatography. A sample of unfixed or HCHO-fixed DNP (0.2 ml) was layered onto a 10-ml siliconized column containing Sepharose 4B (Pharmacia) which was equilibrated with 2 mM Na-EDTA, 10 mM TEA-HCl, pH 7.6. Fractions were collected directly onto glass filters /15,16/.

Isopycnic banding of DNP in CsCl gradients. Fixed DNP samples were centrifuged in the SW50.1 rotor (Beckman) at 45,000 rpm for approximately 75 hr at 15°C /13-16/. Fractions were collected directly onto glass filters. Selected fractions were collected into tubes to determine the density along the gradient /15,16/. In most of the experiments 0.5% Sarcosyl NL97 (Geigy) was present in CsCl gradients to make the recovery of the  $^{14}\text{C}$ ,  $^3\text{H}$ -counts close to 100% /16/. Sarco-

syl did not change the density of the DNP in CsCl as compared with pure CsCl gradients.

Polyacrylamide gel electrophoresis of DNA. It was carried out in 6% polyacrylamide gels. The buffer system was that described in ref.17. DNA bands were visualized by staining with ethidium bromide.

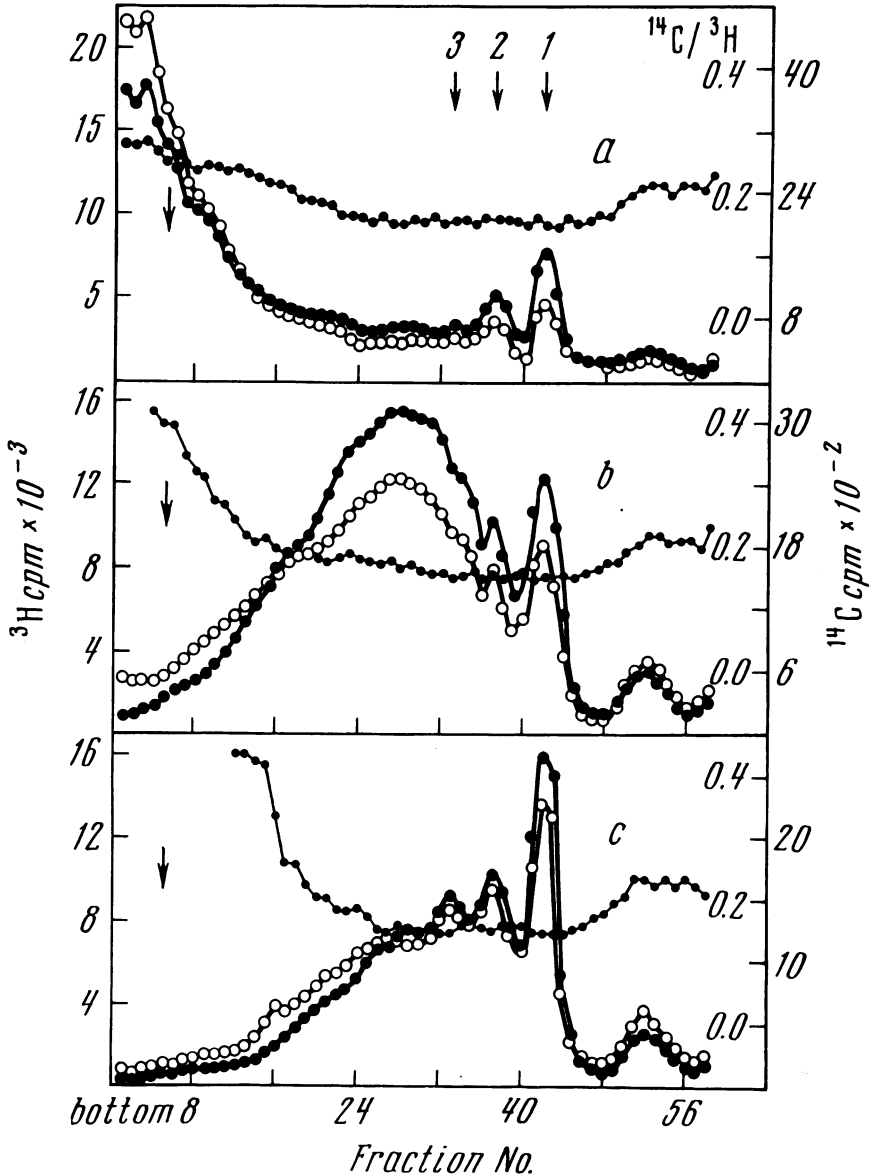
SDS-gel electrophoresis of proteins. It was carried out as described in ref.18 except that no spacer gel was used. Fractions from the preparative sucrose gradient (see above) were made 80 mM in Na-acetate followed by addition of 2.7 volumes of 95% ethanol. After overnight incubation at  $-20^{\circ}\text{C}$  the precipitate was collected by centrifugation. All proteins were contained in the precipitate as no  $^{14}\text{C}$ -counts (corresponding to proteins) were found in the ethanolic supernatant. The precipitate was dissolved in a small volume of the sample buffer containing SDS and dithiothreitol, thereafter dialysed against the same buffer and subjected to gel electrophoresis. Destained gels were scanned at 620 nm with a Joyce Loebble densitometer.

Electron microscopy. All fixed DNP samples were dialysed overnight against 1 mM Na-EDTA, pH 7.0. The time of fixation varied from 0.5 to 20 hr for electron microscopy and from 10 to 60 hr for isopycnic analysis in CsCl gradients. The results did not depend on the time of fixation within these intervals. Dialysed samples were diluted with 1 mM Na-EDTA, pH 7.0 to a required DNP concentration (usually between 3 and 20  $\mu\text{g}$  of DNA per ml). A drop of DNP solution was placed onto a carbon-coated copper grids followed by removal of excessive liquid by a filter paper touched to the edge of the grid. No additional treatments of the sample on the grid were used. In some experiments the purified chromatin subunit (monosome) was applied onto the grid using a pulverizer. The samples were rotatory shadowed with Pt-Pd (3:1) at the angle of  $6^{\circ}$  followed by examination in the JEM-100B electron microscope at an initial magnification of 15,000. Supporting carbon films were prepared on freshly cleaved mica plates followed by a transfer to the copper grids. The films were made hydrophilic by a glow discharge.

Crystallization of purified chromatin subunit (monosomes). Unlabelled chromatin was used. Purified monosomes (see Results) were fixed overnight with 1% HCHO (pH 7.6) followed by extensive dialysis from HCHO and sucrose against 1 mM TEA-HCl, pH 7.6 for several days. The fixed and dialysed monosomes were precipitated with ethanol, dissolved in 1 mM TEA-HCl, pH 7.6 to a final concentration of about 6 mg of DNA per ml followed by overnight dialysis against the same buffer and centrifugation at 30,000 g for 20 min to remove dust and DNP aggregates if existed. Sodium chloride and cetyltrimethyl ammonium bromide (CTAB) were then added and the <sup>monosomes</sup> was crystallized for several weeks as a CTA-salt. No proteins were dissociated from the fixed monosomes in the presence of CTAB and NaCl. The CTAB-technique was developed previously by Mirzabekov et al. /19/ for crystallization of tRNA. It was used also for crystallization of sheared DNA /20/.

#### RESULTS AND DISCUSSION

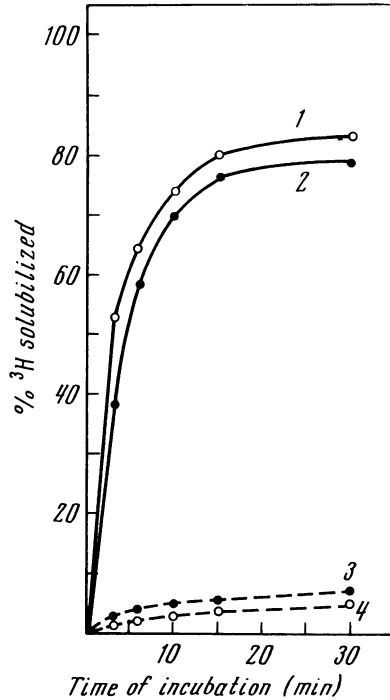
Patterns of nuclease digestion<sup>of</sup> chromatin. Double-labelled chromatin prepared from mouse Ehrlich ascites tumor cells was treated with staphylococcal nuclease and the digestion stopped by addition of EDTA at various points in the course of the reaction. Solubilized portion of chromatin was centrifuged through a steep sucrose gradient (Fig.1). The dependence of chromatin solubilization on the time of nuclease treatment is presented in Fig.2 together with the similar data for whole nuclei. Fig.1a shows that although at low times of incubation the majority of solubilized DNP particles sediment rapidly, a small but significant amount of the DNP is already converted to monosomes and disomes. The  $^{14}\text{C}/^{3}\text{H}$  ratio (corresponding to a protein/DNA ratio) is approximately constant along the gradient, but is strongly increased near the bottom of the tube (Fig.1). Similar and even much stronger effect is observed with digests of the whole nuclei. In the latter case the majority of rapidly sedimenting  $^{14}\text{C}$ -proteins are not bound to the DNP (data not shown). Increase of the time of incubation results in a fur-



**Fig. 1.** Analytical sucrose gradient centrifugation of nuclease-treated chromatin. (a) Nuclease treatment for 2 min; (b) the same, but treatment for 5 min; (c) the same, but for 15 min.

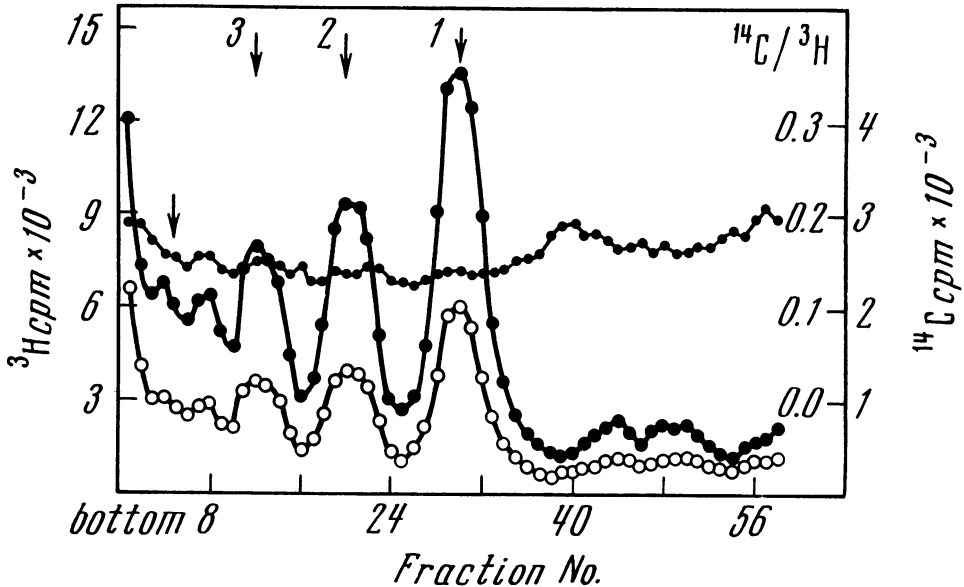
●,  $^3\text{H}$ (DNA); ○,  $^{14}\text{C}$ (protein) ●●●,  $^{14}\text{C}/^3\text{H}$ .

An arrow near the bottom indicates the position of a dense sucrose shelf. The SW40 rotor, 39,000 rpm for 16 hr at  $3^\circ\text{C}$ .



**Fig. 2.** Solubilization of chromatin and nuclei as a function of time of nuclease treatment. 1 - percentage of the total  $^3\text{H}$ (DNA) solubilized from the whole nuclei; 2 - percentage of the total chromatin  $^3\text{H}$ (DNA) solubilized; 3 - percentage of acid-soluble  $^3\text{H}$  radioactivity in the chromatin; 4 - the same but in the nuclei.

ther increase of the proportion of monosomes, disomes etc. at the expense of rapidly sedimenting DNP in the nuclease digest of chromatin (Fig. 1b,c). Although longer than 15-min incubations under these particular conditions did not result in a further solubilization of chromatin (see Fig. 2), they did increase the percentage of monosomes in the digest. A 30-min incubation resulted in the digestion to monosomes of approx. 50% of the material (data not shown). At the same time, the digestion of DNA in the chromatin to acid-soluble oligonucleotides proceeds slowly e.g., only 5-6% of the total DNA was converted to the acid-soluble products during a 15-min incubation (Fig. 2).

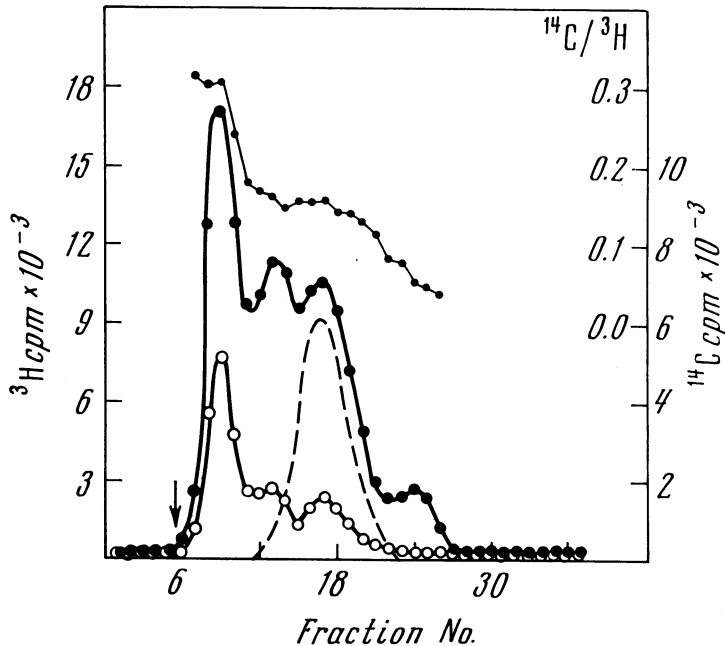


**Fig. 3.** High-resolution analytical sucrose gradient centrifugation of nuclease-treated chromatin. The same sample and rotor as in Fig. 1c but centrifuged for 31 hr. The same designations as in Fig. 1.

Longer centrifugation of the 15-min digest in a sucrose gradient resulted in a complete separation of mono-, di- and trisomes, the larger DNP particles being sedimented to the bottom of the tube (Fig. 3).

We found that a significant amount of the DNP in the digest sedimented more slowly than monosomes (see Figs. 1 and 3). We called this DNP particles "submonosomes". In Fig. 3 submonosomes are resolved into the two partially overlapping peaks. Gel chromatography of the 15-min nuclease digest of the chromatin on Sepharose 4B also reveals submonosomes as the most strongly retarded material which forms a shoulder on the monosome peak (Fig. 4). Polyacrylamide gel electrophoresis of the same digest separates submonosomes into several discrete bands (unpublished data). It should be pointed out that the formation of submonosomes under our experimental conditions is apparently not due to a partial degradation

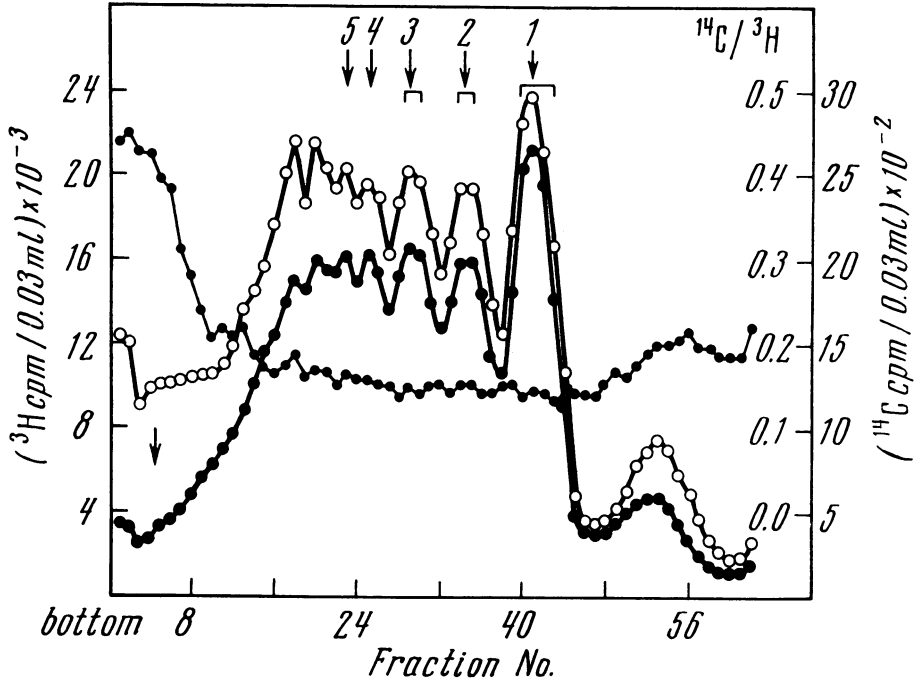




**Fig.4.** Gel chromatography of nuclease-treated chromatin on Sepharose 4B. The same sample as in Fig.1c. The same designations as in Fig.1. A dotted line indicates a pattern of the purified chromatin subunits (monosomes) chromatographed under the same conditions. An arrow indicates the void volume.

of some histones (see above) in contrast with the trypsin-induced submonosomal DNP particles in the experiments of Weintraub /21/. Furthermore, one can notice that Sepharose-purified submonosomes (Fig.4) are much more poor in the protein than submonosomes which were purified by sucrose gradient centrifugation (Figs.1 and 3; cf. Fig.5). Apparently a significant amount of nonhistone proteins (see below) co-sediments with submonosomes in a sucrose gradient but is partially separated from them by gel chromatography.

A striking feature of the submonosomes is that their content in the digest (6-8% of the total chromatin DNA) does not depend on the time of digestion within a wide time interval. For example, the content of submonosomes reaches a



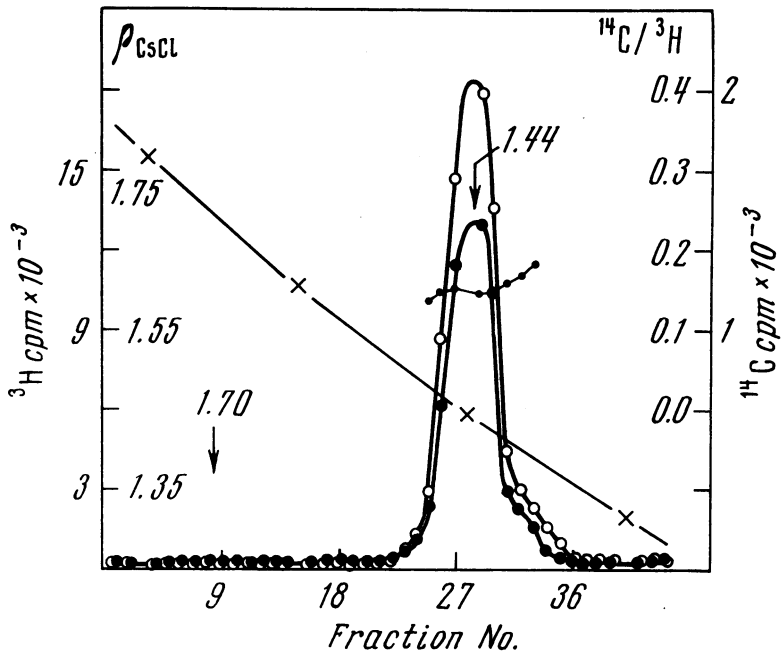
**Fig. 5.** Preparative sucrose gradient centrifugation of nuclease-treated chromatin in the Ti15 zonal rotor. The same sample as in Fig. 1c. The same designations as in Fig. 1.

plateau value at less than 5 min of incubation and remains virtually unchanged up to at least 30 min of incubation (Fig. 1 and unpublished data). At the same time, the content of e.g., monosomes is increased from 15% of the total DNA at 5 min of incubation (Fig. 1b), to more than 50% at 30 min of incubation. This result suggests (but does not prove) that the submonosomes and monosomes are produced from different structures in the chromatin. One of explanations is that submonosomes are the result of a nuclease attack on "active" regions of the chromatin i.e., on those stretches of chromosomal fibers which were transcribed at the moment of chromatin isolation. The fine structure of the DNP in these regions of chromatin may be different from that in inactive regions, the latter being the major, if not the exclusive

source of chromatin subunits (monosomes). Work is now in progress to check the above-mentioned hypothesis. Of course, we considered only those submonosomes which were produced under conditions of a relatively mild nuclease digestion). Much more intensive or longer digestions resulted in the conversion of almost all DNP into submonosome-like particles (ref.5 and our own unpublished data).

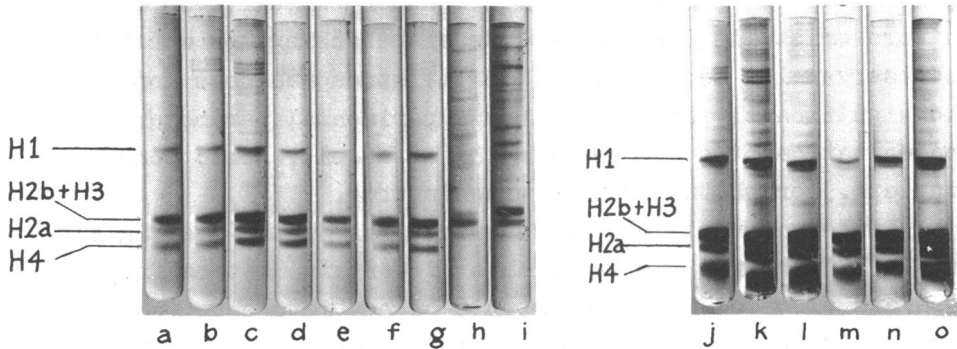
Large-scale fractionation of nuclease-treated chromatin. Sucrose gradient centrifugation of the digest in the Tl15 zonal rotor (Beckman) permitted one to obtain 10-15 mg of purified monosomes and slightly lower amounts of disomes and trisomes in a single run (Fig.5). The resolution obtained with a zonal rotor was close to that obtained in the analytical-scale runs with the SW40 rotor (Figs.1 and 3; cf. Fig.5). When appropriate fractions (indicated by brackets in Fig.5) were pooled followed by isolation of DNA from them and polyacrylamide gel electrophoresis one could see separate DNA bands corresponding to monosome, disome etc. (data not shown). The bands migrated in the gel with mobilities corresponding to an arithmetic progression of molecular weights /1,4/. DNP fractions from the Tl15 zonal rotor were used also for the determination of protein composition of the monosomes and of their "oligomers", for electron microscopy, CsCl centrifugation and also for cristallization of the monosome (see below).

Isopycnic banding of monosomes in a CsCl gradient. Fig.6 shows an equilibrium CsCl pattern of the purified, HCHO-fixed monosome. One can see a single peak with a density of 1.44-1.45 g/cm<sup>3</sup>. The <sup>14</sup>C/<sup>3</sup>H ratio (corresponding to a protein/DNA ratio) remains constant within the peak area except for a few last "light" fractions where the protein/DNA ratio is slightly increased (Fig.6). No free DNA molecules (at a density of 1.70 g/cm<sup>3</sup>) were present in the gradient. However, an increase of the ionic strength of solution to approximately physiological one was found to result in the formation of completely free DNA molecules in the monosome preparation (unpublished data). This phenomenon was already observed in the total hydrodynamically sheared chromatin /15,16/.



**Fig. 6.** CsCl equilibrium pattern of the purified chromatin subunits. The same designations as in Fig. 1. X, density ( $\text{g}/\text{cm}^3$ ).

**Protein composition of chromatin subunits.** Fig. 7 shows SDS-gel electrophoretic patterns of proteins of the original chromatin gel (a), of the insoluble residue after a 15-min nuclease digestion of the chromatin (b and c); of the total soluble DNP after the same digestion (d), of the purified monosome (e), of the purified trisome (f), of the heavier "oligosomes" (g) and of the submonosomes (h-fractions 49-52 in Fig. 5; i - fractions 54-57 in Fig. 5). One can see that purified monosomes as well as trisomes and larger oligosomes contain all five histone fractions (Fig. 7). However, the relative content of histone H1 as measured from densitometer tracings of the gels is not one and the same in different fractions. Specifically, the relative content of H1 in the supernatant after a 15-min nuclease digestion of chromatin (d) was 1.1 times higher than that in the original chromatin (a), whereas the relative content of H1 in the insoluble residue after digestion (b,c) was 1.3 lower than that in the



**Fig. 7.** SDS-polyacrylamide analysis of the protein composition of chromatin subunits. a - proteins from the original chromatin gel (15  $\mu$ g); b and c - proteins from the insoluble pellet after 15-min nuclease digestion of chromatin (20  $\mu$ g and 30  $\mu$ g); d - proteins from the supernatant after the same digestion (22  $\mu$ g); e - proteins from the purified subunits (monosomes) (12  $\mu$ g); f - proteins from the purified trisomes (15  $\mu$ g); g - proteins from "oligosomes" (25  $\mu$ g); h and i - proteins from different zones of "submonosomes" (see text) (16  $\mu$ g and 30  $\mu$ g); j - the same as a but 50  $\mu$ g; k - the same as b but 80  $\mu$ g; l - the same as d but 80  $\mu$ g; m - the same as g but 80  $\mu$ g; n - the same as f but 60  $\mu$ g; o - the same as g but 80  $\mu$ g.

original chromatin (a). Most importantly, the relative content of H1 in the purified monosome (e) was 2.0 times lower than that in the original chromatin (a) and correspondingly, 2.2 times lower than that in the soluble DNP fraction of the whole digest (d). The content of H1 in the purified trisomes (f) and in the oligosome fraction (g) was the same as in the original chromatin gel (a). It should be noted that virtually no degradation of histones (including H1) occurred during and after nuclease digestion of the chromatin. Thus one can conclude that the purified monosome contains only a half amount of histone H1 which is present in the original chromatin. Hence, the population of purified monosomes used in the work is wittingly a heterogeneous one because there is only one H1 molecule per approximately two monosome particles.

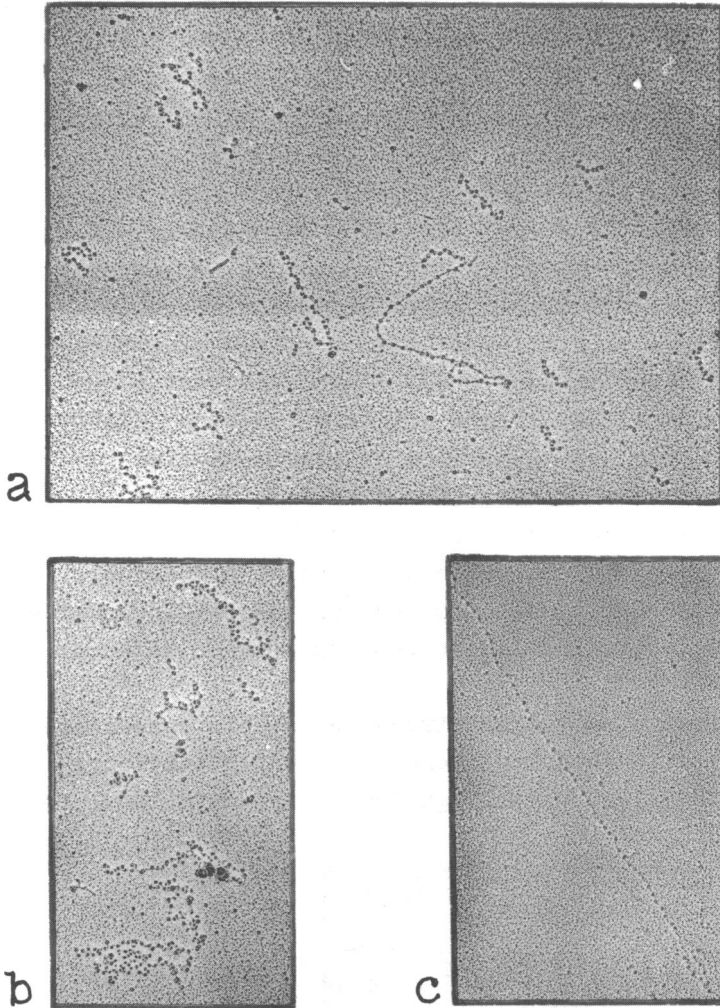
Nevertheless the purified monosomes under certain conditions can form cristalls (see below). We are checking now whether the observed low content of H1 in the monosomes does not depend significantly on the various experimental parameters (concentration of nuclease, time of treatment, ionic conditions etc.) or whether the reverse is true.

Different zones of the "submonosomal" peak (see Fig.5) contain different relative amounts of certain histone fractions and also considerable amounts of nonhistone proteins (Fig.7 h,i). A significant proportion of these nonhistone proteins is not bound to submonosomal DNP particles as could be seen by gel chromatography of the chromatin digest on Sepharose 4B (see Fig.4 and the text above). A reason for a reproducibly lower mobility of submonosomal histones (Fig.7 h, i) as compared with histones of the monosomes, trisomes etc. (Fig. 7 e,f) remains unclear. This question is now under study.

Purified chromatin from mouse Ehrlich ascites tumor cells contains a relatively low amount of nonhistone proteins (less than 10-15% of histones by weight). Therefore to reveal nonhistone proteins the SDS-polyacrylamide gels were strongly overloaded by histones (Fig.7, j-o). One can see that although the total soluble DNP after the 15-min nuclease digestion of the chromatin contains a small but significant amount of nonhistone proteins (Fig. 7,l), the purified monosome does not contain any detectable amount of them (Fig.7, m). One can calculate that the weight content of nonhistone proteins in the monosome is wittingly lower than 1% of the histone content. At the same time, the purified trisome (Fig.7 n) contains very small but detectable amount of nonhistone proteins and the content of nonhistones in the oligosomes (Fig. 7, o) is about the same as in the total soluble DNP (Fig.7, l).

Electron microscopy of nuclease-treated chromatin.

Fig.8 illustrates an electron microscopic appearance of the unfractionated nuclease-treated chromatin. One can see relatively long "beaded" DNP fibers together with occasional trisomes, disomes and monosomes (Fig.8 a,b). The diameter of

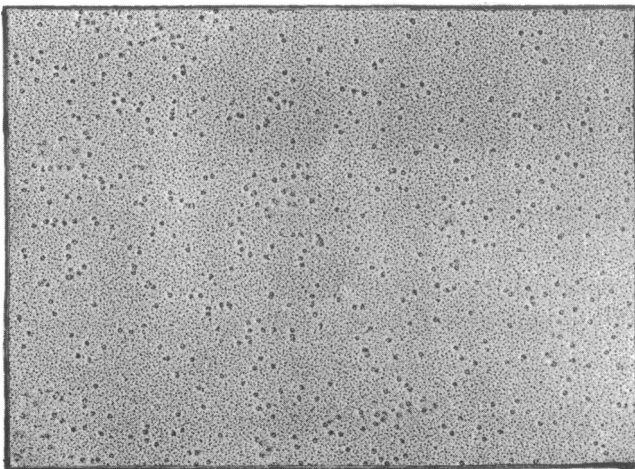


**Fig. 8.** Electron microscopy of nuclease-treated unfractionated chromatin. Magnification 45,000 times. a and b - examples of a typical appearance of the sample; c - example of a stretched fiber with short DNA-like threads between adjacent V-bodies (see also a similar V-shaped fiber in a).

separate metal-shadowed chromatin subunits (V-bodies /2/) equals approx.  $110 \text{ \AA}$ . There are two major kinds of fibers which differ from each other by their degree of stretching on the electron microscopic grid. Unstretched fibers (Fig.

8 a,b) consist of  $\gamma$ -bodies which are closely packed along the fiber; no DNA-like threads connecting adjacent  $\gamma$ -bodies can be seen in such fibers. Notice also a tendency of unstretched fibers to form "two-dimensional" coils on the grid (Fig. 8 a,b). This observation is consistent with the recent results of chromatin analysis by neutron scattering, which suggest the existence of a coil formed by subunit-containing chromosomal fibers /23,24/. On the other hand, stretched DNP fibers consist of alternating  $\gamma$ -bodies and short (50-200 Å in length) DNA-like threads (Fig. 8 c; see also the central V-shaped fiber in Fig. 8 a). It appears that a mechanical stretching of DNP fibers slightly "unfolds"  $\gamma$ -bodies the result being the appearance of short DNA-like threads in the stretched fibers (see also ref. 25). It should be added that Oudet et al. /9/ and ourself /22/ recently observed  $\gamma$ -bodies in chromatin lacking histone H1. The overall picture was similar to that of the original chromatin except for longer DNA-like stretches between adjacent  $\gamma$ -bodies /22/.

Fig.9 illustrates the electron-microscopic appearance of separate chromatin subunits (monosomes) which were purified by sucrose gradient centrifugation in the Ti15 zonal ro-



**Fig.9.** Electron micrograph of purified chromatin subunits (monosomes). Magnification 50,000 times.



tor (see Fig.5). It should be noted that a large fraction of monosomes in electron micrographs contain a small "hole" in the center of the particle (Fig.9). The hole is better seen at higher magnifications (data not shown). We are checking now whether this is simply an electron microscopic artifact or whether the above-mentioned appearance of the monosome indicates its tore-like shape.

Crystallization of monosome. We used a method based on a gradual lowering of the ionic strength of a CTAB-containing solution of the purified monosomes (see Methods). Fig.10 shows relatively small ( $\sim 100 \mu\text{m}$  long) needle-like crystals of the monosome which were formed during two weeks after a relatively rapid lowering of the ionic strength of solution from 0.60 to 0.52. CTAB-technique was used for the first time by Mirzabekov et al. /19/ to obtain crystals of tRNA and later by Osicka et al. /20/ to prepare crystals of sheared DNA.

Preliminary X-ray analysis of small monosome crystals by a powder diffraction method gave one strong reflection at

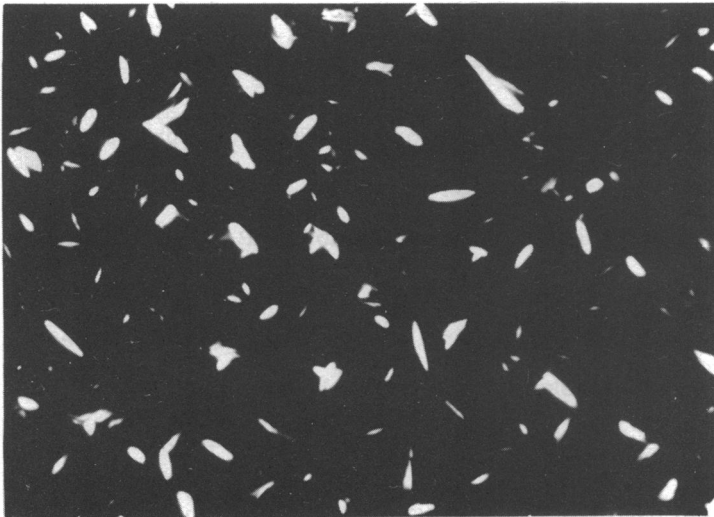


Fig.10. Crystals of purified chromatin subunits (monosomes) photographed with the use of polarizing microscope. Magnification 140 times. Largest crystals in the photograph have a length of  $\sim 100 \mu\text{m}$ .

51 Å and a number of weaker reflections at larger spacings. Work is now in progress to grow larger crystals suitable for the X-ray analysis of single monocrystals.

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