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

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ABSTRACT
        Earlier findings /1-10/ bearing on a subunit organiza-
tion 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 puri-
fied chromatin subunit contains all five histones, the rela-
tive content of histone H1 in it is two times lower than
that in the original chromatin. It is shown that a mild di-
gestion 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 frag-
ments which are integral multiplies of a unit length. Fur-
ther 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 "oli-
gomers" of this repeating DNP particle. Each monosome con-
tains 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-^{3}H/thymidine and L-/^{14}C/lysine or a hydrolysate of Chlorella ^{14}C-proteins /12,13,16/. Chromatin was prepated 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 ^{3}H-DNA ranged from 10,000 to 25,000 cpm/μg; the specific radioactivity of proteins - from 3,000 to 8,000 cpm/μg. Histones contained 60-70% of the total ^{14}C-counts. ^{3}H-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 μg/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 su-
pernatant to determine the total and acid-soluble radioacti-
vity. 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 suc-
rose 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% CCl_3COOH and ethanol and thereafter coun-
ted 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 Ti15 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 col-
lected 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/. Frac-
tions 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 $^{14}C, ^{3}H$-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 car-
ried out in 6% polyacrylamide gels. The buffer system was
that described in ref.17. DNA bands were visualized by stai-
ning 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°C the precipitate was collected by centrifugation. All
proteins were contained in the precipitate as no ^{14}C-counts
(corresponding to proteins) were found in the ethanolic su-
pernatant. The precipitate was dissolved in a small volume
of the sample buffer containing SDS and dithiothreitol, there-
after dialysed against the same buffer and subjected to gel
electrophoresis. Destained gels were scanned at 620 nm with
a Joyce Loeble densitometer.

Electron microscopy. All fixed DNP samples were dia-
lized overnight against 1 mM Na-EDTA, pH 7.0. The time of fi-
xation 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 µg of DNA per ml). A drop of DNP solution was placed
onto a carbon-coated copper grids followed by removal of ex-
cessive 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 (mo-
nosome) was applied onto the grid using a pulverizer. The
samples were rotatory shadowed with Pt-Pd (3:1) at the angle
of 6° followed by examination in the JEM-100B electron micro-
scope at an initial magnification of 15,000. Supporting car-
bon films were prepared on freshly cleaved mica plates fol-
lowed by a transfer to the copper grids. The films were made
hydrophillic by a glow discharge.

Crystallization of purified chromatin subunit (mono-
some). 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 dialys-
ed 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 re-
move dust and DNP aggregates if existed. Sodium chloride and
cetyltrimethyl ammonium bromide (CTAB) were then added and
the monosome was crystallized for several weeks as a CTA-salt. No pro-
teins were dissociated from the fixed monosomes in the pre-
sence 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 of chromatin. Double-la-
belled chromatin prepared from mouse Ehrlich ascites tumor
cells was treated with staphylococcal nuclease and the di-
gestion 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 nuc-
lease treatment is presented in Fig.2 together with the si-
milar data for whole nuclei. Fig.1a shows that although at
low times of incubation the majority of solubilized DNP par-
ticles sediment rapidly, a small but significant amount of
the DNP is already converted to monosomes and disomes. The
$^{14}C/^{3}H$ ratio (corresponding to a protein/DNA ratio) is ap-
proximately constant along the gradient, but is strongly in-
creased 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}C-proteins are not bound to the DNP (data not
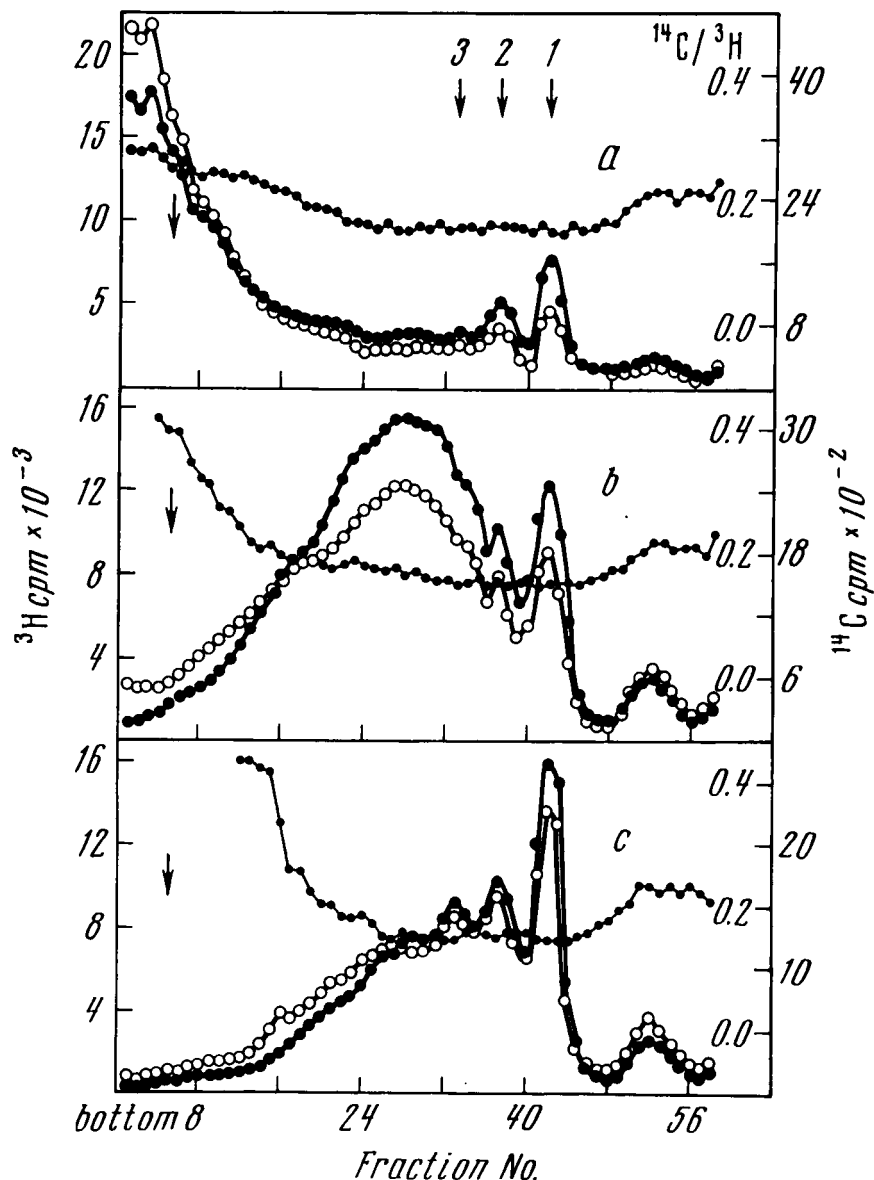shown). Increase of the time of incubation results in a fur-

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}H(DNA); —O— , ^{14}C(protein) ◆◆◆ , ^{14}C/^{3}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^oC.

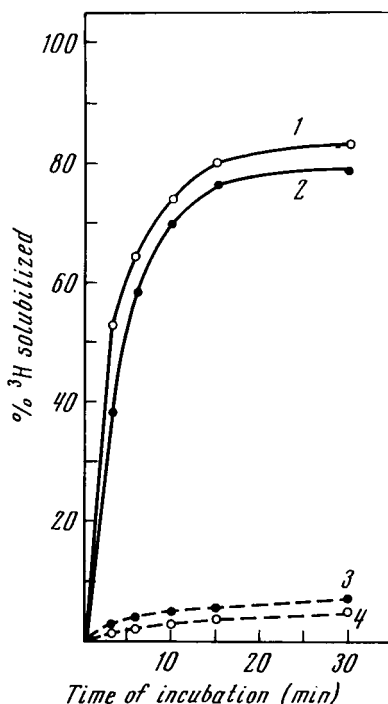Fig.2. Solubilization of chromatin and nuclei as a func-
tion of time of nuclease treatment. 1 - percentage of the
total ^{3}H(DNA) solubilized from the whole nuclei; 2 - percen-
tage of the total chromatin ^{3}H(DNA) solubilized; 3 - per-
centage of acid-soluble ^{3}H radioactivity in the chromatin;
4 - the same but in the nuclei.

ther increase of the proportion of monosomes, disomes etc.
at the expence of rapidly sedimenting DNP in the nuclease digest
of chromatin (Fig.1b,c). Although longer than 15-min incuba-
tions 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 oligonuc-
leotides proceeds slowly e.g., only 5-6% of the total DNA
was converted to the acid-soluble products during a 15-min
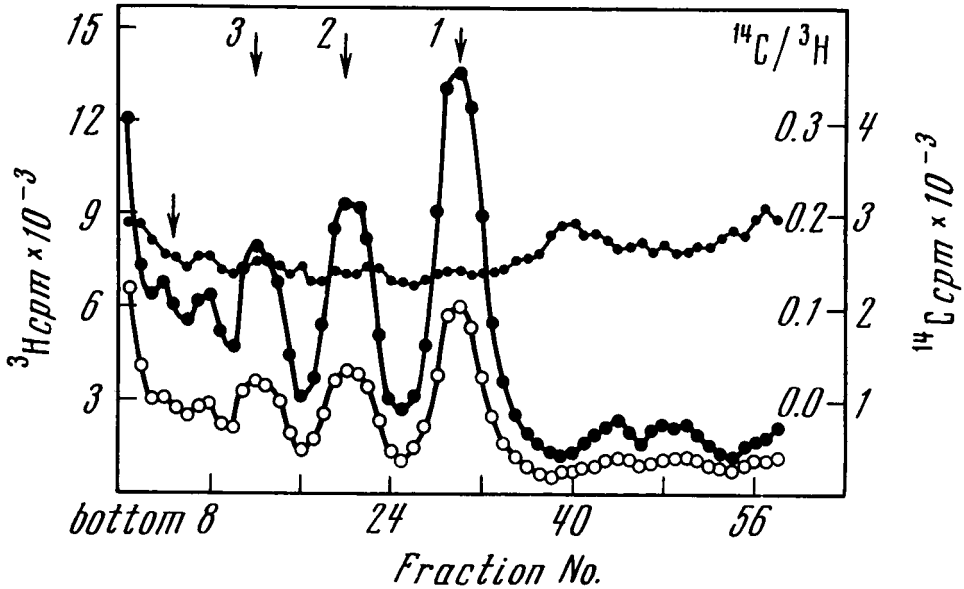incubation (Fig.2).

Fig.3. High-resolution analytical sucrose gradient cent-
rifugation of nuclease-treated chromatin. The same sample and
rotor as in Fig.1c but centrifuged for 31 hr. The same desig-
nations as in Fig.1.

Longer centrifugation of the 15-min digest in a suc-
rose 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 electropho-
resis 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
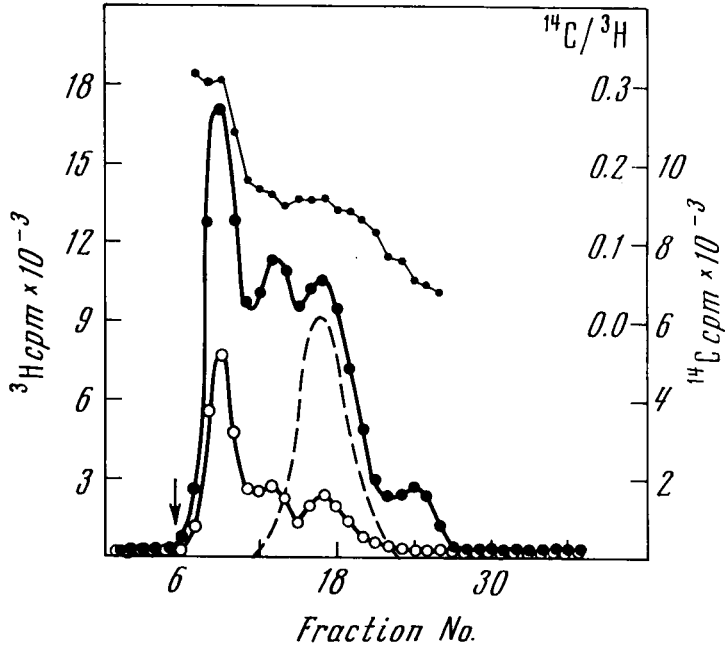conditions is apparently not due to a partial degradation

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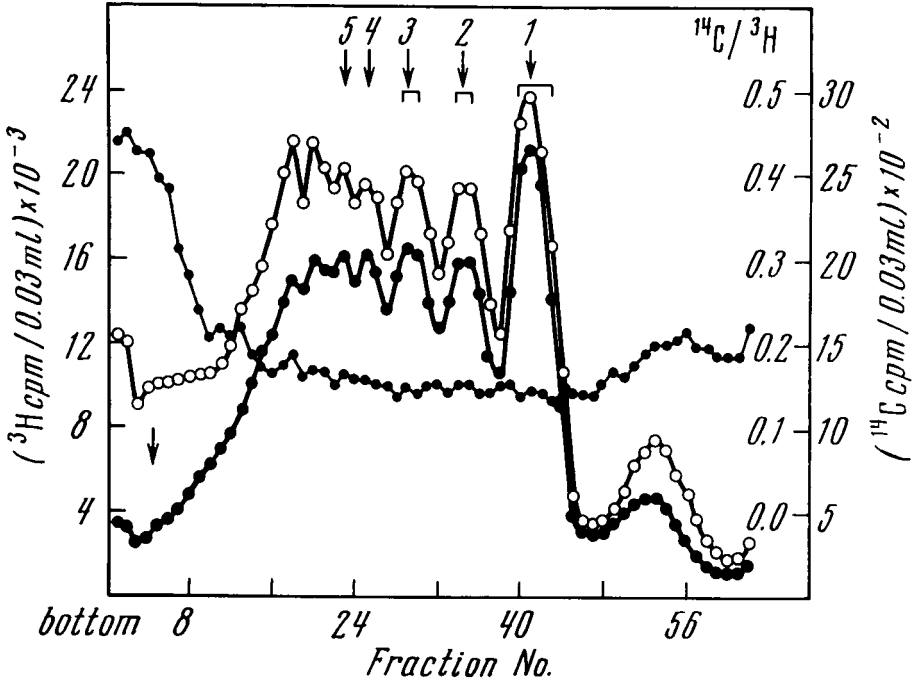
Fig.5. Preparative sucrose gradient centrifugation of nuclease-treated chromatin in the Ti15 zonal rotor. The same sample as in Fig.1c. The same designations as in Fig.1.

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 con-
version of almost all DNP into submonosome-like particles
(ref.5 and our own unpublished data).

Large-scale fractionation of nuclease-treated chroma-
tin. Sucrose gradient centrifugation of the digest in the
Ti15 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 ob-
tained 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 cor-
responding to an arithmetic progression of molecular weights
/1,4/. DNP fractions from the Ti15 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 mo-
nosome (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 densi-
ty of 1.44-1.45 g/cm^3. The ^{14}C/^{3}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 molecu-
les (at a density of 1.70 g/cm^3) were present in the gradi-
ent. 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 mono-
some preparation (unpublished data).This phenomenon was al-
ready observed in the total hydrodynamically sheared chroma-
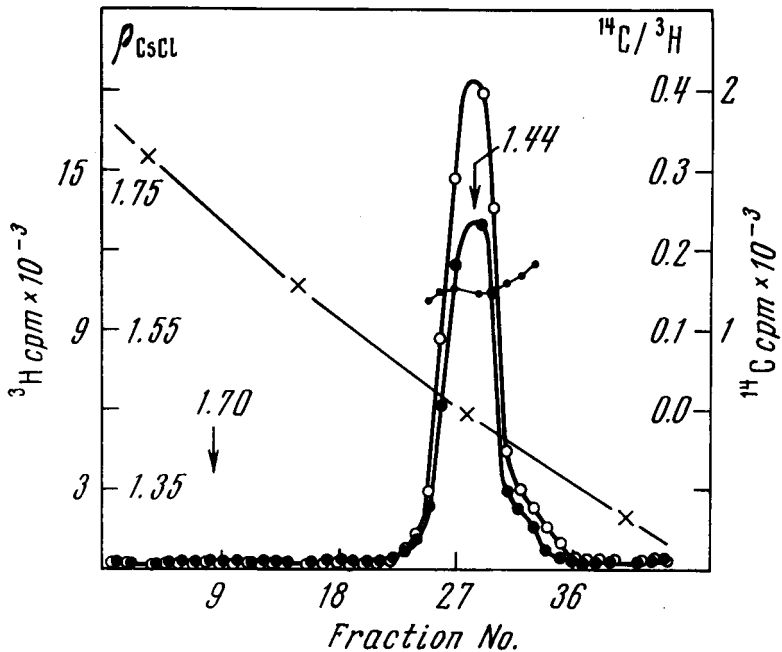tin /15,16/.

Fig.6. CsCl equilibrium pattern of the purified chromatin subunits. The same designations as in Fig.1. X, density (g/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
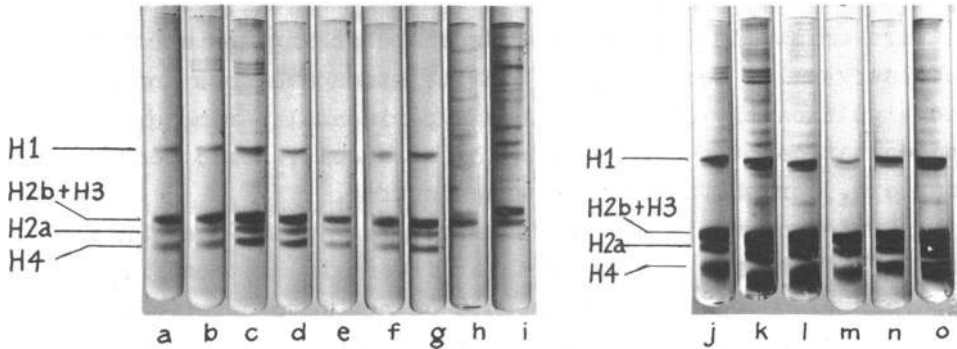
Fig.7. SDS-polyacrylamide analysis of the protein composition of chromatin subunits. a - proteins from the original chromatin gel (15 μg); b and c - proteins from the insoluble pellet after 15-min nuclease digestion of chromatin (20 μg and 30 μg); d - proteins from the supernatant after the same digestion (22 μg); e - proteins from the purified subunits (monosomes) (12 μg); f - proteins from the purified trisomes (15 μg); g - proteins from "oligosomes" (25 μg); h and i - proteins from different zones of "submonosomes" (see text) (16 μg and 30 μg); j - the same as a but 50 μg; k - the same as b but 80 μg; l - the same as d but 80 μg; m - the same as g but 80 μg; n - the same as f but 60 μg; o - the same as g but 80 μg.

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 de-
pend significantly on the various experimental parameters
(concentration of nuclease, time of treatment, ionic condi-
tions etc.) or whether the reverse is true.

Different zones of the "submonosomal" peak (see Fig.5)
contain different relative amounts of certain histone frac-
tions 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 Se-
pharose 4B (see Fig.4 and the text above). A reason for a re-
producibly 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 re-
veal 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 mono-
some 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 non-
histone proteins and the content of nonhistones in the oligo-
somes (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 rela-
tively long "beaded" DNP fibers together with occasional
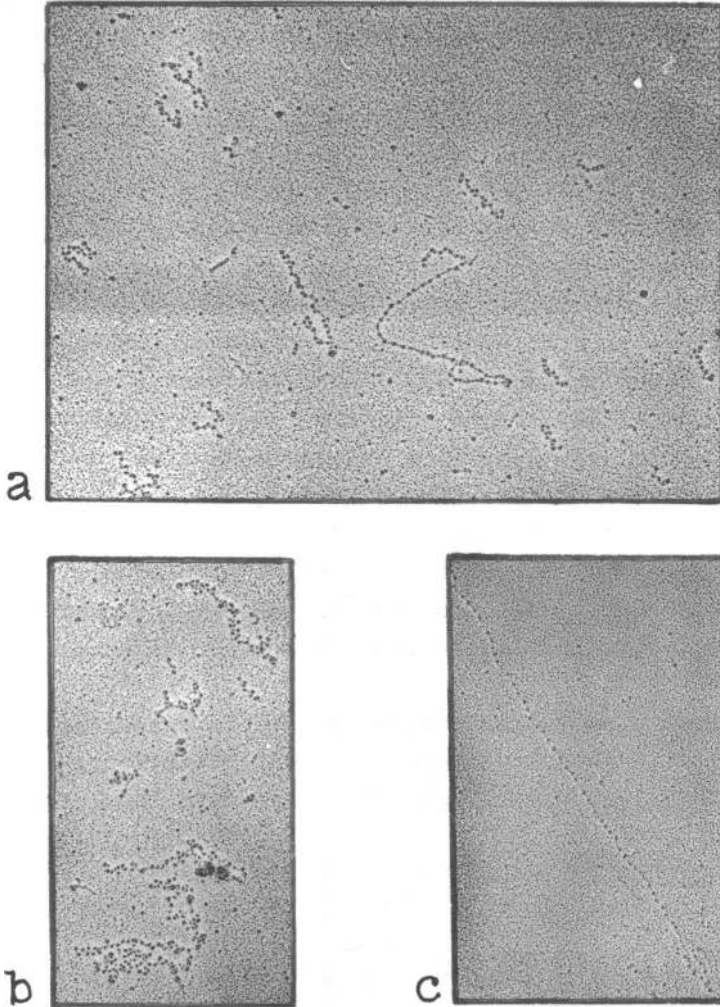trisomes, disomes and monosomes (Fig.8 a,b). The diameter of

Fig.8. Electron microscopy of nuclease-treated unfrac-
tionated 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 ad-
jacent 𝛾-bodies (see also a similar V-shaped fiber in a).

separate metal-shadowed chromatin subunits (𝛾-bodies /2/)
equals approx. 110 Å . 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 γ-bodies which are closely packed along
the fiber; no DNA-like threads connecting adjacent γ-bodies
can be seen in such fibers. Notice also a tendency of un-
stretched 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-contain-
ing chromosomal fibers/23,24/. On the other hand, stretched
DNP fibers consist of alternating γ-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 me-
chanical stretching of DNP fibers slightly "unfolds" γ-bo-
dies 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/ re-
cently observed γ-bodies in chromatin lacking histone H1.
The overall picture was similar to that of the original chro-
matin except for longer DNA-like stretches between adjacent
γ-bodies /22/.

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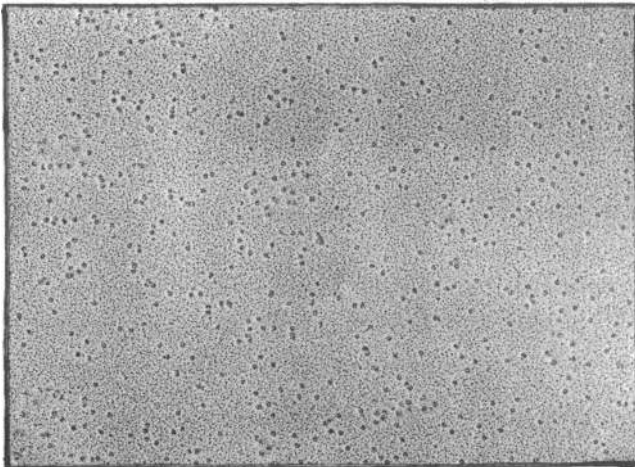fied by sucrose gradient centrifugation in the Ti15 zonal ro-

Fig.9. Electron micrograph of purified chromatin sub-
units (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 (~100 μm long) needle-like cristalls 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
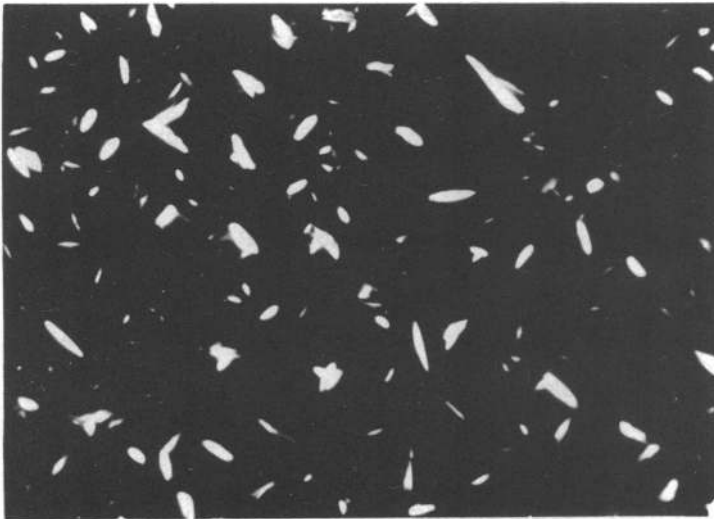

Fig.10. Crystals of purified chromatin subunits (monosomes) photographed with the use of polarizing microscope. Magnification 140 times. Largest cristalls in the photograph have a length of ~100 μm.

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.

ACKNOWLEDGEMENTS

We thank Prof. G.P.Georgiev for helpful discussions,
Drs A.S.Tichonenko, V.A.Kadykov and Y.Y.Vengerov for teach-
ing one of us (A.J.V.) the ways of electron microscopy, Drs
A.D.Mirzabekov and D.Sanko for advice on the CTAB-technique,
Dr. O.F.Polivtcev for obtaining debaegrams of monosome cris-
talls and T.G.Bakayeva and L.R.Pittina for their help in
some experiments.

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