The Submicroscopic Basophile Structure of Chromosomes in Ehrlich Ascites Tumor Cells

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SUMMARY

The cytochemistry and morphology of interphase and condensed mitotic chromosomes are described in ultrathin-sectioned specimens prepared by freezing and drying, postfixation with protein-sensitive bifunctional reagents of low density, basophile staining with an electron-dense dye lake, and enzymatic digestions. In specimens not subjected to enzymes, interphase chromatinic threads were observed as paired bodies with a seemingly granular structure which may represent cross-sectioned fibrils. At the nucleolus-organizer region, the chromatin is extremely dense and consists of occasional, large, true granules. Metaphase chromosomes consist of tightly crowded, strongly basophilic fibers about 160 Å wide, with no evidence of fibrillar subunits; the chromosomal mass also comprises an extremely fine, longitudinally oriented substructure of obscure morphologic features. Sister chromatids have been observed in cross-section at one of the earliest stages of a split. The chromatinic nature of stainable basophile structure was in each instance established by its resistance to RNase and digestibility with DNase. The residuum after DNase action on metaphase chromosomes consists mainly of 60–80 Å particles. These occur among tubular elements measuring about 250 Å in width. Trypsin was capable of digesting neither the chromatin nor the 60–80 Å particles but the tubules were not visualized after enzymatic extraction. The trypsin-resistant particles correspond to those observed in DNase-digested chromosomes and probably consist, for the most part, of non-histone protein together with smaller amounts of basophile substance than are present in the chromatin and in ribosomes.

INTRODUCTION

The ultrastructure of condensed, mitotic chromosomes has been studied by several methods: fixation with fluids containing metals or aldehydes (5, 8, 15, 20, 23, 28, 33, 38, 39, 42, 43, 48, 55, 57–59), whole-mounts of more or less intact chromosomes, or of chromosomes treated so as to unravel their fine structure from an initially compacted condition (11, 14, 19, 56), improved embedding media (5), and the examination of hydrated, unstained chromosomes (47). These approaches have yielded valuable information on certain items for which agreement exists among most workers.

In most recent electron micrographs, chromosomes appear to consist of both fibrillar and granular elements. Dissensions exist on the precise dimensions of fibrils and on interpretation of the spatial relationships of elementary subunits (55, 56) and as to whether the chromosomal mass is exclusively fibrillar or comprises granules as well (8). In addition, divergent interpretations of subchromatid structure have resulted from work employing widely different methods of chromosome analysis at the autoradiographic, cytogenetic, light- and electron microscopic levels (summarized in Ref. 3). There is clearly a need for a reliable consensus on the submicroscopic morphology and cytochemistry of chromosomes; and it may eventually be reached by utilizing a variety of procedures of specimen preparation in addition to those which have already served usefully in one respect or another.

The results now reported were obtained by means of cytologic freeze-drying. They involve postfixation of specimens with anhydrous vapors of organic reagents possessing very low electron density but high reactivity with proteins. The special features of anhydrous vapor postfixation, and the particular reagents used by them are discussed by the originators of this refined method (16, 17). One of these is the bifunctional reagent FFDNB. Additional protein reagents such as the bifunctional FNPS have been used either in anhydrous vapor form directly after freeze-drying or, as in the case of DMH, in liquid form in earlier studies on nuclear structure in Ehrlich cells carried out by the present author (30, 31) The electron-dense dye lake GC is now used both before and after the specific extractive action of nucleases and of trypsin in order to localize basophile structure for which this dye lake has a pronounced affinity. Questions of selectivity of GC for nucleic acids at the levels of both the light and electron microscope have been examined by several workers (6, 10, 12, 13, 18, 24, 27, 29, 32, 36, 37, 41, 44, 52).

These applications are sufficiently distinct as to produce electron micrographs differing from those yielded by fluid metallic fixation or other commonly used methods. Membranes, for instance, are invisible, although frozen-dried or frozen-substituted specimens stained for general morphology with heavy metals

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such as osmium do reveal them (50, 53). The conditions leading
to image contrast in frozen-dried cells also differ when purely
organic, low-density reagents like FFDNB or FNPS are used
alone, i.e., without subsequent application of electron-dense
basic dye (31); or, as shown in this work, when structures stained
primarily by low-density reagents are to be visualized in the
presence of vicinal, electron-dense matter. Being primarily cyto-
chemical, these procedures cast fresh light on certain aspects of
organization of basophilic structure in mitotic chromosomes.
In addition, they have enabled the production of electron micro-
graphs at considerably high, useful magnification. Reports on
the morphology of the Ehrlich ascites tumor cell based on other
electron microscopic methods are numerous (7, 22, 35, 40, 45,
46, 51, 54, 57-59, 61) and well supplemented by pertinent refer-
ences (cited above) on chromosome structure in other material.

MATERIALS AND METHODS

The procedures adopted in propagating Ehrlich tumor cells,
freezing and drying of specimens, postfixation, staining, and
enzymatic digestions with the nucleases and with trypsin were
essentially similar to those used earlier (30, 31). Anhydrous
vapor postfixation (17) was carried out according to an original
suggestion by Dr. I. Gersh especially pertinent to FFDNB
(personal communication). This involves the sublimation in
vacuo of FFDNB over the specimens for 4-5 hr at 50°C, fol-
lowed by removal of excess reagent overnight by raising the tem-
perature to 60°C. FNPS was applied in a similar manner, but
under the conditions described previously by the present author
(31).

The following enzymatic digestions were performed on frozen-
dried specimen pellets after postfixation with the above reagents:
1. Ribonuclease (Worthington Chemical Co.; crystallized
3 times from ammonium sulfate and finally salt-free in etha-
nol), 1 mg/ml of distilled water, at pH 6.2, and incubated for
4 hr at 37°C. Control specimens were incubated concurrently
in distilled water alone.
2. Deoxyribonuclease (Worthington; crystallized once, beef
pancreatic), 0.05 mg/ml of Gomori's tris (hydroxymethyl)-
aminomethane buffer with 0.2 M MgSO4·7H2O, pH 5.7, and in-
cubated at room temperature for 9 hr. Control specimens were
treated concurrently under identical conditions except for the
omission of enzyme from the incubation mixture.
3. Tryptsin (Worthington; salt-free; crystallized twice),
0.1% in phosphate buffer, at pH 9.0, and incubated for 15 min
at 37°C. Control specimens were incubated concurrently, with
enzyme omitted from the buffer.

The conditions of incubation in each of the above cases were
judged as optimal on the basis of numerous variations of incu-
bating times and pH, aided with light microscope controls so
to confirm recognition of obviously under- or overdigested
specimens at the electron microscope level. The digestions were
in each case followed by distilled water rinses and staining with
GC (aqueous, pH 1.68) for 24 hr at room temp., and the speci-
mens compared with similar treated, but enzymatically un-
digested, controls. Tryptic digestions were, in addition, studied
with the aid of alkaline fast-green and chloramine T-Schiff
controls at the light microscope level.

The embedding material was Epon 812. Ultrathin sections
were examined at 40 or 60 kv in a Philips 100B electron micro-
scope after careful adjustment of the stigmator. The micro-
scope was equipped with a 20-μ objective aperture.

Micrographs were made on Kodak Spectroscopic type 649
GH film, at initial magnifications not exceeding × 30,000 and
enlarged photographically as desired.

RESULTS

Basophile Staining of Enzymatically Unextracted Cells

The interphase nucleoplasm is characterized by the presence
of randomly strewn threads. These minute chromatinic bodies,
from which the highly condensed metaphase chromosomes origi-
nate, stain densely with GC against a very light background.
Fig. 1 shows a portion of such a thread from an Ehrlich tumor
in late interphase (Fig. 2). Both illustrations represent dif-
ferent sectioning planes through the same cell and were selected
to reveal sufficient longitudinal profile of the thread and the typ-
ical appearance of the entire cell at an identifiable stage in the
mitotic cycle. At late interphase, or early prophase, the nucleoli
characteristically have lost the dense coats of perinucleolar
(nucleolus-associated) chromatin. In addition, as a prelude to
the complete disappearance of the nucleoli at prophase, the nu-
cleolar ribosomes lose their prominence and appear as though
organized in ill-defined, strandlike array as compared to their
more or less uniform disposition in strictly interphase nucleoli
(30).

It may be that chromatinic threads such as those in Fig. 1
represent a stage in genetic replication, believed to occur during
interphase-prophase, since they are characterized by the pres-
ence of a narrow furrow separating densely stained masses of
chromatin. Probably owing to twists of the thread in the ultra-
thin sections, the furrow is rarely distinct throughout its length.
The furrow varies in width between 130 and 400 Å, but it is pos-
sible that it is uniformly wide and that the variation seen is due
to twists of the chromatin threads in the sectioning plane.
Neither sufficient lengths of medial longitudinal-sections nor
precise cross-sections through chromatin threads have been
encountered, despite meticulous search, to verify this possi-
bility. The chromatin is not equally distributed on opposite
points along the furrow, nor uniformly massed along its length.
In relatively uncrowded regions of these densely basophile
masses, granules (cross-sections through fibrils?) measuring
115-150 Å are sometimes visible. The difficulty of resolving
interphase chromatin threads of this nature at the light micro-
scope level is obvious from Fig. 1.

Prior to commencement of premiotic disintegration of the
nucleolus the perinucleolar chromatin at interphase is very
prominent and so densely basophile as to appear solid or poorly
differentiated (Fig. 3). Higher magnification (Fig. 4), of the long
chromatinic processes that issue from the nucleolar surface at
restricted points reveals bodies (about 300 Å) most of which
appear to be true granules rather than sections of fibrils. Chro-
matinic processes like these probably exemplify the known asso-
ciation of nucleoli with certain specific (nucleolus-organizer)
regions of the chromosome—a morphologic feature not easily
visualized by light microscopy of Ehrlich ascites cells. A note-
worthy feature in Figs. 3 and 4 is the abundance of minute par-
articles which, together with less numerous but larger particles, occur in the nucleoplasm in the vicinity of the nucleolus. Neither type of particle is as densely basophilic as the cytoplasmic and nucleolar ribosomes or the chromatinic granules.

Fig. 5 portrays a cell in metaphase. The fully condensed chromosomes, some of which show abnormal orientation on the metaphase plate, stain with the same intensity characteristic of chromatin in earlier mitotic stages and, at this relatively low magnification, appear uniformly dark. Part of a metaphase chromosome is shown at intermediate magnification in Fig. 6. Its structure contrasts with the very numerous bodies of granular appearance revealed in metaphase chromosomes by other means, even at much lower magnification (8, 15, 54). In addition, there is little obvious correspondence in morphologic disclosures by the present methods and by conventional ones.

Under the present experimental conditions, a finer order of structural organization in the metaphase chromosome is discernible only at far higher magnification (Fig. 7). In longitudinal section, it is characterized by a tight and apparently disorderly crowding of dense elements, with only occasional, restricted regions where the structure is less tightly packed. The dense elements show little evidence of strikingly circular outlines such as might be expected of spherical particles in section. On close inspection the dense basophilic structure appears to consist of discrete, mostly short, oblong bodies the variable lengths of which suggest the random orientation of a convoluted thread-like mass in the sectioning plane. This conclusion is strengthened by the occasional presence of unusually long threadlike segments (arrowed in Fig. 7). Such elongated threads are almost uniformly about 160 Å in width—a dimension in agreement with that of the great majority of more or less circular, cross-sectioned threads measured. Where image contrast is clearest, these basophilic components of the chromosome are not solidly dense, but appear to contain the very finest particles of about 15 Å diameter. These are generally inconspicuous, faint entities the dispersion and arrangement of which are difficult to describe. Nevertheless, they are consistent in occurrence and seem to create a diffuse rather than a totally solid image of chromatinic threads in the sectioned chromosome.

Close scrutiny of Fig. 7 will also reveal a definite “overall” orientation of structure. It is very difficult to convey an adequate description of this extremely complex effect. It may best be visualized (but not without some effort) by quick scanning of the entire micrograph, top to bottom, particularly the left hand side part. The effect may roughly be described as the manifestation of a “stretch” similar to that prevailing among the minute, unidirectionally oriented dichroic crystals in the matrix of Polaroid polarizing filters. The effect seems to involve primarily the background of the sectioned threads. It is created by innumerable, minute, elongated, discontinuous bodies (about 20 Å wide) of medium density which are disposed unidirectionally in closely parallel array. Whether or not these are discrete entities cannot easily be ascertained, for they are visualized in ultrathin section, and it may be that the effect described is due to continuous, extremely tenuous fibrils which weave in and out of the plane of the section. They follow the long axis of metaphase chromosomes (top to bottom in Fig. 7) seemingly cutting across the randomly oriented, 160 Å, convoluted basophile threads which form the more obvious mass of the chromosome.

Fig. 8 shows a cross-section of a chromosome from a metaphase plate. Its bipartite nature is evident from a medial zone where basophile material is markedly sparse as compared to the dense compactions on either side of it. It is an obvious representation of the bivalent stage. The width of the faintly stained space between the two chromatids (“half-chromatids”) is well beyond the limit of light microscopic resolution (0.2 μ), although a widening of it at a later stage in the mitotic cycle could reveal its doubleness better (cf. controversies examined in Ref. 3). The dense compactions of basophile substance in each chromatid display the same lack of orderliness noticed in the longitudinal section in Fig. 7, while the interchromatid zone is reminiscent of the furrow in the interphase chromatinic thread in Fig. 1. Two features of appreciable clarity amidst the generally confusing aspects of internal morphology in Fig. 8 are the smooth outline of most of the surface of the bivalent and the lack of precise symmetry in shape and size of each chromatid.

It should be noted that 40 Å particles possessing the characteristics described earlier (31) were observed in none of the examples described above. These 40 Å particles, which appear to be mainly proteinaceous in composition, were disclosed with low-density reagents under special conditions. Even if they were lightly stained by GC in the present work, it is probable that they are overshadowed by neighboring structures which are far more intensely reactive to GC.

**Basophile Structure after Enzymatic Digestions**

The efficacy of GC in localizing cellular basophilia with a high degree of selectivity is indicated by the complementary effects yielded by prior extractions with RNase and DNase, and by the pattern of staining after digestions with trypsin.

**Extraction with RNase.** Digestion with RNase drastically abolished image density of the cytoplasm but the chromosomes were largely unaffected. Fig. 9 exemplifies these effects in a cell in which the metaphase plate is viewed equatorially. The individual chromosomes are as dense as those in the undigested controls. However, there are occasional instances where RNase has laid bare limited regions showing structure unnoticeable in the enzymatically unextracted controls. These regions (arrowed in Fig. 9) consist of more or less circular areas from which sufficient density has been abolished to disclose 1 or 2 small bodies. Higher magnification reveals no further useful detail, for these bodies are uniformly dense and the partially digested zone surrounding them does not differ (except in density) from the rest of the chromosome.

Fig. 10 shows basophile staining in one member of a chromatid pair after exposure to RNase. There is some disruption of morphologic sharpness as compared to the undigested control in Fig. 8, but the integrity of the densely basophilic compactions is clearly maintained.

These observations permit the general conclusion that the basophile portions of the condensed mitotic chromosome are constituted overwhelmingly of RNase-insensitive substance.

**Extraction with DNase.** The digestive action of DNase was drastic; there was complete obliteration of image density of chromatin threads in the interphase nucleoplasm and, more spectacularly, in condensed chromosomes at all stages in the mitotic cycle.
Fig. 11 shows that GC has localized the cytoplasmic ribosomes as distinct, dense individual particles while the chromosomes are almost thoroughly denuded of structure normally visible in enzymatically undigested controls and in RNase-digested specimens. The DNase-insensitive residuum in chromosomes consists of a sparse structure of inconstant form. It is much lower in density than the surrounding, undigested ribosomes. In over-digested specimens, the residuum is often diffuse in appearance with ill-defined contents (Fig. 12). Even so, the internal structure is characterized by the presence of clearly tubular, enzyme-resistant elements which traverse the chromosome in indiscriminate directions. Their numbers, frequencies, and patterns bear no relation to the mitotic stage. They are generally few but constant in occurrence and are often conspicuously wide (about 250 Å at most) and straighter than any visible in undigested controls. It is very doubtful that they correspond to the spindle tubules which have so clearly been demonstrated by other methods (20, 39). Besides the randomness of their occurrence, they are not found in the cytoplasm at any time and they never pierce through metaphase or anaphase chromosomes in closely arrayed, more or less parallel formations as do the spindle tubules.

The interior of the DNase-digested chromosome consists predominantly of distinct particles which measure about 60–80 Å and yield low image densities (Fig. 11). As these particles are quite resistant to the electron beam owing to postfixation with bifunctional protein reagents, they may be held considerably longer in the beam to enhance their contrast (31), but this results in inevitably strong density and photographic "fusion" of the cytoplasmic ribosomes (Fig. 13). The chromosomal particles occur in dense aggregates or in looser, randomly scattered groups, or in strandlike array. Like the tubular elements which traverse them, they show no consistent characteristics relatable to different stages in the mitotic cycle.

**Extraction with Trypsin.** At low magnification (Figs. 14, 15) the effects of trypsin were indistinguishable from those of RNase. Cytoplasmic density attributable to GC was abolished with equal severity while the intensity of staining was undiminished in the chromatin. The latter, especially in condensed chromosomes at anaphase (Fig. 15), was at times pitted with small, clearly unstained zones. However, higher magnification (Fig. 16) of the immediately surrounding fine structure disclosed no differences from sections of chromatin lacking in such clear areas.

The efficacy of tryptic digestion is evident from the displacements and distortions wrought in the fine structure of chromatin as compared with undigested controls as well as independent, light microscopic controls using specific protein-localization methods (H. Greenwood and B. Mundkur, unpublished observations). Provided that the enzyme incubation was not obviously extremely prolonged, the basophilic substance of condensed chromosomes was remarkably resistant to extraction or reduction in staining intensity even after digestion periods well in excess of those required to abolish cytoplasmic density completely. Apart from morphologic distortion, the most noticeable alteration from the normal aspect of the chromosome was a greatly enhanced visibility of particulate entities reminiscent of, and presumably corresponding to, those seen after DNase digestions. In metaphase chromosomes, they were of approximately the same size and distribution as those seen in metaphase chromosomes digested by DNase, though, in the sections examined, they tended to be more numerous in anaphase. However, this may be a normal variation in their distribution as seen in thin section and without real significance. The tubular elements, also prominent in the DNase-digested chromosomes, were not observed at any time after incubation in trypsin.

**DISCUSSION**

The usefulness of the electron-dense, basic dye lake GC in localizing chromatin and ribosomal basophile substance is evident from the effects of specific digestions with the nucleases; in addition, considerable differences in morphology of the Ehrlich tumor cell are produced by purely organic as well as organometallic reagents which are highly sensitive to protein but apparently ineffective for the visualization of nucleic acids or lipid [(30, 31), and unpublished observations]. As might be expected, the morphologically preponderant basophilic component of chromosomes is deoxyribonucleoprotein; for cellular DNA (bindable with GC) exists only as a protein conjugate. This component is too tightly packed and too intensely stained to show details except at considerably high magnifications.

In contrast, others (54, 59) have shown numerous, distinct entities of particulate appearance in Ehrlich cell chromosomes studied at much lower magnification after fixation with the usual metallic fluids. A similar, seemingly particulate appearance may also be noted in other material (8, 39). It is possible that a partially or exclusively granular appearance, especially at low magnifications, is an illusion, enhanced, on occasion, by surface tension, partially solubilizing, aggregating, or other effects of liquid fixatives which may tend to disrupt an originally fibrillar, fragile substructure. Distinct fibrils, however, have been clearly demonstrated in sections by means of the common fluid fixatives (33, 38, 55), and in mounts of whole chromosomes (11, 14, 19, 56) but there are marked disagreements on the dimensions and spatial relations of the fibrils. Others (8) report observing "a mixture of granules 100–150 Å, and of what appears to be a network of filaments of 45–100 Å" in relatively low-power micrographs.

Some of the less desirable attributes of fixation with liquids, which in the past have evoked comments on the problems of disclosing chromosome structure satisfactorily, have perhaps now been mitigated by resort to freeze-drying. The potential hazard of the method—ice crystal artifacts—was, in general, successfully averted in this work, and there is no recognizable evidence of them in the micrographs chosen for discussion. As is well known, this is the preferred biochemical method of preserving protein concentrates in a largely undenatured state. It is also an excellent means of maintaining cellular morphology by minimizing the spatial displacement or chemical alteration of protoplasmic constituents from the condition prevailing during life. In addition, postfixation in sacco with vapors of protein reagents, anhydrously, immediately after freeze-drying, could be expected to stabilize the frozen-dried structure by excluding contact with liquids until after completion of postfixation.

Yet, despite these measures, or perhaps because of them and the intense staining, the structure seen in condensed chromo-
somes is far too tightly organized to reveal any structure at magnifications of, say, 50,000 or lower. These levels have often sufficed to show distinct granules or fibrils in other work (8, 15, 23, 54).

However, at much higher magnification, in extremely thin sections (Fig. 7), considerable lengths of 160-Å-wide filaments are seen in the compacted chromosomal mass. The low frequencies of equally long filaments in a given field of view suggests that they are highly convoluted. No obvious filaments of lesser width have been observed. This is a major difference between these findings and reports of fibers of varied widths (15–20, 40, 100, 45–100, 200–250, and 400–600 Å) in material fixed or stained with liquids. Apart from the failure to detect finer "elementary fibrils" or obvious subunits of the 160-Å-wide fibrils observed in this work, there are some noteworthy observations:

The unidirectional, longitudinal orientation of straight, extremely fine, parallel fibril-like elements which traverse apparently most portions of the chromosome, as though unhindered by the denser basophile compactions of 160 Å filaments. Because of the obscure nature of this phenomenon and the necessity of using magnifications of about 300,000, it is unlikely that they correspond to preferentially oriented parallel fibers reported in other observations (8) made at far lower magnification.

The chromatins which surrounds interphase nuclei (Fig. 3), and especially that part of the chromatin which may represent the nucleolus-organizer region of the chromosome, is probably truly granular. The masses of chromatin at the nuclear boundary also consist of large granular bodies amid densely staining chromatin.

The mitotic metaphase chromosome has a generally smooth surface, and does not possess lateral loops of the lampbrush type such as are believed to occur in almost all metazoan chromosomes at nearly all times (33).

Condensed chromosomes contain large numbers of extremely fine particles which stain very much less intensely with GC than the larger, basophilic components.

The sister chromatids in cross-section (Fig. 8) represent one of the earliest stages of split as yet observed in the electron microscope. The space separating them is made up mainly of the extremely fine particles just mentioned.

Some interphase chromatin threads (Fig. 1) seem to have a double structure resembling sister chromatids, but their study is made difficult by their minuteness and randomness of orientation in the sections.

The occurrence of RNA in chromosomes of Ehrlich ascites cells has been well-established by light microscopic cytochemistry (25) and is pertinent to current views on the association of RNA and DNA in the transcription of genetic information (21, 49). However, the results of the present work show that the morphology and electron image density of chromosomes are much less severely affected after digestion with RNase than after DNase. This must not be interpreted as indicative of the ineffectiveness of the RNase treatment; it was sufficiently prolonged and its efficacy in degrading RNA is evident from the drastic decrease in image density of the cytoplasm. Moreover, in parallel light microscopic controls of thick sections stained by GC there was an appreciable loss of color intensity in metaphase chromosomes as compared to undigested specimens. The sparse residue seen in electron micrographs of DNase digested chromosomes (Fig. 11) is believed to represent RNA-containing basophile matter. A fuller discussion of this substance, in terms of image density characteristics of thin sections, appears below. It is sufficient now to note that electron microscopic visualization of fine structural alterations is obscured in RNase-treated chromosomes by residual, DNA-containing material. The massive proportions of the latter, relative to RNA-containing basophile substance, is evident from comparisons with the effects of digestion with DNase.

The morphology of the DNase-resistant structure in metaphase chromosomes is of interest inasmuch as similar details in its pattern are not seen in previous work (5, 34). This structure, as has also been shown by others (5) is much weaker in image density than the enzymatically unaffected ribosomes. It probably corresponds to the structures stained faintly blue by GC in light microscopic preparations of chromosomes digested by DNase. If this residue of DNase digestion consisted solely of proteins stainable by low-density organic reagents like FNPS or FFDBN, but not by the electron-dense dye GC, it is unlikely that even the weak contrast exhibited by the particles constituting this residue would be visualized in Fig. 11. However, the fact that the contrast can be enhanced considerably under certain conditions (31) may be attributed largely to the use of organic protein reagents as postfixatives preceding basophile staining (Fig. 13). For these reasons, it would appear that DNase-resistant structure within chromosomes comprises largely protein with smaller amounts of basophile matter. It also explains why this structure is normally indistinguishable amid the dense masses of chromatin in undigested chromosomes.

The present results with trypsin differ from reports on the complete losses of chromatins produced by trypsin digestions after brief aldehyde fixation of specimens. These losses occurred following certain embedding procedures for electron microscopy, and only erratically after others (5). Complete disruption of nuclear structure, leading to gel-like residues, has also been reported (2). However, the specimens in this case were nuclei isolated in hypotonic sucrose solutions and digested with trypsin without prior fixation. But in agreement with the present findings, it has been reported that trypsin, at least in gross effects where particulate structure is not visualizable, did not extract DNase-sensitive structure from cells fixed with osmium tetroxide (34). Thus, morphologic discrepancies may stem from widely differing methods of specimen preparation.

The disclosures now effected with trypsin provide certain suggestive indications of the composition of the structure remaining after digestion. The drastic losses of density in the cytoplasm reflect the absence of binding of GC. The effects observed, including those yielded by buffer and light microscopic controls, are in accord with the expectation that contaminating traces of RNase, even if they were to occur in the crystalline sample of trypsin used, would be incapable of producing these drastic losses of image density in the cytoplasm. The trypsin digestions were very brief and at high pH—conditions which overwhelmingly favor trypsin but not the RNase impurity. The effects, moreover, are consistent with biochemical data on the disintegration of polyribosomes with trypsin (1, 9, 26, 60). The remnants of trypsin digestion of chromosomes are, on the one hand, particles which seemingly correspond to those that also resist DNase and, on the other, densely basophilic, fiber-like
structures which are sensitive to DNase. There can be little question about the chromatinic nature of this basophile matter.

But it is harder to explain definitively why the particulate residuum of trypsinized chromosomes (in which it appears to be almost as electron-dense as the chromatin) cannot be distinguished in undigested, control chromosomes. It is possible that trypsin, for unknown reasons, enhances the basophilia of these particles and that a certain degree of loosening-up of the chromatin following trypsinization gives them greater prominence. However, for reasons relating to image contrast discussed above, it is more likely that they consist partly of GC-stainable RNA but mostly of protein preserved through postfixation with the low-density reagents FNPS or FFDB. This explanation, moreover, is consistent with their invisibility in RNase digested chromosomes (Fig. 10). The resistance of these particles to trypsin (Fig. 16) suggests that their protein moiety is primarily non-histone. For, otherwise, it is unlikely that they would withstand the preferential hydrolysis by trypsin of peptide bonds between the carboxyl group of lysine or arginine and the amino group of another amino acid. Both lysine and arginine are particularly prominent in histone, and it is known that the bulk of this protein is enzymatically very easily degraded and released from nuclei while non-histone losses are relatively minor (2). A clearer characterization of the trypsin-insensitive particles visualized in this work must await further tests with proteases of relatively broader activity, such as $\alpha$-chymotrypsin, papain, and pepsin, as well as newer cytochemical approaches currently under study.

There is little to be gained at this time by correlating in undue detail the resemblances and differences in dimensions and morphology revealed in this study and in other observations based on quite unrelated, primarily morphologic, electron microscopic procedures. A comparative analysis would entail controlled, quantitative observations where factors such as selectivity of localization, retention of submicroscopic structure free from undesired extraction or chemical alteration, shrinkage, or swelling are necessary to assess precisely the dimensions, spatial interrelationships, and chemical properties of chromosomal components in situ. Improvement of procedures for submicroscopic cytochemistry with these ends in view would have relevance not only to questions of fundamental significance but also to research on chromosomes of living cells subjected to mitotic inhibitors, stimulants, or other agents.

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All illustrations are of frozen-dried preparations, postfixed anhydrously, in vacuo, with vapors of p,p′-difluoro-m,m′-dinitrodiphenyl sulfone (FNPS) or 1,5-difluoro-2,4-dinitrobenzene (FFDNB).

FIGS. 1–8. Basophile staining with gallocyanin-chromalum in the absence of enzymatic digestions.

FIGS. 9–16. Basophile staining with gallocyanin-chromalum preceded by an enzyme digestion step indicated separately for each figure.

FIG. 1. Individual chromatinic element from late interphase nucleoplasm. Note its doubleness due to a furrow, marked by arrows directed against each other. The seemingly granular appearance at the position of the larger arrow may be caused by cross-sectioning of fibrils. Verification of this is difficult owing to the rarity of such sections and the general crowding of structure over most of the element. × 90,000.

FIG. 2. Serial section of the cell in which the chromatinic thread in Fig. 1 was found. It is shown to reveal the image quality at medium magnification, and to identify the stage in the mitotic cycle as late interphase. This is marked, in general, by loss of the perinucleolar chromatin. The dense patch above the nucleolus on the right may be a remnant of chromatin associated earlier with nucleoli. Note the nucleolar ribosomes, the general density distribution in the cell and the fine structure in the nucleoplasm. Mitochondria are not disclosed by the procedure used. CYT, cytoplasm; No, nucleolus. × 38,000.

FIG. 3. General view of an interphase nucleolus (No) before the onset of the mitotic cycle. The ribosomes in it are enclosed by a dense coat of perinucleolar chromatin (PC) which at one end tapers into an elongate body (arrowed). This structure probably represents the nucleolus-organizer region of a chromosome. Despite its almost uniform density, prominent granules (about 300–400 Å) are often found in the latter at this stage. Chromatin (Ch) at nuclear boundary is equally dense. Note that ribosomal particles in the cytoplasm (CYT) are more intensely basophilic than those in the nucleoplasm. × 85,000.

FIG. 4. Higher magnification of chromatin at the nucleolus-organizer region. × 130,000.

FIG. 5. Basophile staining pattern after digestion with RNase. This equatorial view of a metaphase plate shows that the enzyme has abolished staining ability of the cytoplasm without at all reducing the basophilia of the chromosomes. Compare with Fig. 5. Arrow points to a region of a chromosome where the enzyme action has bared two dense bodies of unidentified nature (shown in inset at higher magnification). × 10,000.

FIG. 6. Part of a sister chromatid pair (cut somewhat obliquely as cross-sections) after RNase digestion and basophile staining. Although the enzyme has affected the morphology to some degree, the chromatinic components have resisted enzymatic extraction almost completely. Compare with the enzymatically untreated control in Fig. 8. × 200,000.

FIG. 7. Basophile staining pattern after digestion with DNase. This equatorial view of a metaphase plate shows that the enzyme has abolished staining ability of the cytoplasm without at all reducing the basophilia of the chromosomes. Compare with Fig. 5. Arrow points to a region of a chromosome where the enzyme action has bared two dense bodies of unidentified nature (shown in inset at higher magnification). × 10,000.

FIG. 8. Polar view of an almost perfectly sectioned metaphase plate. The disposition of some of the chromosomes is irregular. The chromosomes are highly condensed and very strongly basophile; their internal structure cannot be visualized at this magnification. × 300,000.

FIG. 9. Basophile staining pattern after digestion with RNase. This equatorial view of a metaphase plate shows that the enzyme has abolished staining ability of the cytoplasm without at all reducing the basophilia of the chromosomes. Compare with Fig. 5. Arrow points to a region of a chromosome where the enzyme action has bared two dense bodies of unidentified nature (shown in inset at higher magnification). × 10,000.

FIG. 10. Basophile staining pattern after digestion with DNase. Intensity of staining in the three metaphase chromosomes is severely reduced (compare with Figs. 1–8). Extraction of chromatin by the enzyme leaves a residue of particles which normally appear much fainter than the cytoplasmic ribosomes. The integrity and stainability of the latter are not at all affected. The weakly stained residue consists mainly of particles (60–80 Å) amidst infrequent tubular elements (arrowed). × 75,000.

FIG. 11. Part of DNase-digested chromosome stained as in Fig. 11. The tubular elements (one is arrowed) resist extraction by enzyme after periods of incubation which are too excessive for the maintenance of rigidity in the particles in their vicinity. The latter, nevertheless, are visible as a diffuse substance which is not easily degraded by DNase. × 130,000.

FIG. 12. DNase-digested metaphase chromosome. Although the 60–80 Å particles remaining in the chromosome after enzyme action are normally less intensely basophile than the ribosomes (see Fig. 11) their image contrast may be enhanced by slightly longer exposure to the electron beam. This is attributable to the stabilizing effect of the protein reagents used in postfixation. However, the cytoplasm (CYT) surrounding the chromosome is unnaturally overexposed. The pattern exhibited by the particles is highly variable. A chromosomal tubule is marked by arrows. × 130,000.

FIG. 13. Chromosome Cytochemistry

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