

# Low Angle X-ray Diffraction Studies of HeLa Metaphase Chromosomes: Effects of Histone Phosphorylation and Chromosome Isolation Procedure

J. R. PAULSON\* and J. P. LANGMORE‡

\**Medical Research Council Laboratory of Molecular Biology, Cambridge, England CB2 2QH; ‡Biophysics Research Division and Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan 48109. Dr. Paulson's present address is Department of Chemistry, St. Olaf College, Northfield, Minnesota 55057.*

**ABSTRACT** To test whether gross changes in chromatin structure occur during the cell cycle, we compared HeLa mitotic metaphase chromosomes and interphase nuclei by low angle x-ray diffraction. Interphase nuclei and metaphase chromosomes differ only in the 30–40-nm packing reflection, but not in the higher angle part of the x-ray diffraction pattern. Our interpretation of these results is that the transition to metaphase affects only the packing of chromatin fibers and not, to the resolution of our method, the internal structure of nucleosomes or the pattern of nucleosome packing within chromatin fibers. In particular, phosphorylation of histones H1 and H3 at mitosis does not affect chromatin fiber structure, since the same x-ray results are obtained whether or not histone dephosphorylation is prevented by isolating metaphase chromosomes in the presence of 5,5'-dithiobis(2-nitrobenzoate) or low concentrations of p-chloromercuriphenylsulfonate (ClHgPhSO<sub>3</sub>).

We also compared metaphase chromosomes isolated by several different published procedures, and found that the isolation procedure can significantly affect the x-ray diffraction pattern. High concentrations of ClHgPhSO<sub>3</sub> can also profoundly affect the pattern.

The goal of structural studies of chromatin is first to understand the static structure of the chromatin fiber, and then to understand how that fiber structure may change during gene activation, organismal development, chromosome replication and repair, and throughout the cell cycle.

In this study we asked the question: Can changes in bulk chromatin structure during the mitotic cell cycle be detected by low angle x-ray diffraction? More specifically, can we detect differences between interphase nuclei and metaphase chromosomes?

Comparison of interphase and metaphase chromatin structures is the obvious first experiment for two reasons. First, metaphase is one of the few points in the cell cycle at which cells can be efficiently arrested or synchronized; second, and more importantly, mitotic metaphase is the stage of the cell cycle at which detectable differences would be most likely to occur, since at this stage the cells and nuclei undergo several dramatic structural and biochemical transformations. These include breakdown of the nuclear envelope, shut-off of transcription, condensation of chromosomes into their familiar

compact form, and extensive phosphorylation of histones H1 and H3 and other chromatin proteins.

Our current understanding of the structure of metaphase chromosomes is summarized by the radial loop model (24), which states that the 25–30-nm thick chromatin fiber is folded into loops and that the bases of these loops are anchored at the axis of the chromatid by nonhistone proteins (reviewed in reference 27). The underlying structural organization of interphase chromosomes is probably very similar, since interphase chromatin is organized into supercoiled domains about the same size as the loops in metaphase chromosomes (4, 9, 17), and since there is evidence for similar metalloprotein interactions in the higher-order structure of both interphase and metaphase chromosomes (21–23).

A number of previous studies have compared interphase and metaphase chromatin fibers by different techniques. Morphological studies in the electron microscope, using surface-spreading or thin-sectioning, show both interphase and metaphase chromosomes to consist mainly of 20–30-nm fibers (1, 2, 12, 24). Careful measurements by Golomb and Bahr (3, 15) showed

both interphase and metaphase fibers to be 20 nm in diameter in surface spread, critical-point-dried preparations, although metaphase fibers appear to be 15–22% thicker after colchicine arrest (3, 14). Reports of 50-nm fibers in metaphase appear to be due to coiling of the 20–30-nm fiber on itself in the presence of hexylene glycol (2, 11, 24).

Nuclease digestion studies of chromatin have shown that nucleosomes exist in metaphase chromosomes and that the chromatin repeat remains the same between interphase and metaphase (8, 16, 36, 37). In the electron microscope, nucleosomes can be observed to be packed into 20–30-nm fibers (29–31) both in interphase and metaphase, but dehydrated electron microscope specimens do not show enough regularity to allow comparison of the internal structure of the fibers. In one study, interphase chromatin and metaphase chromosomes were compared by x-ray diffraction and both were found to give diffraction peaks at 6.0, 3.8, 2.7, and 2.1 nm (25).

Thus, despite the dramatic changes in chromosomes between interphase and metaphase, no differences in chromatin fiber structure have yet been observed, either by x-ray diffraction, electron microscopy, or nuclease digestion.

A serious difficulty with previous studies of metaphase chromatin was pointed out by D'Anna et al. (10), who found that histones H1 and H3 become dephosphorylated during isolation of metaphase chromosomes by conventional procedures. One might expect that, if structural differences exist between interphase and metaphase chromatin, they might result from the phosphorylation of H1 and H3; but none of the early studies took account of histone phosphorylation, and the chromosomes studied were very likely dephosphorylated.

To make a more meaningful comparison of interphase and metaphase chromatin, we looked for methods to prevent histone dephosphorylation. We found that certain sulfhydryl reagents, such as p-chloromercuriphenyl sulfonate (ClHgPhSO<sub>3</sub>), effectively inhibit the phosphohistone phosphatase present in the chromosomes, and they also inactivate the endogenous proteases in the chromosome preparations (26).

In the present study, we improved on previous x-ray studies in three respects. First, we studied metaphase chromosomes containing phosphorylated histones. Second, we directly compared interphase nuclei with metaphase chromosomes under the same ionic conditions. Third, we used an improved x-ray camera with which all of the reflections characteristic of chromatin *in vivo* (at 30–40, 11.0, 6.0, 3.8, 2.7, and 2.2 nm) can be observed (20). In particular, we were interested in observing the 30–40-nm and 11.0-nm reflections, since they are probably the most sensitive indicators of the side-by-side packing and internal structure of the fibers, and had not yet been observed from metaphase chromosomes.

We show, first, that interphase and metaphase fibers differ only in their packing, not in their internal structure, and that phosphorylation does not detectably affect the fiber structure. Second, we show that some of the isolation methods and some of the sulfhydryl reagents used *can* alter the x-ray patterns and therefore should be used with caution in structural studies of metaphase chromatin fibers.

## MATERIALS AND METHODS

**Chemicals and Buffer Solutions:** The basic isolation buffer (IB) for HeLa aqueous chromosomes, chromosome clusters, and interphase nuclei consisted of 10 mM HEPES, pH 7.3, 10 mM NaCl, and 5 mM MgCl<sub>2</sub>.

Colcemid, thymidine, and monosodium ClHgPhSO<sub>3</sub> were obtained from Sigma Chemical Co. (St. Louis, MO). Methylmercury (II) hydroxide (1 M aqueous solution) was obtained from Ventron (Danvers, MA), and 5,5'-di-

thiobis(2-nitrobenzoic acid) (Nbs<sub>2</sub>) was obtained from British Drug Houses, Ltd. (Poole, Dorset, England). Tissue culture media and components were obtained from Flow Laboratories, Inc. (McLean, VA).

**Isolation of Metaphase Chromosomes and Interphase Nuclei:** HeLa S3 cells were grown and arrested in metaphase as described in the previous paper, which accompanies this (20). Aqueous chromosomes were isolated either as individual chromosomes by the method of Marsden and Laemmli (24) (except that modified isolation buffers were used) or as chromosome clusters by the method of Paulson (26) from cultures which had been arrested to 90–95% in metaphase. In both methods, cells were lysed in IB plus 0.5 M sucrose, 0.5 mM CaCl<sub>2</sub>, and 0.1% Nonidet P-40 (NP-40), and the chromosomes were subsequently resuspended and washed in IB plus 0.5 mM CaCl<sub>2</sub> and 0.1% NP-40. No differences in the x-ray patterns were observed between the two methods, nor did the additional use of 0.05% sodium deoxycholate in the isolation buffers make any difference in the diffraction patterns.

Hexylene glycol chromosomes were isolated by a modification of the method of Wray and Stubblefield (38) as described by Paulson (26). The isolation buffer consisted of 0.1 mM PIPES, pH 6.7, 1 M hexylene glycol (2-methylpentan-2,4-diol), and 0.5 mM CaCl<sub>2</sub>.

Polyamine chromosomes were isolated by a modification of the method of Lewis and Laemmli (23) as described by Paulson (28). HeLa interphase nuclei were isolated as described in the accompanying paper (20).

For x-ray experiments involving sulfhydryl reagents to prevent dephosphorylation, chromosomes were isolated as chromosome clusters as previously described (26) with 5 mM ClHgPhSO<sub>3</sub>, 1 mM methyl mercury, or 5 mM Nbs<sub>2</sub> present in all solutions. In the experiments using 0.015 mM ClHgPhSO<sub>3</sub>, the volumes of solutions used were increased to ensure that the phosphatase in the chromosomes was completely inactivated. The cells were suspended to no >4 × 10<sup>6</sup> cells/ml in the lysis solution (with 0.015 mM ClHgPhSO<sub>3</sub>) and after pelleting, the chromosome clusters were washed twice in 40 ml of IB containing 0.5 mM CaCl<sub>2</sub>, 0.1% NP-40, and 0.015 mM ClHgPhSO<sub>3</sub>.

To separate the possible structural effects of phosphorylation from the effects of the sulfhydryl reagents themselves, chromosomes were isolated as chromosome clusters, allowed to become dephosphorylated by incubation for 3 h at 4°C, and finally treated with ClHgPhSO<sub>3</sub> by pelleting and resuspending in IB + 0.5 mM CaCl<sub>2</sub> + 0.1% NP-40 + 5 mM ClHgPhSO<sub>3</sub>. In another experiment, interphase nuclei were isolated and then finally resuspended and washed in IB + 0.5 mM CaCl<sub>2</sub> + 0.1% NP-40 + 5 mM ClHgPhSO<sub>3</sub>.

**Handling of X-ray Specimens and Analysis of Diffraction Patterns:** Our previous paper (20) describes the handling of the x-ray specimens and the analysis and presentation of the diffraction data. All figures are presented as log *s*<sup>2</sup> vs. *s*, with arbitrary vertical positioning of the curves to prevent overlap. All of the experiments reported here were done with fresh material. It should be noted that the x-ray diffraction pattern of chromosomes or nuclei changes with time if the material is stored in the absence of inhibitors for several days after isolation, even at 4°C. These changes are presumably due to proteolytic or nucleolytic degradation, since ClHgPhSO<sub>3</sub>, Nbs<sub>2</sub>, and methyl mercury, which are known to prevent such degradation (26), completely prevent the time-dependent changes in the x-ray diffraction patterns. Specimens were monitored for proteolysis before and after the x-ray exposure by running 15% SDS polyacrylamide gels according to Laemmli and Favre (18), and in all cases there was little or no proteolysis. Specimens were also monitored for phosphorylation or dephosphorylation of histone H1 by extracting a sample of chromosomes (or the contents of a specimen capillary after the x-ray exposure) with 0.2 M H<sub>2</sub>SO<sub>4</sub> and analyzing the acid-extractable proteins on acid/urea gels (26).

## RESULTS

### Comparison of Interphase Nuclei and Metaphase Chromosomes

We sought first to answer the question: Can any differences in structure between HeLa interphase nuclei and mitotic chromosomes be detected using low angle x-ray diffraction? To ensure that any differences observed resulted from real differences in the chromatin rather than from differences in the conditions or method of preparation, the isolation procedures and the ionic conditions were kept as similar as possible throughout.

Fig. 1 shows composite patterns for nuclei (Fig. 1*a*) and chromosome clusters (Fig. 1*b*). It is clear that there are no significant differences in the higher angle parts of the pattern (i.e., in the 11-, 6.0-, 3.8-, 2.7-, and 2.2-nm reflections) between nuclei and metaphase chromosomes. The precise spacings of

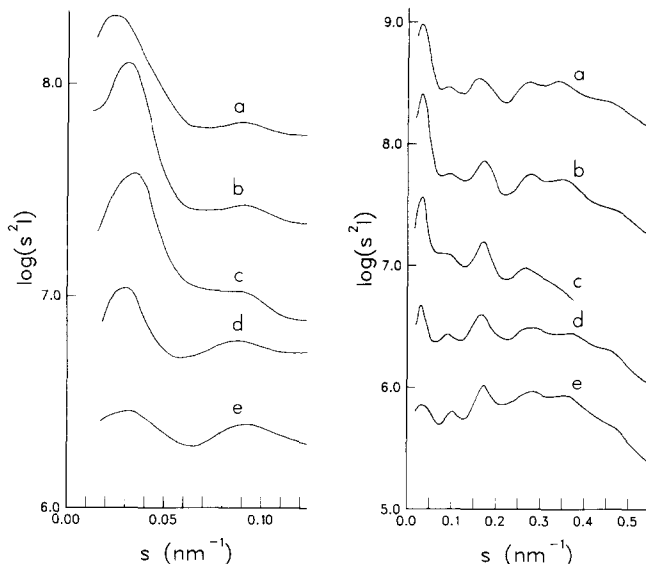


FIGURE 1 Comparison of diffraction patterns from (a) HeLa interphase nuclei and (b) metaphase chromosome clusters under the same conditions. (c, d, and e) Chromosome clusters under the same conditions, except with 0.015 mM ClHgPhSO<sub>3</sub>, 5 mM Nbs<sub>2</sub>, and 1 mM methyl mercury, respectively, continuously present during the isolation to prevent dephosphorylation of histones H1 and H3. Acid urea gels showed that in b, histone H1 was dephosphorylated, but in c, d, and e it was fully phosphorylated. The same data is shown in both panels but on the left the scale is expanded to show better the very low angle region.

the peaks are the same (cf. Table I), and the overall shapes of the plots are also the same (Fig. 1 a and b).

There are differences, however, in the 30–40-nm peak. Metaphase chromosomes consistently give a strong peak at 32 nm under these conditions, but for interphase nuclei the peak is usually between 36 and 40 nm and it is significantly broader (compare the left parts of Fig. 1 a and b).

### Phosphorylation of Histones H1 and H3 in Metaphase Has No Detectable Effect on the Chromatin Structure

To test whether phosphorylation of histones H1 and H3 makes a difference in the structure of metaphase chromatin fibers, chromosomes were isolated in the presence of sulfhydryl reagents to prevent dephosphorylation (26). X-ray diffraction patterns were recorded from metaphase clusters which were isolated with 0.015 mM ClHgPhSO<sub>3</sub> (Fig. 1 c), 5 mM Nbs<sub>2</sub> (Fig. 1 d), or 1 mM methyl mercury hydroxide (Fig. 1 e) and the results were compared with those from chromosomes which had been allowed to become dephosphorylated (Fig. 1 b). In each experiment involving metaphase chromosomes, we verified whether histone H1 was phosphorylated or dephosphorylated by running acid urea gels. We assume that histone H3 will be dephosphorylated if and only if histone H1 is also dephosphorylated (26).

As can be seen from Fig. 1 and Table I there is no significant difference between the diffraction patterns of metaphase chromosomes with dephosphorylated histones and those isolated with ClHgPhSO<sub>3</sub> or Nbs<sub>2</sub> to prevent dephosphorylation. The pattern for chromosomes isolated with 1 mM methyl mercury (Fig. 1 e) is also the same except that the 32-nm reflection is relatively weaker. Because the higher angle reflections are identical we conclude that histone phosphorylation makes no

significant difference in the structure of nucleosomes or the arrangement of the nucleosomes in the chromosome fibers.

### High Concentrations of ClHgPhSO<sub>3</sub> Destroy the 32-nm Reflection But Do Not Affect the Higher Angle Pattern

Originally, most of our biochemical studies of phosphorylation were done using 5 mM ClHgPhSO<sub>3</sub> (26), but we found that this concentration completely destroys the 32-nm reflection. This can be seen by comparing the diffraction patterns from chromosomes isolated with a low concentration (0.015 mM) of ClHgPhSO<sub>3</sub> (Fig. 2 a) and those isolated with 5 mM ClHgPhSO<sub>3</sub> (Fig. 2 b). Surprisingly, the higher angle part of the x-ray diffraction pattern is not noticeably affected.

This effect seems to have nothing to do with phosphorylation since it is the same if we first allow chromosomes to become dephosphorylated by storage for 3 h at 4°C and then transfer them to buffer solutions containing 5 mM ClHgPhSO<sub>3</sub> (Fig. 2 c). A similar effect is seen when interphase nuclei are isolated in the presence of 5 mM ClHgPhSO<sub>3</sub> (Fig. 2 d).

### Differences Are Observed between Chromosomes Isolated by Different Procedures

For all of the experiments mentioned above, metaphase chromosomes were isolated with IB (10 mM HEPES, pH 7.3, 10 mM NaCl, and 5 mM MgCl<sub>2</sub>) plus 0.5 mM CaCl<sub>2</sub>, either as chromosome clusters or as aqueous chromosomes. We also isolated chromosomes by two other procedures to see how

TABLE I  
Measured Periodicities from X-ray Diffraction of HeLa Interphase Nuclei and Metaphase Chromosomes

Specimen*	Reflections					
	30–40 nm	11 nm	6.0 nm	3.7 nm	2.7 nm	2.1 nm
Interphase nuclei	38.0	11.0	6.2	3.7	2.9	2.2
Interphase nuclei + 5 mM ClHg-PhSO <sub>3</sub>	—	11.5	5.9	3.5	2.7	2.1
Chromosomes	31.7	11.1	6.0	3.6	2.8	2.1
Chromosomes, isolated with 0.15 mM ClHgPhSO <sub>3</sub>	30.1	10.7	5.8	3.7	ND†	ND
Chromosomes, isolated with 5 mM Nbs <sub>2</sub>	33.8	11.4	5.9	3.6	2.6	2.1
Chromosomes, isolated with 1 mM methyl mercury	31.8	10.8	5.7	3.6	2.7	2.1
Chromosomes, isolated with 5 mM ClHgPhSO <sub>3</sub>	—	11.3	6.0	3.6	2.7	2.1
Chromosomes, 5 mM ClHgPhSO <sub>3</sub> added 3 h after isolation	—	11.1	6.0	3.5	2.7	2.1
Hexylene glycol chromosomes	—	10.6	5.8	3.7	2.8	2.2
Polyamine chromosomes	37.6	11.8	5.9	3.6	2.7	2.1

\* Except for hexylene glycol chromosomes and polyamine chromosomes, all specimens were in IB + 0.5 mM CaCl<sub>2</sub>.

† ND, not determined.

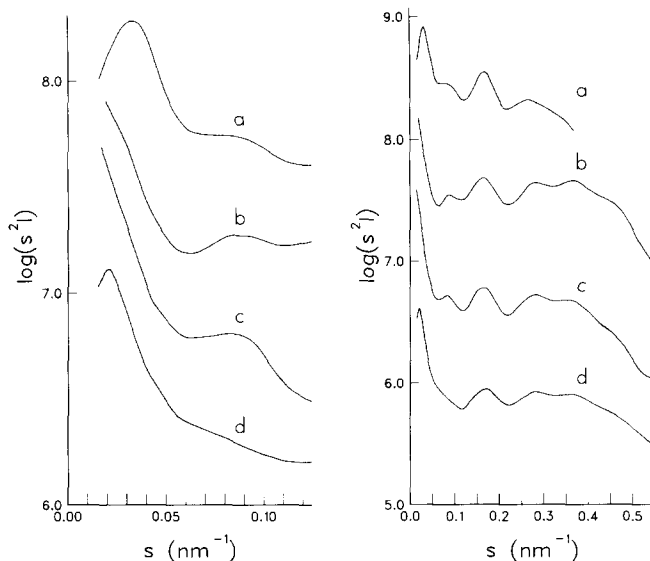


FIGURE 2 High concentrations of ClHgPhSO<sub>3</sub> destroy the 32-nm reflection but do not affect the higher angle pattern. (a) Chromosome clusters isolated in IB + 0.5 mM CaCl<sub>2</sub> + 0.015 mM ClHgPhSO<sub>3</sub>. (b) Chromosome clusters isolated in IB + 0.5 mM CaCl<sub>2</sub> + 5 mM ClHgPhSO<sub>3</sub>. (c) Chromosome clusters isolated in IB + 0.5 mM CaCl<sub>2</sub> and then shifted to the same buffer plus 5 mM ClHgPhSO<sub>3</sub> after incubation for 3 h at 4°C. (d) Interphase nuclei in IB + 0.5 mM CaCl<sub>2</sub> + 5 mM ClHgPhSO<sub>3</sub>. Note that the 32-nm reflection, which is present in a, is apparently lost or shifted to much lower angles in the other patterns.

much, if at all, the isolation procedure could affect the diffraction pattern. In all cases, only fresh material was used to make x-ray specimens, but in these experiments we made no effort to prevent dephosphorylation.

The results are shown in Fig. 3. Metaphase chromosome clusters in IB plus 0.5 mM CaCl<sub>2</sub> (Fig. 3a) are compared with polyamine chromosomes in buffer A (15 mM Tris-Cl, pH 7.4, 0.2 mM spermine, 0.5 mM spermidine, 2 mM potassium EDTA, and 80 mM KCl) plus 0.1% digitonin isolated by the method of Lewis and Laemmli (23) (Fig. 3b), and hexylene glycol chromosomes in 0.1 mM PIPES, pH 6.7, 0.5 mM CaCl<sub>2</sub>, and 1 M hexylene glycol isolated by the method of Wray and Stubblefield (38) (Fig. 1c). Metaphase chromosome clusters isolated in IB + 0.5 mM CaCl<sub>2</sub> + 5 mM ClHgPhSO<sub>3</sub> are shown in Fig. 3d. Slight differences may exist in the higher angle part of the pattern; for instance, the 11-nm reflection is significantly weaker in relation to the 6.0-nm reflection in the pattern from hexylene glycol chromosomes.

The most striking differences, however, are in the low angle region. In the case of polyamine chromosomes the 32-nm reflection is much weaker and is shifted to 38 nm. In the case of the hexylene glycol chromosomes, it is completely absent. In this respect, the hexylene glycol chromosomes are very similar to chromosomes in IB plus 5 mM ClHgPhSO<sub>3</sub>.

## DISCUSSION

One of the primary aims of our research has been to compare interphase and metaphase chromatin by various techniques to see whether any structural differences can be observed. Such a comparison might give clues to the function of histone phosphorylation at mitosis and it might yield important information on the mechanism of such mitotic events as chromosome condensation and the shut-off of transcription.

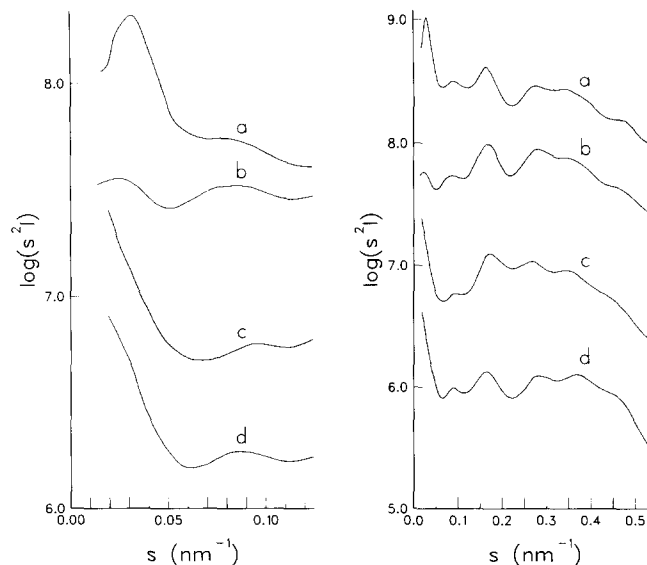


FIGURE 3 Comparison of the x-ray diffraction patterns of metaphase chromosomes isolated by several different procedures. (a) Chromosome clusters isolated in IB + 0.5 mM CaCl<sub>2</sub> + 0.1% NP-40. (b) Polyamine chromosomes isolated in buffer A + 0.1% digitonin. (c) Hexylene glycol chromosomes isolated in 0.5 mM CaCl<sub>2</sub>, 0.1 mM PIPES, pH 6.7, and 1 M hexylene glycol. (d) Chromosome clusters isolated in IB + 0.5 mM CaCl<sub>2</sub> + 0.1% NP-40 + 5 mM ClHgPhSO<sub>3</sub>.

In this paper we have compared interphase nuclei and metaphase chromosomes from HeLa cells using low angle x-ray diffraction. Metaphase chromosomes have been studied by low angle x-ray diffraction by Pardon et al. (25), but we have made several significant improvements over their study. First, we isolated chromosomes under conditions that were fairly close to physiological and, in particular, close to neutral pH, whereas Pardon et al. used chromosomes isolated at pH 3.2 which might have extracted a significant amount of the histone H1 (7). Second, we have compared interphase nuclei and metaphase chromosomes directly by isolating them by procedures as similar as possible, and under conditions as near as possible, and by handling the x-ray specimens identically throughout. Third, we have used an improved low angle x-ray camera to observe reflections in the 30–40-nm region and at 11 nm. Finally, we have directly tested the possible effects of phosphorylation of histones H1 and H3. The possible effect of this phosphorylation on chromatin structure is of interest because histone H3 is essential to the nucleosome core and because H1 plays an important role in the formation of thick fibers (34).

Our results show that there is no detectable difference in the internal structure of the chromatin fiber between interphase and metaphase but that there is a difference in the packing of the fibers. The 11-, 6.0-, 3.8-, 2.7-, and 2.2-nm reflections, which have been shown to come from the internal structure of the fibers (19, 20), are the same from interphase nuclei and metaphase chromosomes. The 32-nm reflection from metaphase chromosomes, however, which comes from the side-to-side packing of the fibers (19, 20), is broader and shifted to 36–40 nm with interphase nuclei. Thus, metaphase chromatin fibers are on average more tightly packed than interphase fibers. This is not surprising, since one would expect that during condensation of chromosomes at mitosis the loops of 30-nm fiber would be drawn more tightly together than they

are during interphase, probably by means of interconnections between the "loop fastening" proteins which anchor the bases of the various loops (see e.g., reference 27). An alternative possibility is that condensed interphase and metaphase fibers are equally tightly packed but that in interphase only a fraction of the chromatin is condensed, thus biasing the low angle reflection to lower angles.

Our results show further that phosphorylation of histones H1 and H3 does not make any detectable difference in the diffraction pattern of metaphase chromosomes. Chromosomes in which these proteins have been allowed to become dephosphorylated (e.g., Fig. 1*b*) give the same pattern as chromosomes in which dephosphorylation has been prevented by carrying out the isolation in the presence of 0.015 mM ClHgPhSO<sub>3</sub> or 5 mM Nbs<sub>2</sub>.

Our failure to detect any difference in the internal structure of chromatin fibers which might be attributable to phosphorylation of histones H1 and H3 is disappointing. Perhaps phosphorylation of the histones does not affect internal structure of chromatin fibers at all but serves to promote or inhibit interactions between the fibers and one another, as suggested by Bradbury et al. (5), or between the fibers and other cell components. H1 is thought to be on the inside of the thick fiber (33), but the fact that it is readily phosphorylated, and therefore accessible to the kinase, suggests that it might also be accessible for such interactions. In our experiments, however, we have been unable to detect any change in the side-to-side fiber spacing in metaphase chromosomes as a result of total dephosphorylation of H1 and H3.

On the other hand, histone phosphorylation at mitosis might affect the kinetics of condensation or the thermodynamic stability of the condensed state. A stabilizing function of phosphorylation on the internal structure of the chromatin fibers might be detectable by determining the salt-dependence of the structure of chromatin fragments, as has been done for rat liver chromatin fragments using electron microscopy (34) and using analytical sedimentation (6, 35). A stabilizing function of phosphorylation on side-to-side packing of fibers in chromosomes might also be detected by studying the x-ray packing reflections as a function of ionic conditions.

To test whether isolation conditions could make differences in the x-ray diffraction pattern, we examined metaphase chromosomes isolated by four different procedures. Not surprisingly, since they are prepared in the same buffers, aqueous chromosomes and metaphase chromosome clusters gave identical x-ray diffraction patterns. Polyamine chromosomes, however, differ significantly from aqueous chromosomes in the 30–40-nm region. With hexylene glycol chromosomes, the 11-nm reflection is much weaker relative to the 6.0-nm reflection, and the 32-nm reflection is eliminated completely. A possible clue to understanding the disappearance of the 32-nm reflection in this case comes from the electron microscopic work of Daskal et al. (11). They showed that hexylene glycol chromosomes contain many 52-nm thick "microconvules," which can be interpreted as loops of 30-nm chromatin fiber twisted back to form stubby projections (2, 24). Such a structural change in the chromosomes probably reduces the amount of ordered packing of 30-nm fibers and gives rise to increased x-ray scattering in the very low angle region corresponding to spacings >50 nm.

We conclude that isolation conditions can have significant effects on the higher order structure of chromatin, and these effects must be borne in mind when carrying out more detailed

investigations of chromatin fiber structure.

Caution must also be exercised when sulfhydryl reagents (26) are used to study the structure or other properties of chromatin containing phosphorylated H1 and H3 histones, since these reagents can themselves affect the structure of the chromatin. For instance, even though low concentrations of ClHgPhSO<sub>3</sub> (such as 0.015 mM) have no adverse effect on the chromatin x-ray pattern, high concentrations (such as 5 mM) have a drastic effect. The effect is intriguing because 5 mM ClHgPhSO<sub>3</sub> seems to affect only the packing of the chromatin fibers but not their internal structure.

Three possible structural changes which could result in disappearance of the 30–40-nm reflection come to mind: (a) decompaction of the chromosome fibers (cf. reference 20); (b) excessive compaction, similar to that suggested by Langmore and Paulson (20), to explain the lack of a 30–40-nm reflection from sea urchin sperm nuclei; and (c) formation of "microconvules," similar to those induced by hexylene glycol. The first of these possibilities is unlikely because phase-contrast microscopy shows that chromosomes are still compact in IB + 0.5 mM CaCl<sub>2</sub> + 5 mM ClHgPhSO<sub>3</sub> (data not shown). The other two possibilities could be tested by thin-sectioning chromosomes isolated in the presence or absence of 5 mM ClHgPhSO<sub>3</sub>.

How could 5 mM ClHgPhSO<sub>3</sub> cause a structural change? One possibility, suggested by the work of Earnshaw and Fujimori (13), is that ClHgPhSO<sub>3</sub> exerts its effect by a detergentlike action at high concentrations. Earnshaw and Fujimori (13) found that certain sulfhydryl groups in rhodopsin were accessible to ClHgPhSO<sub>3</sub> at high concentrations but not at low concentrations. At a concentration of 5 mM, ClHgPhSO<sub>3</sub> might be able to penetrate to the sulfhydryls of histone H3 (or other sulfhydryls which it does not reach when used at low concentrations), thus causing a structural change. Alternatively, its action might be less specific, for instance similar to the effect of hexylene glycol discussed above.

Of course, since the low angle resolution of our x-ray camera is limited to spacings of less than ~70 nm, we are unable to test the validity of models for higher order structures with very large periodicities (e.g., reference 32). Likewise, even though our results are consistent with the radial loop model for the third level of chromosome structure, they are unable to distinguish whether that particular model or any alternative model is correct.

The most important result of these studies is the confirmation that nucleosome structure and the pattern of nucleosome packing within thick chromatin fibers are essentially the same in both interphase and metaphase chromosomes in their native state. This conclusion could not be reached from earlier studies due to the poor low angle resolution of the x-ray cameras used, the harsh isolation procedures used for earlier work, and the failure of electron microscopy to give any definitive data on the packing of nucleosomes in thick fibers. From our results we conclude that the structural differences between interphase and metaphase chromosomes are limited to the side-to-side packing and higher orders of folding of the chromatin fibers.

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