RELATIONSHIP BETWEEN CHROMOSOME CONDENSATION AND METAPHASE LYSINE-RICH HISTONE PHOSPHORYLATION

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ABSTRACT

Treatment of metaphase HTC cells with $ZnCl_2$ inhibits histone phosphatase activity and leads to an increase in the hyperphosphorylated forms of the lysine-rich (F_1) histone. Under normal conditions a massive phosphatase activity is triggered as the cells shift from M into G_1 phase. In the presence of $ZnCl_2$ this activity is abolished and the hyperphosphorylated form of F_1 persists intact into G_1. We have asked the simple question of whether the chromosome can still extend during the M-G_1 transition even if the F_1 histone is maintained in the hyperphosphorylated form. We observe an apparently normal extension of the chromosomal material under these conditions, though it is evident that high levels of $ZnCl_2$ have rather substantial effects on other cell functions.

Phosphorylation of the lysine-rich F_1 histone is intimately associated with cell division. Two general types of F_1 phosphorylation have been identified and to some extent distinguished: these are (*a*) the interphase phosphorylation occurring most rapidly in S phase and (*b*) that occurring in metaphase cells. This latter type of F_1 phosphorylation has been documented in HTC cells (3), Chinese hamster cells (CHO line) (10), Chinese hamster cells (V-79) (12, 13), HeLa cells (14, 16) and *Physarum polycephalum* (7). This type of F_1 phosphorylation is evidently characteristic of metaphase and is not an artifact of the drugs used to obtain the metaphase cells (3, 10).

There are several ways in which metaphase F_1 phosphorylation differs from that occurring in interphase. First, in metaphase HTC cells there are four to six phosphorylated F_1 species in contrast to interphase which shows the presence of only two to three phosphorylated forms (3). Second, in HTC cells the half-life of metaphase F_1 phosphate is 2 h in metaphase and one-half hour in G_1 phase, whereas that of interphase F_1 phosphate is 4½–5 h in every phase except in metaphase (3). Third, in HTC cells and Chinese hamster cells (V-79), the sites of the metaphase phosphorylation include several of the interphase phosphorylation sites, but there are also other different sites (3, 11).

There is compelling evidence that is consistent with the idea that the function of interphase F_1 phosphorylation is related to the deposition of the newly synthesized F_1 histone onto DNA and that the bulk level of interphase phosphorylated F_1 histone may not be physiologically significant (4, 15). On the other hand, several groups have proposed that the gross amount of the phosphorylated form of F_1 histone in metaphase may play a role in either the initiation (11, 12) or the maintenance (5–7) of chromosome condensation. Such a model can be criticized on the grounds that it is based purely on correlative observations and that there may be no direct interrelationship between chromosome condensation and the attainment of the high bulk level of metaphase phosphorylated F_1 species. A direct test of this model would

involve inhibiting the F_1 phosphorylation and assaying for the effect on mitotic chromosome condensation. Unfortunately, at this time there is no specific inhibitor in vivo for histone phosphorylation (nor, for that matter, for chromosome condensation). However, we have found that $ZnCl_2$ can substantially inhibit the activity of histone phosphate phosphatase (22). Accordingly, we have asked whether the chromosome could extend upon going from metaphase to the G_1 phase if the high bulk levels of interphase phosphorylation were maintained intact by the use of $ZnCl_2$. In this way, we can probe the role of metaphase phosphorylation in the maintenance of the condensed state, though it throws little light on the ideas which suggest that histone phosphorylation is only a trigger to chromosome condensation (5–7).

MATERIALS AND METHODS

$ZnCl_2$ Treatment of Metaphase HTC Cells and their Release into G_1 Phase

$ZnCl_2$ (10 mM) was added to 1.8 liter of metaphase cells (density 200,000 cells/ml) prepared as described previously (2). After incubation for 4 h, an aliquot (600 ml) of these cells was collected and frozen. The rest of the cells were pelleted by gentle centrifugation (500 *g*). One portion of the cells was resuspended in fresh S-77-S medium (lacking Colcemid) containing $ZnCl_2$ (10 mM), the other in fresh S-77-S medium without $ZnCl_2$. 2 h after resuspension, the cells were collected and frozen. Histones were isolated as described earlier (17).

^{32}P-Labeling of $ZnCl_2$-Treated Metaphase HTC Cells

An aliquot of metaphase cells (1.5 liter) of density 200,000 cells/ml was prepared as described above. $ZnCl_2$ (10 mM) and ^{32}P (20 mCi) were then added simultaneously. At the end of the 4th h, cells were collected and frozen. Histones were isolated and ^{32}P radioactivity was determined as described earlier (4).

Electron Microscopy

Fresh cells (10 ml) were treated with 20–30 ml of 3% glutaraldehyde in 0.1 M sodium cacodylate pH 7.2 as described by Sabatini et al. (19). After this initial fixation, the cells were washed with 0.1 M sodium cacodylate at pH 7.2 and postfixed with 1% osmium tetroxide and then gradually dehydrated in ethanol. Finally, they were suspended in Spurr's plastic and embedded in B.E.E.M. capsules (Better Equipment for Electron Microscopy, Inc., Bronx, N.Y.) (20). The polymerized blocks were trimmed and sectioned on a Sorvall Porter-Blum Ultramicrotome model MT-2 (DuPont Instruments, Sorvall Operations, Newtown, Conn.). The grids were stained with 5% uranyl acetate (23) and washed with distilled water. Secondary staining was performed on a petri dish with lead citrate (18). The grids were washed with 0.02 N NaOH and distilled water and allowed to dry in air. The grids were observed with a Hitachi HU 125E electron microscope at 50 kV.

Determination of Amount of Phosphorylated F_1 Histone

F_1 histones were electrophoresed on high-resolution polyacrylamide gels as described previously (1). Stained gels were scanned, and the phosphorylated F_1 peaks were resolved with a curve analyzer.

RESULTS

Bulk Levels of Phosphorylated F_1 Histone

The electrophoretic patterns and attendant scans of F_1 histone isolated from metaphase cells treated with $ZnCl_2$ are very similar to those obtained from untreated metaphase cells (Fig. 1). The F_1 histone contains multiple levels of modified species that move more slowly than the parental species on the high-resolution polyacrylamide gel electrophoresis system. These slower moving bands have incorporated ^{32}P-label when F_1 histone was isolated from $ZnCl_2$-treated cells incubated with [^{32}P]phosphate (Fig. 2). The level of F_1 phosphorylation of $ZnCl_2$-metaphase cells appears to reach at least four and perhaps as much as five phosphate groups per F_1 molecule.

The extent of phosphorylation of F_1 species can be determined from the scans of the electrophoretic pattern of F_1 histone, using a curve analyzer. We find that 85% of the F_1 molecules contain at least one or more phosphate groups in untreated metaphase cells and up to 93% in $ZnCl_2$-treated metaphase cells. Although the increase is small, it is significant and presumably arises from the inhibitory effect of $ZnCl_2$ on histone phosphate phosphatase. If it is assumed that there was no change in the phosphorylating capacity in these cells, the capacity for F_1 dephosphorylation is reduced to at least one-half of that in the untreated metaphase cells.[1]

Fig. 3 A and B shows the electrophoretic patterns and attendant scans of F_1 histones from cells

[1] The details of this type of calculation were documented in an earlier paper by Balhorn et al. (3).

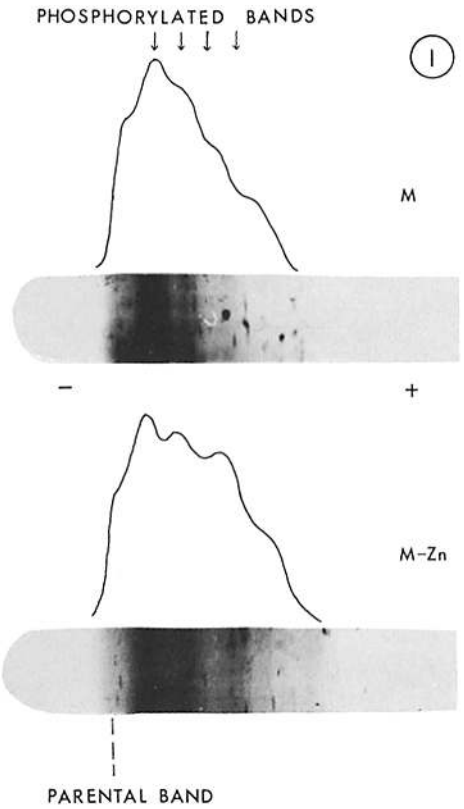

FIGURE 1 Microheterogeneity of F_1 histones isolated from untreated metaphase cells and $ZnCl_2$-treated cells. HTC cells were trapped in metaphase as described earlier (2). 4 h after the addition of Colcemid, metaphase cells were gently shaken from the monolayer surface, and the density of these cells was adjusted to about 200,000 cells/ml by centrifugation. Zinc chloride was added directly to these cells. After 4 h, cells were collected and frozen. A control experiment was performed without $ZnCl_2$.

that were released from a Colcemid-$ZnCl_2$ block either in the presence or absence of $ZnCl_2$. After removal of $ZnCl_2$, only 30% of the F_1 complement is in the phosphorylated form. This observation is identical to that obtained with the untreated G_1 phase cells (which have never been treated with $ZnCl_2$) and suggests that the cells can eliminate $ZnCl_2$ reasonably efficiently and that the turnover rate of the metaphase F_1 phosphate at the M-G border is approaching its normal value ($t_{1/2} \leq 30$ min) (3). On the other hand, if metaphase cells were released from the Colcemid block in the continued presence of $ZnCl_2$, the level of phosphorylated F_1 species remains very high, similar to

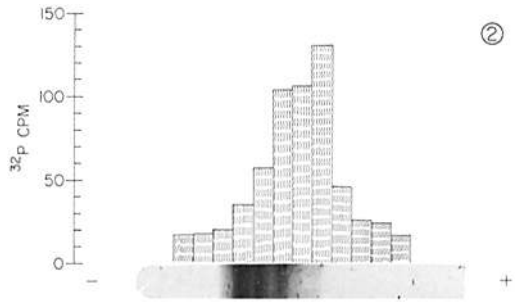

FIGURE 2 Incorporation of ^{32}P into slower moving bands of F_1 histone from $ZnCl_2$-treated metaphase cells. $ZnCl_2$-treated metaphase cells were prepared as described in the legend of Fig. 1, except that [^{32}P]phosphate was added at the same time as $ZnCl_2$.

that of control metaphase cells. We interpret this result as due to a massive inhibition of the turnover of the metaphase F_1 phosphate as the cells shift into G_1 phase. We have previously shown that the high level of microheterogeneity is not due to inhibition of the normal G_1 phosphate phosphatase, as insufficient time had elapsed to permit the levels of G_1-phosphorylated histone to increase to these high values (22).

The Morphology of $ZnCl_2$-Treated Cells

As shown in Fig. 4, the morphology of the cells in thin sections of Colcemid-trapped metaphase cells treated with $ZnCl_2$ for 4 h is similar to that found in cells of parallel culture lacking $ZnCl_2$. In both cases, the chromosomes are condensed in the middle region of the cell, the nuclear membrane has disappeared, and both types of cells lack spindle fibers as expected during Colcemid treatment.

Upon release from the Colcemid block, the cells were allowed to grow either in the presence or in the absence of $ZnCl_2$ as described above. The chromosomes appear to be able to extend fully, and the nuclear membrane is seen in both instances (Figs. 5 and 6). However, cytokinesis does not occur as is indicated by the data in Table I. This appears to be a function of $ZnCl_2$ treatment *per se* and is independent of whether or not $ZnCl_2$ was removed along with Colcemid. If $ZnCl_2$ treatment is continued for 24 h, all of the cells are destroyed and the suspension becomes turbid. On the other hand, when $ZnCl_2$ is removed at the same time as Colcemid, the cells remain intact and viable but do not increase in number, even 24 h afterward.

Although the chromosomes decondense after

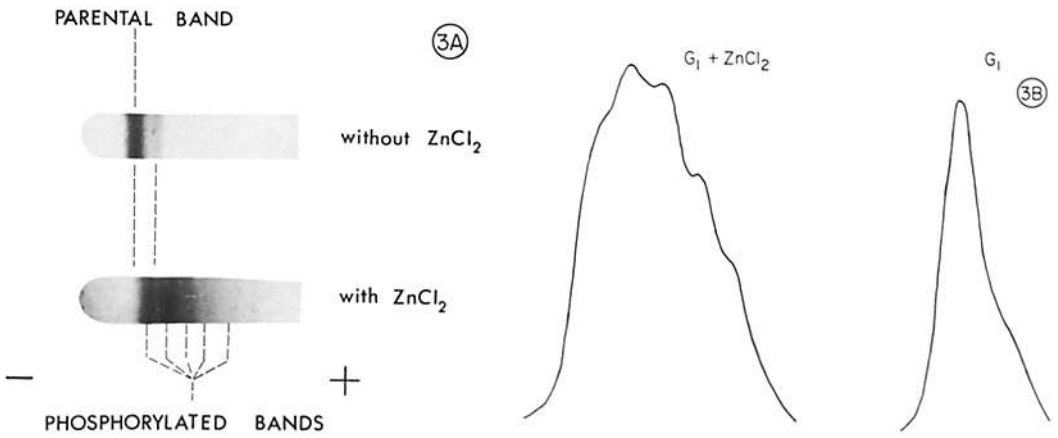

FIGURE 3 Level of phosphorylated F_1 histone 2 h after release from Colcemid block both in the presence and in the absence of $ZnCl_2$. $ZnCl_2$-treated metaphase cells were prepared as described in the legend of Fig 2. 4 h after the $ZnCl_2$ treatment, Colcemid was removed by centrifugation (900 gpm for 5 min.). Cells were resuspended and incubated either in the presence or in the absence of $ZnCl_2$ (10 mM final concentration) for 2 h. At the end of this period, cells were collected and frozen. Histones were isolated as described previously (17). (A) Electrophoretic patterns; (B) Microdensitometer scans of the polyacrylamide gels.

release from $ZnCl_2$-Colcemid block, the appearance of these cells 2 h later (whether subsequently grown in the absence or presence of $ZnCl_2$) is not completely identical to that of control "G_1 phase" cells. About one-half of the cells contain more vacuoles than normal, and they have a swollen nuclear membrane (Figs. 5 *b* and 6 *b*). Furthermore, most of the cells are bigger than normal G_1-phase cells, and the nuclei are often highly convoluted. These features are most probably due to the failure of the cells to undergo cytokinesis and to the consequent polyploidy.

DISCUSSION

Several groups of investigators have surmised that there might be a direct relationship between chromosome condensation and the extensive metaphase F_1 phosphorylation (5–7, 10, 11–14). Our results, however, do not support this notion. Cells maintained in the presence of $ZnCl_2$ after release from a Colcemid block yield an F_1 histone with an extensive phosphorylation pattern typical of the metaphase state. Yet, these cells proceed apparently normally through chromosome decondensation and nuclear membrane formation. The lack of correlation between chromosome condensation and extensive metaphase F_1 phosphorylation is also observed in micronuclei of *Tetrahymena pyriformis* (8), in which mitosis is triggered and accomplished in the absence of the F_1 histone and its attendant phosphorylation. The results described in this paper argue against an involvement of massive phosphorylation in the maintenance of the condensed state of metaphase chromosomes, leaving open the possibility that such phosphorylation might act as a trigger for initiating condensation, though the results of Gorovsky et al. leave even this possibility open to some criticism (8, 9). Interstingly, Gorovsky et al. (9) have also shown that extensive phosphorylation of the F_1 histone does occur in the amitotic *Tetrahymena* macronucleus.

Finally, we have recently observed (21, 22) that $ZnCl_2$ treatment completely inhibits the substantial phosphorylation of F_3 characteristic of and specific to metaphase cells, presumably by interfering with the act of phosphorylation rather than phosphate hydrolysis. Thus, in this specific case, we can further conclude that the maintenance of the compact conformational state of the chromosome in metaphase does not require phosphorylation of F_3 histone.

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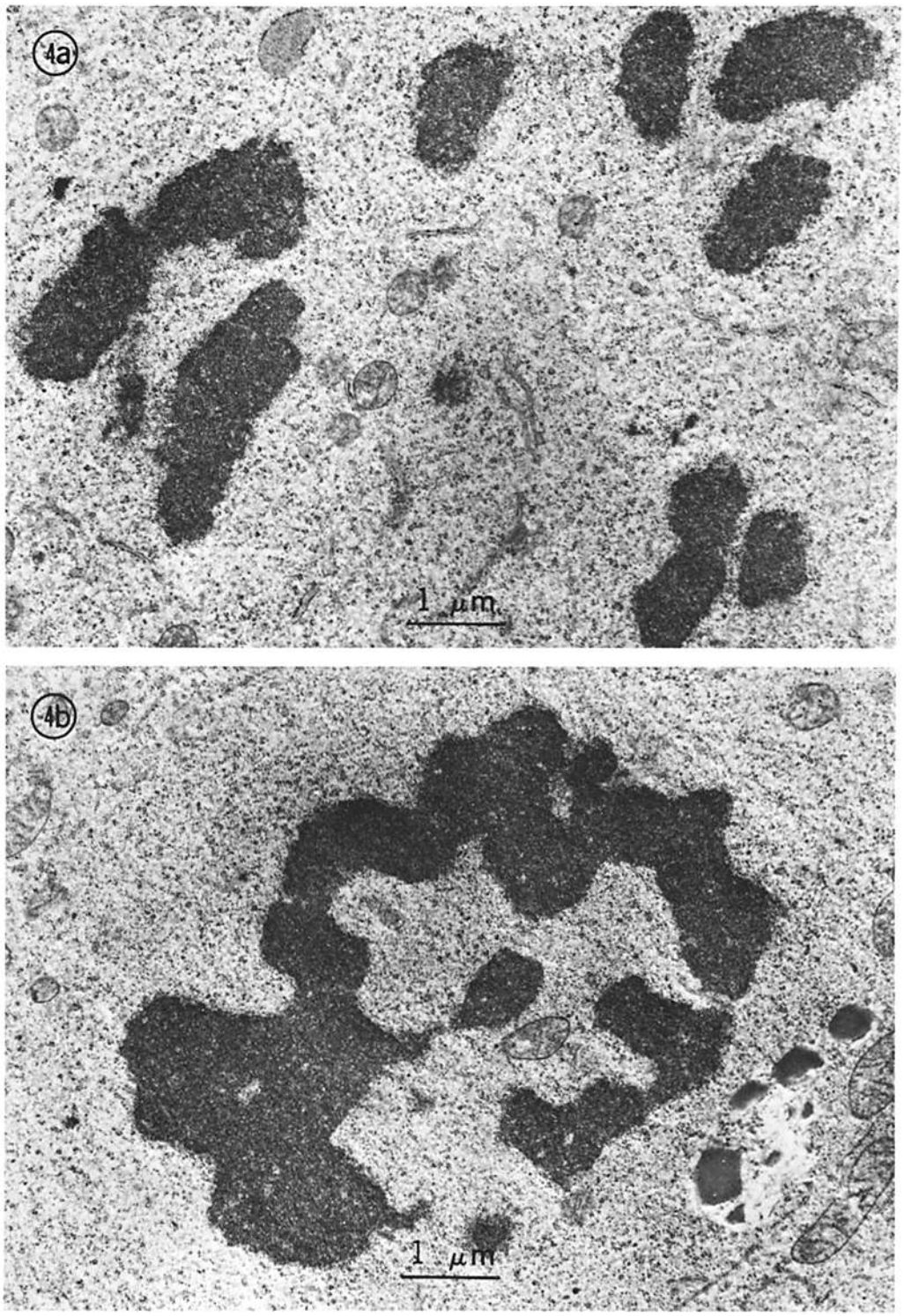

FIGURE 4 Electron micrographs of untreated metaphase cells and $ZnCl_2$-treated metaphase cells. (*a*) Without $ZnCl_2$, × 15,000. (*b*) With $ZnCl_2$, × 15,000. Metaphase and $ZnCl_2$-treated metaphase cells were prepared as in the legend of Fig. 1.

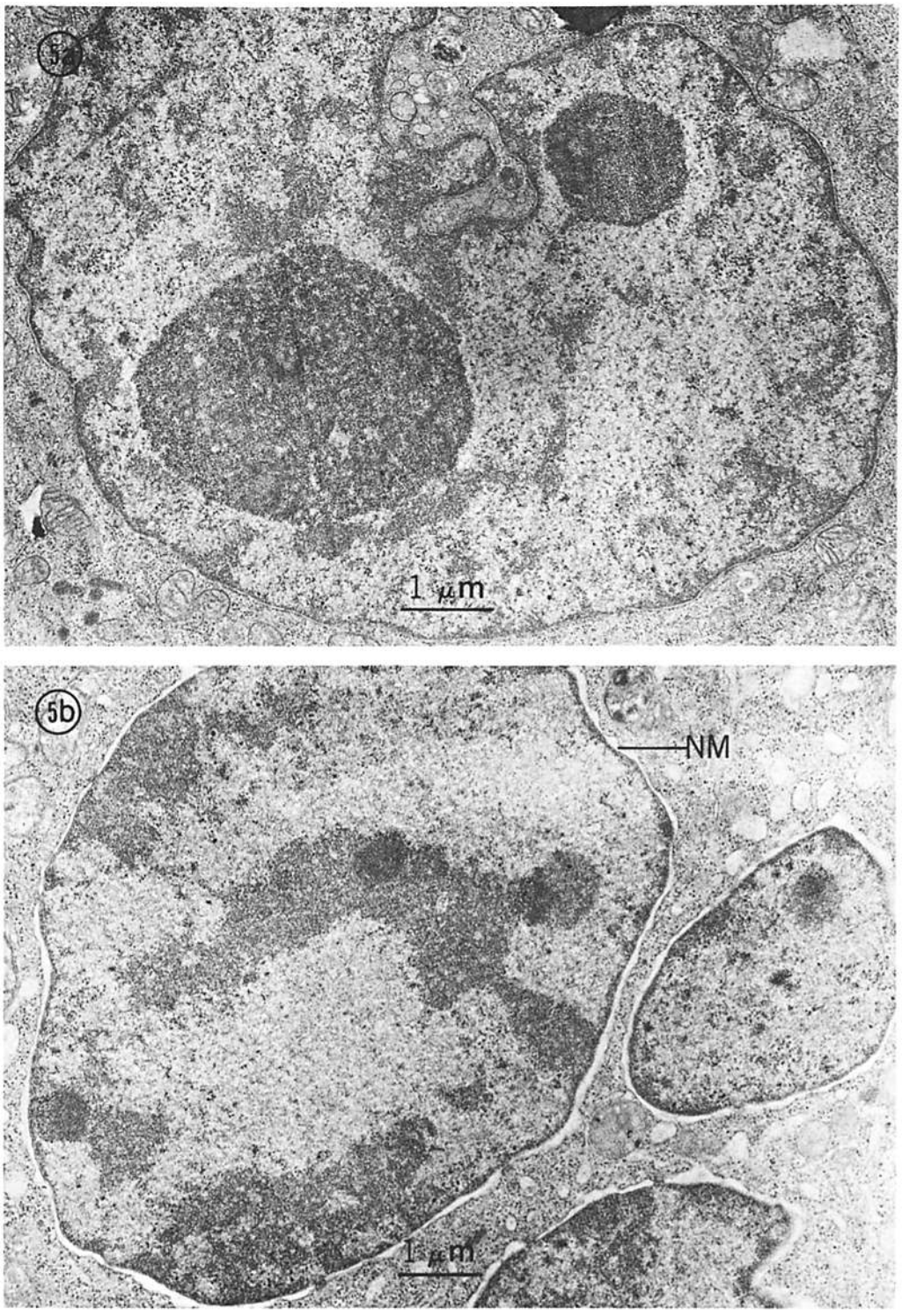

FIGURE 5 Electron micrographs of G_1 phase $ZnCl_2$-treated cells. (*a*) Normal-looking cells, × 14,000. (*b*) Cells with a swollen nuclear membrane (*NM*), × 13,000. G_1 phase $ZnCl_2$-treated cells were prepared as described in the legend of Fig. 2.

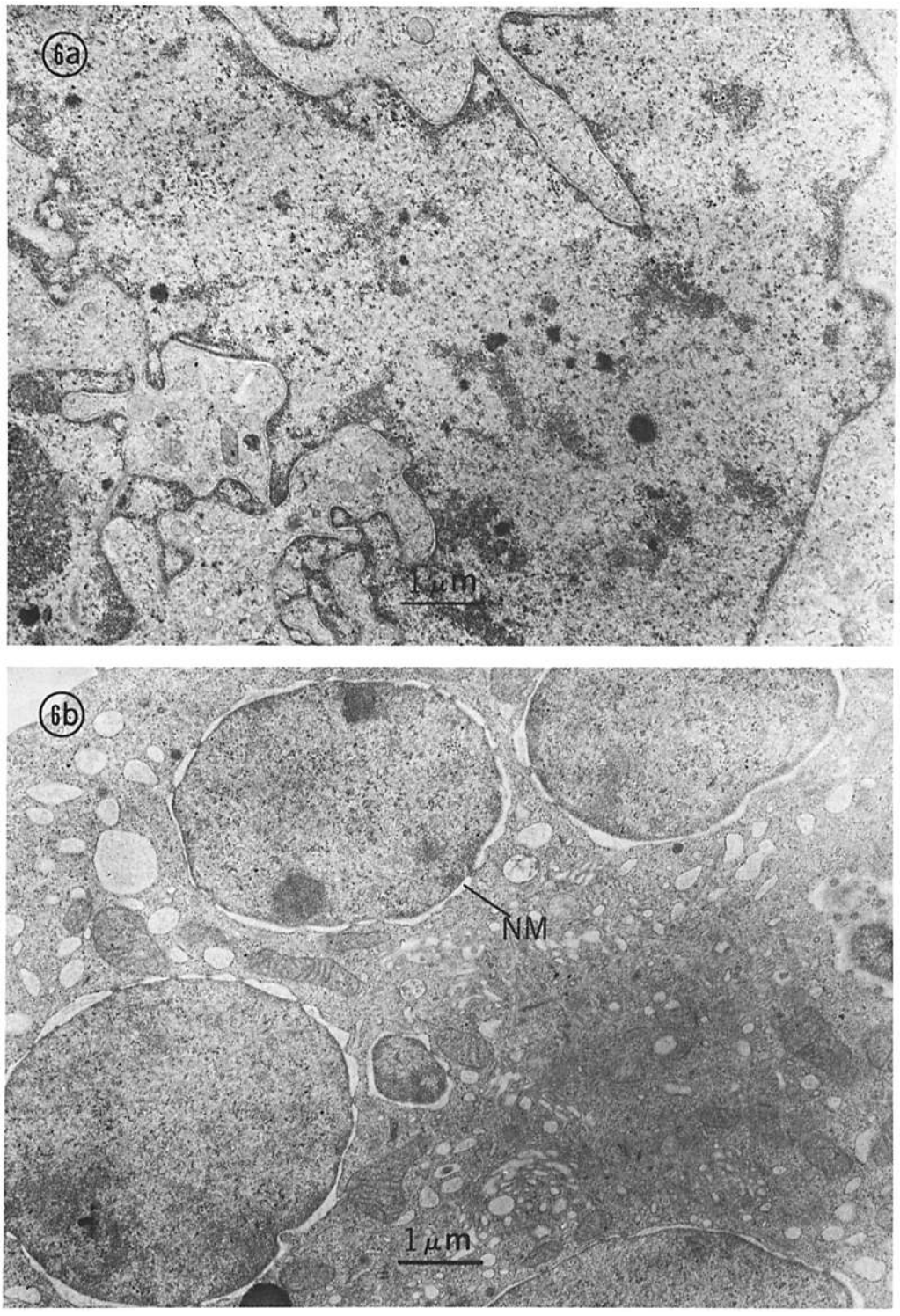

FIGURE 6 Electron micrographs of G_1 phase $ZnCl_2$-pretreated cells. (*a*) Normal looking cells, × 13,000. (*b*) Cells with a swollen nuclear membrane (*NM*), × 13,000. G_1 phase $ZnCl_2$-pretreated cells were prepared as described in Fig. 2.

TABLE I

Number of Surviving Cells after Release from Colcemid, as a Function of Time after Release

Hours after being released from $ZnCl_2$-Colcemid block	Cells resuspended in fresh medium ($\times 10^{-3}$)*	Cells resuspended in $ZnCl_2$ medium ($\times 10^{-3}$)*
0	376 ± 34	376 ± 34
1	365 ± 30	321 ± 18
2	338 ± 18	321 ± 16
24	332 ± 22	0

* Data are presented with standard deviation from three cell counts from each aliquot of cells.

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