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Effects of Divalent-Cation Chelators and Chloramphenicol on the Spatial Relationship of the Nuclear Envelope to Chromatin in Micronuclei of Chinese Hamster Cells

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In the presence of the spindle poison Colcemid in the culture medium to prevent anaphase, approximately 20% of Chinese hamster metaphase cells were converted to micronucleated cells during 7 h. In the micronuclei the chromosomes had become enclosed by a nuclear envelope (NE). In the light-microscope the micronuclei were of two kinds: with either visible chromatids or with decondensed chromosomes. In the electron microscope (EM) the spatial relationship of the NE to the chromatin was of two kinds only in the presence of Colcemid. In about 90% of the micronucleated cells the spatial relationship was normal, ie, the NE was immediately adjacent to the chromatin. In the remaining cells, the NE was distended so that the outer NE was separated from the inner one. In the presence of the divalent cation chelator, (ethylenedinitrilo) tetraacetic acid (EDTA) or the Ca^{2+}-chelator [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA), in addition to Colcemid, the amount of cells with micronuclei increased to 40%. The light-microscope appearance was the same as that found in the absence of the chelating agents. However, after Colcemid plus EGTA, EM revealed that only about 50% of the micronucleated cells had NE that was immediately adjacent to the chromatin and about 10% of them had distended outer NE. In the remaining 40% a third kind of spatial relationship was seen: the NE was intact but most of it was not adjacent to the chromatin. Furthermore, this type of micronucleus often contained mitochondria within the confines of NE. Thus, Ca^{2+} and possibly Mg^{2+} may regulate the rate of formation of the NE and also its ultrastructural relation to the chromatin. Mitochondrial function also appears to be involved in this relationship. In the presence of chloramphenicol (CAP), an inhibitor of mitochondrial protein synthesis, in addition to Colcemid, only about 50% of the micronucleated cells exhibited the normal relationship. The outer NE was separated from the inner NE in about 46% of the micronucleated cells and the third kind of NE-chromatin relationship was observed only in 2%. In the case of the third kind of relationship produced by CAP, inclusion of mitochondria within the micronuclei was not observed, in contrast to the finding with EGTA.

Key words: nuclear envelope-chromatin relationship, chromosomes, micronuclei, mitochondria, Colcemid, EDTA and EGTA, calcium magnesium.

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INTRODUCTION

Cellular proliferation can be influenced by environmental Ca^{2+} and Mg^{2+} [1, 2], and there is mounting evidence that the mitochondria are involved in Ca^{2+} homeostasis [3]. With regard to the cell cycle, the effects of Ca^{2+} on the formation of the mitotic spindle may be influenced by the Ca^{2+}-sequestration function of the mitochondria [4]. We have been examining the effects of divalent cations on another mitotic event, the NE formation. In mitotic Chinese hamster cells reformation of the NE begins in early telophase [5], and occurs immediately adjacent to the chromosomes [5–8]. In the presence of Colcemid, anaphase and telophase do not occur but metaphase cells accumulate [9]. With passage of time in the continued presence of Colcemid, even though anaphase does not occur, the metaphase chromosomes acquire NE and are transformed into micronuclei [10]. This phenomenon affords a means to study NE formation as a distinct event apart from the other mitotic events. Under the usual culture conditions the mitotic period lasts no more than one hour and it would be difficult to synchronize cells in anaphase and then study the formation of the NE in telophase reproducibly under controlled conditions. In a prelimary report [11] we have observed that the rate of micronuclei formation was accelerated in the presence of the divalent cation chelating agents, EDTA and EGTA, in agreement with earlier observations made with Ca^{2+}-depleted fused interphase-metaphase cells [15]. CAP, which specifically inhibits mitochondrial protein synthesis [12–14], retarded formation of micronuclei and also blocked the effects of the chelators [11]. The effects indicated that divalent cations and mitochondria may be involved in regulation of the NE.

In the light microscope the micronuclei which formed under one set of conditions were indistinguishable from those which formed under other conditions. In this report we present the results of EM examination of the micronuclei. Under all the experimental conditions, NE was evident in the micronuclei that were examined. However, the nature of the spatial relationship of the NE to the chromatin depended on the experimental condition under which the micronuclei were formed.

MATERIALS AND METHODS

Chemicals and Media

RPMI 1640 medium, heat-inactivated bovine fetal calf serum, and 0.25% trypsin were obtained from Grand Island Biological Co. (GIBCO), Grand Island, New York. CAP was obtained from Calbiochem, and used from a stock solution containing 10μg/ml in 30% ethanol. EDTA and EGTA (Eastman Organic) were dissolved separately in water to 0.1 M with a minimum quantity of NaOH.

Cells and Culture

The Chinese hamster cell line, Don, was used. The growth of monolayers at 37° in RPMI medium 1640 [16] supplemented with 10% fetal calf serum has been described [5]. Replicate log-phase monolayer cultures were prepared from a single seed culture, each flask receiving about 10^6 cells and 15 ml of medium.

Metaphase Cells

To obtain metaphase (M) cells the log-phase cultures were treated with Colcemid, 0.06 μg/ml, for 3 h at 37°. M-cells collected by shaking them loose (9) from the culture,

followed by centrifugation at 600 X g at room temperature for a few minutes. All the M-cell populations were at least 85% pure.

Experimental Procedures

The conditions under which M-cells were obtained varied. In a typical experiment, four T-60 flasks with log-phase monolayers were prepared. After 18 h at 37° Colcemid was added to the cultures. A few minutes after Colcemid addition, the first flask received EGTA, 0.1 mM, or EDTA, 0.1 mM; the second flask received CAP, 0.3 mM; the third flask received the combination of the same concentrations of EGTA plus CAP or EDTA plus CAP; and the fourth flask had only Colcemid. After 3 h at 37°, the M-cells from all four flasks were obtained as described above. Each batch of cells was washed once with fresh medium containing Colcemid and the corresponding supplement: EGTA, now 1.0 mM; or CAP, 0.3 mM; or 1.0 mM EGTA plus 0.3 mM CAP; or Colcemid alone, and recovered by centrifugation. Each batch of cells was resuspended in fresh prewarmed medium of the same composition as the respective wash. All suspensions were incubated further at 37°. After 4 h the cells were collected by centrifugation and processed for light and electron microscopy. It should be noted that in all procedures, the total time of exposure to Colcemid was 7h.

Light Microscopy

The cell pellets from each flask were resuspended in 15 or 20 mM sodium citrate for a few minutes at room temperature and then fixed by adding an equal amount of Carnoy's fixative (acetic acid/methanol, 1:3). After centrifugation, the supernates were discarded and the cells were exposed to fresh fixative once more, resuspended in 0.3 ml of fixative and spread on glass slides. The air-dried cells were stained with Giemsa and examined in the light microscope. The number of cells with micronuclei and single nuclei among a minimum of 300 total cells were recorded.

Electron Microscopy

The cell pellets, without resuspending in sodium citrate, were fixed at room temperature in either of two ways [5]: 4% glutaraldehyde which contained 0.2 M Millonig phosphate buffer at pH 7.3 was layered over them. Fifteen minutes later the supernatant fluid was removed and replaced by a layer of the Millonig phosphate buffer. Ten minutes later this was replaced by 0.2 M Millonig phosphate-buffered one percent osmium for 2 h. Alternatively, the cell pellets were directly fixed with one percent chromosmium for 2h. Dehydration and embedding procedures were the same as previously described [5]. The specimens were sectioned with glass knives in an LKB Ultramicrotome. The sections were stained with uranyl acetate followed by lead citrate [17] and examined with a JEM-7 at 80 KV.

RESULTS

Light Microscopic Observations

Light-microscopic observations in the present study revealed that the presence of Colcemid for 7 h in the culture medium produced micronuclei in about 20% of the cells, as we have described earlier [18]. Single nuclei were present in less than 10% of the cells. However, when either EDTA or EGTA was present in addition to Colcemid, micronuclei were observed in about 40% and single nuclei in about 10% of the cells in the current experiments.

In the presence of CAP, or CAP plus EGTA, in addition to Colcemid, fewer than 20% of the cells contained micronuclei. Similar results were obtained with the same concentration of EDTA. Regardless of kind of treatment, there were two kinds of micronuclei: those with visible chromatids or others with decondensed chromosomes. Examples are given in Figures 1 and 2. With the light microscope the micronuclei appeared to be of similar appearance regardless of the treatment used.

Even though not illustrated here, cells with a large nucleus also resembled that of an early telophase. The characteristics of the single nuclei also could not be distinguished with respect to the kind of treatment.

Electron Microscopic Observations

Whereas light microscopic observations were made when either EDTA or EGTA were used, EM observations were made with EGTA only.

The first group of cells with micronuclei that were examined by EM were obtained after 7 h exposure to Colcemid only (control). Two kinds of chromatin in the micronuclei were seen. One was electron-dense and resembled condensed chromatin of metaphase or very early telophase (Fig. 3). The other kind was decondensed chromatin with occasional nucleoli (Fig. 4). The structure had inner and outer membranes and was indistinguishable in this regard from the NE of the interphase nuclei of cells obtained from log-phase cultures which had not been exposed to Colcemid [5] additionally, nuclear pores were present as in the interphase NE (Figs. 3 and 4). These features were present whether the chromatin of the micronuclei was condensed or decondensed (Figs. 3 and 4). Thus, the micronuclei which were formed without the chelators in the absence of anaphase possessed typical NE.

Of primary interest was how the experimental conditions affected the spatial relationship of the NE to the chromatin. This relationship could be classified into three different types. The results are summarized in Table I.

In type I, the NE was localized immediately adajcent to the chromatin as illustrated in Figures 3 and 4. Occasionally, a cell with a single nucleus was observed (not illustrated); the association of the NE with the chromatin in the mononucleus was the same as for the NE of the micronuclei. As shown in Table I, with Colcemid alone almost 90% of the micronucleated cells exhibited type I NE-chromatin relation. The other treatments reduced the frequency of type I to about 50%. The results given in Table I are the combined total of four separate experiments.

Type II is illustrated by Figure 5. The NE was distended, ie, the outer membrane was separated from the inner one. The distention of the outer membrane occurred in all the treatments but the presence of CAP produced four times as many micronuclei with this type of NE chromatin relationship as was observed when only Colcemid was present (Table I). The chromatin of the micronuclei with distended NE was observed in either the condensed or decondensed state. When EGTA was present simultaneously with CAP the frequency of cells with the type II relationship was about half of that seen with CAP alone (26% vs 46%).

Examples of type III appear in Figure 6. The NE was intact but most of it was not adjacent to the chromatin. This type appeared most frequently in the presence of EGTA (Table I). With EGTA, about 40% of cells exhibited this type of relationship, but the simultaneous presence of CAP reduced the frequency to about half this value (Table I).

Among the micronuclei and single nuclei with type III NE-chromatin relationship were some which had mitochondria included within the confines of the NE, regardless of the state of the chromatin (Fig. 6). The statistics of this occurrence are presented in Table II, and are the combined total of four separate experiments.

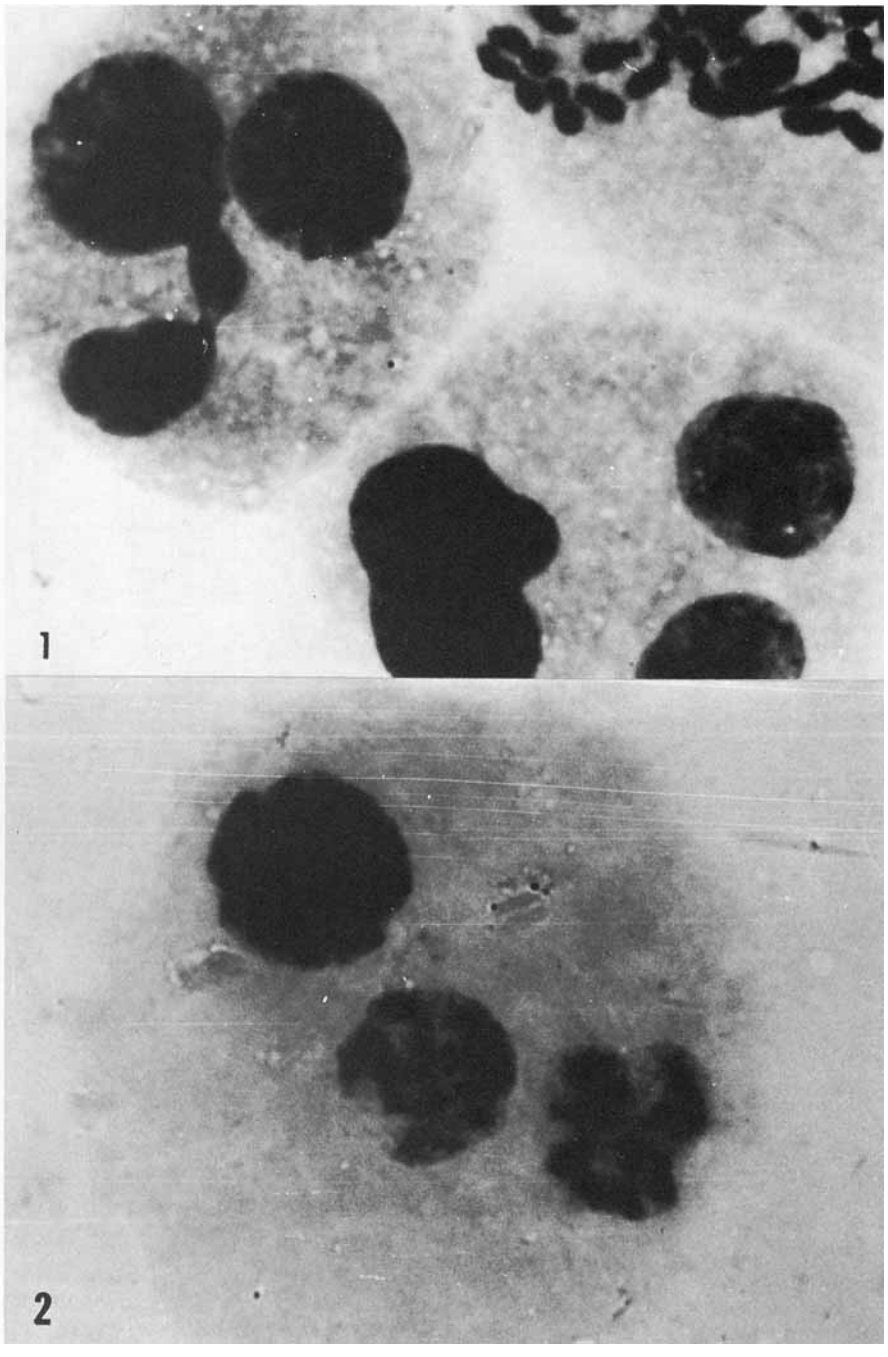

Fig. 1. Micronuclei formation in the presence of Colcemid for 7 h. A portion of a metaphase cell seen at upper right shows chromosomes without NE, while the other two cells show early and advanced micronuclei. × 3,300.

Fig. 2. Micronuclei formed in the presence of Colcemid plus EGTA for 7 h. A micronucleus with NE and an intermediate stage of chromosome decondensation is shown at the top. The other two micronuclei show an early stage of NE formation with recognizable chromatids. × 3,300.

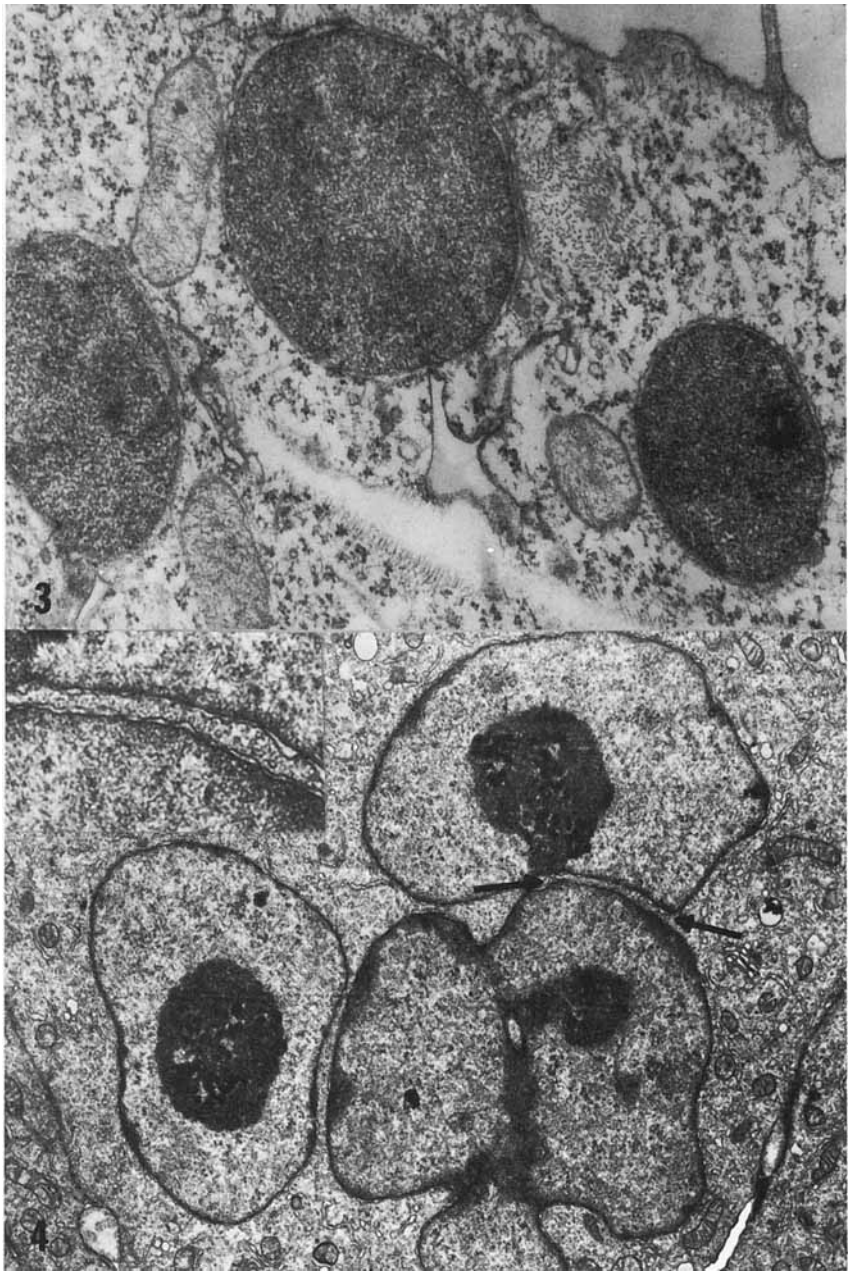

Fig. 3. An electron micrograph of micronuclei formed in the presence of Colcemid alone for 7 h and fixed with chromosmium. Condensed chromatin is immediately associated with NE (type I). Mitochondria are located in the immediate vicinity of the micronuclei. × 40,000.

Fig. 4. Micronuclei formed in the presence of Colcemid alone for 7 h and fixed with chromosmium. Decondensed chromatin shows characteristics of interphase nuclei with prominent nucleoli. NE appears intact and immediately adjacent to the chromatin (type I). Mitochondria are numerous and scattered throughout the cytoplasm. × 8,000. Insert is a higher magnification of an area indicated by arrows. NE-chromatin relationship is similar to that of an interphase nucleus. Nuclear pores are evident. × 24,000.

TABLE I. Occurrence of Various Types of NE-Chromatin Relationship*

Treatment	Cells with micronuclei	Micronucleated cells exhibiting types					
		I[a]		II[b]		III[c]	
	Total[e]	No.	%	No.	%	No.	%
Control[d]	68	59	(88)	9	(12)	0	(0)
EGTA	134	67	(50)	16	(12)	51	(38)
CAP	55	29	(53)	25	(46)	1	(2)
EGTA + CAP	62	33	(53)	16	(26)	13	(21)

*In this analysis, 400 cells were examined in each treatment.
[a]I, normal.
[b]II, outer NE separated from inner NE.
[c]III, Parts of the NE unadjacent to the chromatin.
[d]Colcemid only.
[e]The numbers are the combined total of four separate experiments.

In the case of cytoplasmic membraneous structures, CAP caused vesiculation and distention. Examples are given in Figures 5 and 7. Statistics of one experiment are given in Table III. EGTA treated cells were no different than those exposed to Colcemid only. However, EGTA blocked the effect of CAP. In two additional experiments, the average yield of vesiculated cells that occurred with EGTA was 11% and with CAP 72%. The average for the combined agents was 24%.

DISCUSSION

The NE of most of the micronuclei which formed in M-cells in the prolonged presence of Colcemid in RPMI 1640 medium closely resembled the NE of the normal nucleus, including the presence of nuclear pores. About 12% of the cells had micronuclei which exhibited separation of the outer from the inner NE (type II). When EGTA was present, in about 50% of the cells, the micronuclei appeared to be normal; however, about 40% of the cells had micronuclei where tracts of the NE were not adjacent to the chromatin (type III); the remainder showed the separation of the outer NE. Thus, whereas EGTA accelerated the formation of micronuclei, their occurrence with an abnormal NE-chromatin relationship was increased about fourfold. The results indicate that Ca^{2+} can regulate the rate of formation of the NE and also regulate its ultrastructural relationship to the chromatin.

In the current experiments the concentrations of Ca^{2+} and Mg^{2+} in the medium were 0.42 mM [16]. Addition of EGTA at 1.0 mM to the medium in which the isolated M-cells were incubated for 4 h is sufficient to bind all the ionic calcium, since the association constant of the Ca^{2+}-EGTA complex is 10^{11} ML [19] (for the Mg^{2+}-EGTA complex it is about 10^{5} ML [19]).

Washing of cells with 1.0 mM EGTA in the absence of Ca^{2+} has been reported to remove part of the cellular Ca^{2+} [20], presumably the surface Ca^{2+}. The effects of the chelators as used in this laboratory on the distribution of cellular Ca^{2+} and Mg^{2+} are under investigation.

When CAP was present in the medium there was a disturbance in the NE-chromatin relationship, in that the frequency of separation of the outer from the inner NE (Type II)

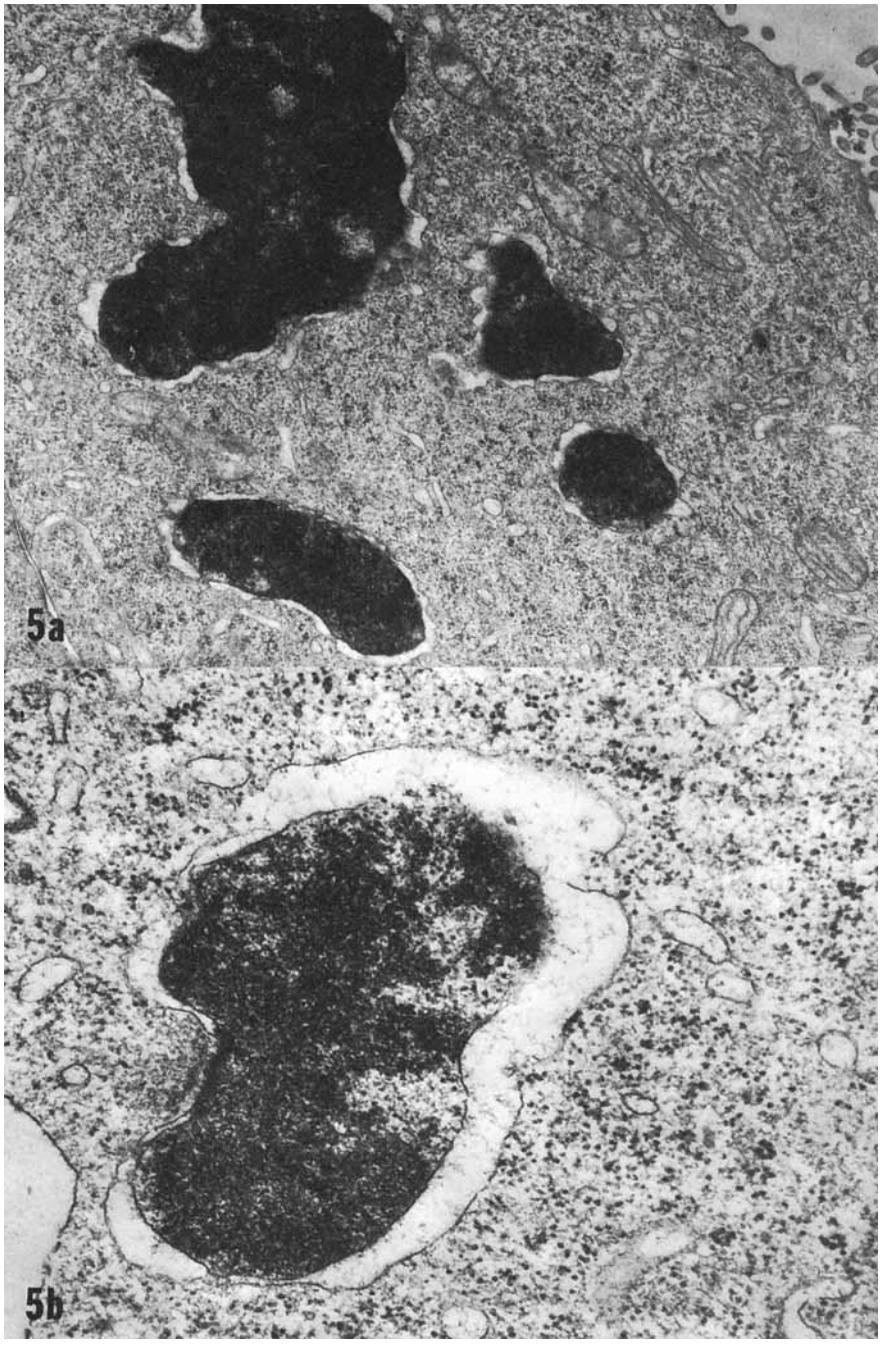

Fig. 5a. A micronucleated cell formed in the presence of Colcemid plus CAP for 7 h and fixed with glutaraldehyde-osmiums. The outer membrane of the NE is distended (type II). Small vesiculated membranous structures are scattered throughout the cytoplasm. × 12,000.

Fig. 5b. Another cell after the same treatment as in Figure 5a shows prominently distended outer NE. Membrane elements in the cytoplasm are vesiculated. × 42,000.

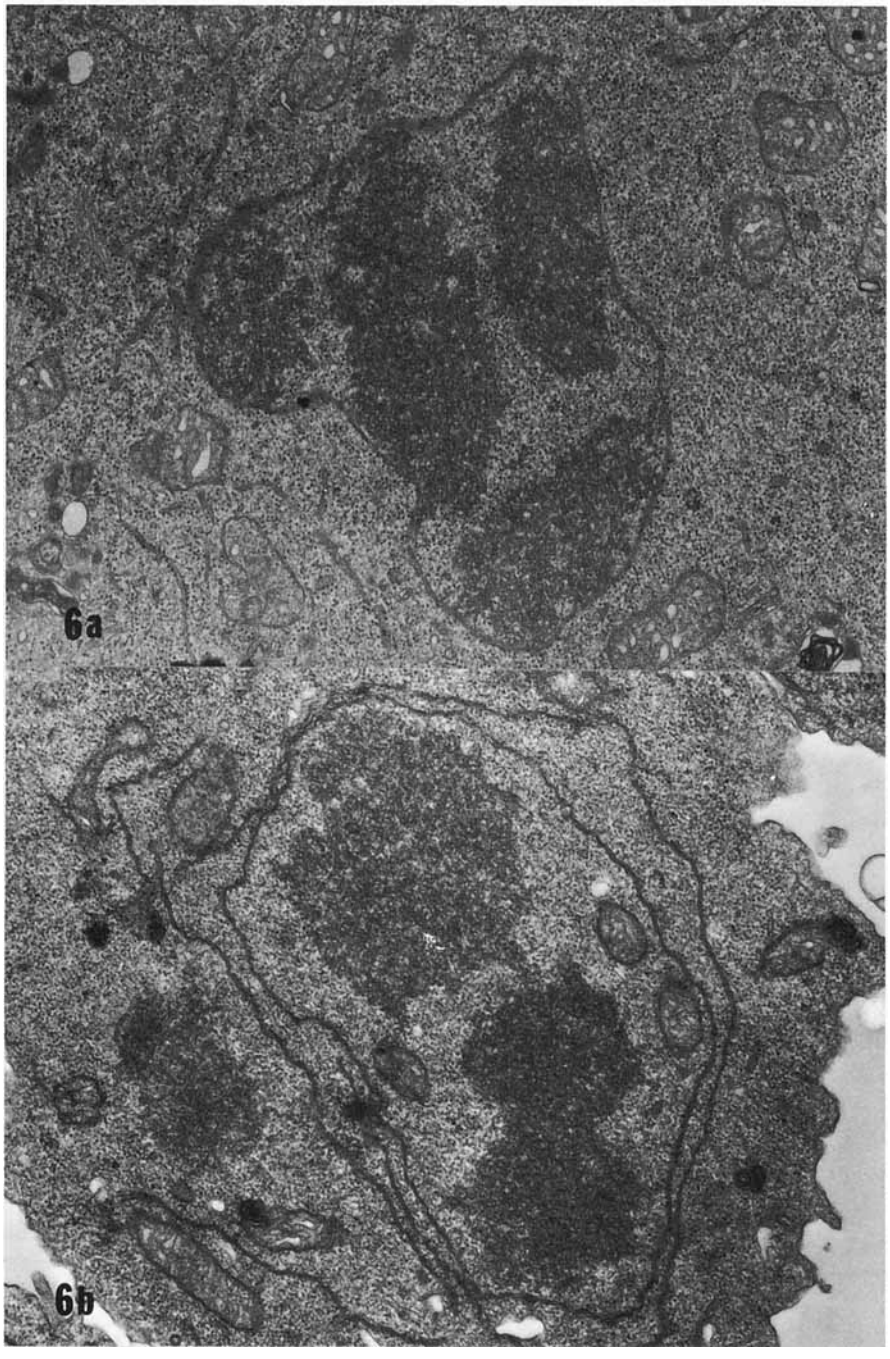

Fig. 6a. Cell treated with Colcemid plus EGTA for 7 h and fixed with chromosmium. Some portions of the chromosomes are closely associated with newly formed NE, but most are not (type III). Cristae of mitochondria appear to be in a condensed state. × 18,000.

Fig. 6b. Another cell under the same treatment as that in Figure 6a, showing a similar characteristic. However, in this cell mitochondria are enclosed within the confines of newly formed NE. Mitochondrial cristae also appear condensed. × 18,000.

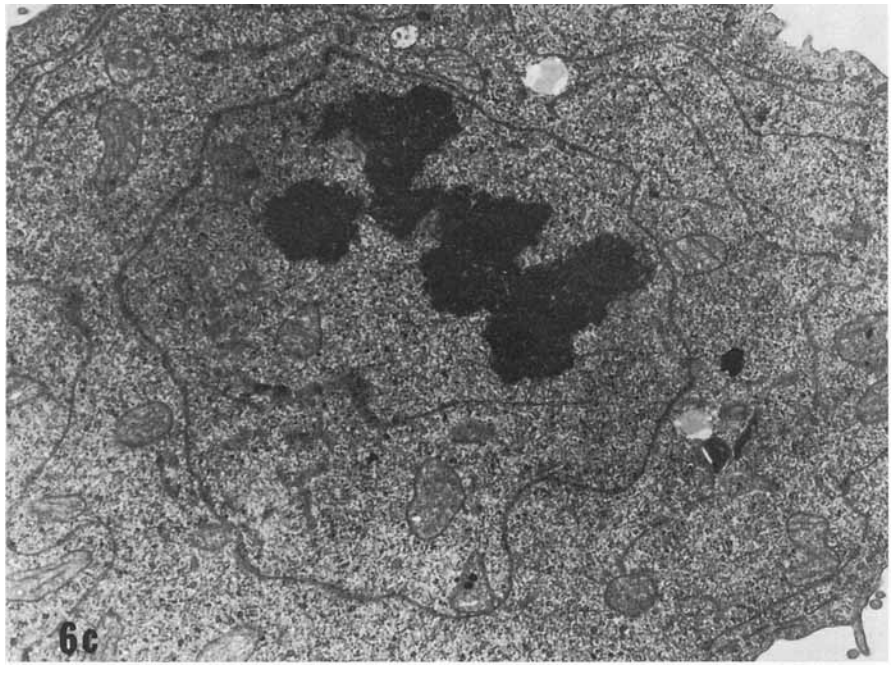

Fig. 6c. Another cell under the same treatment as that of Fig. 6a and fixed with glutaraldehyde-osmium showing similar characteristics as in Fig. 6b. This cell also contains mitochondria within the confines of the NE. Some of the mitochondria contained electron dense granules within their matrix. × 12,000.

increased approximately two- to fourfold. In only about 2% of the cells were micronuclei present where tracts of nuclear membrane were not adjacent to the chromatin (type III). Thus, EGTA and CAP had different effects: with the former the type III abnormality predominated and with the latter type II. Additionally, micronuclei with mitochondria were not observed when cells are exposed to CAP only.

When EGTA plus CAP was used the frequencies of the type II and III abnormalities were intermediate between those found when the agents were used separately, making it

TABLE II. Frequency of Cells Whose Type III Micronuclei and Single Nuclei Contained Mitochondria

Treatment	Number of cells[a]	With mitochondria inclusion		Without mitochondria	
		No.	%	No.	%
EGTA	51	38	(73)	13	(27)
CAP	1	0	(0)	1	(100)
EGTA + CAP	13	7	(52)	6	(48)

[a]Taken from Table I.

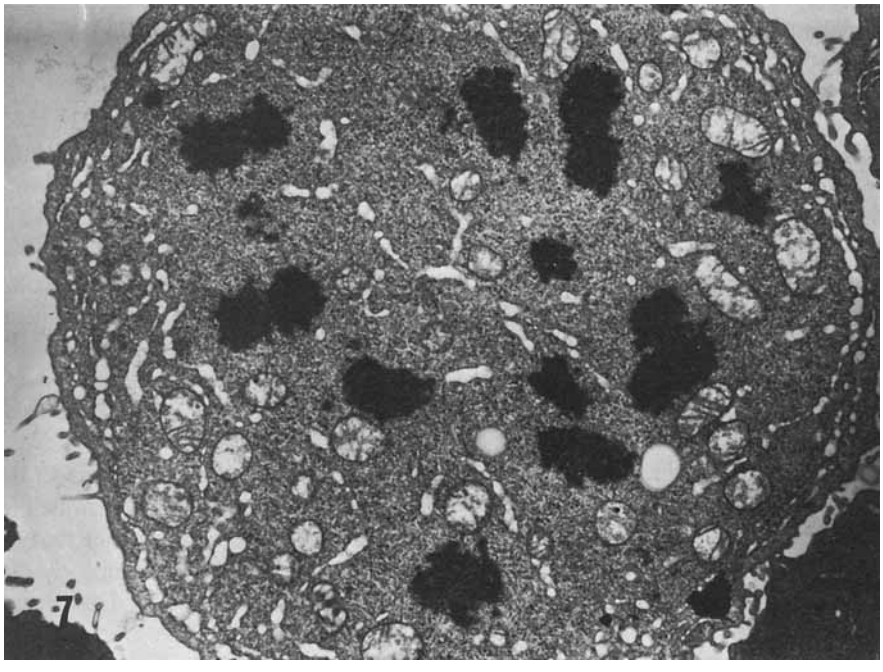

Fig. 7. A cell exposed to Colcemid plus CAP for 7 h and fixed with glutaraldehyde-osmium. Metaphase chromosomes are seen without any evidence of NE formation. The mitochondria contain fewer cristae and the matrices are pale. Even though membrane elements are abundant, they are bead-like and vesiculated. × 8,000.

difficult to assess whether EGTA blocked the effect of CAP or vice versa. Nevertheless, there is some evidence that EGTA blocked the effect of CAP. CAP by itself had an additional effect, namely, vesiculation of cytoplasmic membranous structures in 70% of the cells, a sixfold increase over the frequency seen in its absence. CAP did not produce this effect in the presence of EGTA, ie, the numbers of vesiculated cells were greatly reduced. Thus, there is a suggestion that both these effects of CAP, an inhibitor of mitochondrial protein synthesis, are dependent on a certain distribution of cellular calcium which can be altered

TABLE III. State of Cytoplasmic Membrane Structure in the Total Cell Populations*

Treatment	Normal	Vesiculated
	%	%
Control	88	12
EGTA	89	11
CAP	27	73
EGTA + CAP	77	23

*In this analysis 200 cells were examined in each treatment.

by EGTA. Possibly, CAP by itself caused release of mitochondrial Ca^{2+} into the cytoplasm raising the Ca^{2+} concentration such that micronuclear and cytoplasmic membranes were perturbed, whereas the simultaneous exposure to EGTA depressed the rise of Ca^{2+} and so prevented these membrane alterations. This hypothesis is currently being tested.

An unexpected finding was the inclusion of mitochondria in some of the micronuclei and single nuclei that resulted from the presence of the chelators in the medium. A search of the literature revealed three reports prior to 1977 which described the occurrence of mitochondria in nuclei of malignant and other abnormal cells. They were seen in a tumor which originated from an adrenal cortical carcinoma of the Syrian golden hamster [21] and in ascites leukemia cells L-1210 which occurred in solid clumps in the abdominal cavity of mice [22]. Mitochondria were also seen in the nuclei of myopathic extraocular muscle [23]. In the latest report, Smetana et al [24] described this phenomenon in cells obtained from the peripheral blood and skin lesions of human patients with Sézary syndrome. Whether there is a relationship between the current findings and the previous ones is difficult to assess. Certain lines of cells transformed by oncogenic viruses are known to have different requirements for Ca^{2+} for growth [25]. There was a recent report [20] indicating that the intracellular and surface distributions of Ca^{2+} differ between Balb/c3T3 mouse cells and the Simian virus 40-transformed counterpart. There is evidence that tumor mitochondria are different with respect to Ca^{2+} transport from normal cell mitochondria [3]. To our knowledge there have been no reports of mitochondria in nuclei of transformed cells. The characteristics of NE formation in transformed cells may differ from those of the untransformed counterpart. It will be of interest to compare such cells with respect to the quantitative relationship of divalent cation to NE formation and their relative sensitivities to antimitonchondrial agents in this regard.

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