

CONTRAST BETWEEN THE ENVIRONMENTAL pH DEPENDENCIES OF PROPHASING AND NUCLEAR MEMBRANE FORMATION IN INTERPHASE-METAPHASE CELLS

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ABSTRACT

In Chinese hamster Don cells, fusion of an interphase cell with a metaphase cell resulted either in prophasing of the interphase nucleus, including loss of the nuclear envelope (NE), or in the formation of a double membrane around the metaphase chromosomes. Only one of these phenomena occurred in a given interphase-metaphase (I-M) binucleate cell. At pH 7.4, there was about an equal probability that either event could occur amongst the population of I-M cells. The effect of pH changes in the medium containing the fused cells was examined. At pH 6.6, prophasing was the predominant event; at pH 8.0, membrane formation predominated. It was found that the rate of progression of a mononucleate cell from G₂ to metaphase was appreciably faster at pH 6.6 than at pH 8.0. Conversely, the progression from metaphase to G₁ was faster at pH 8.0 than at pH 6.6. These results with the mononucleate cells strengthen the hypothesis that structural changes in I-M cells are reflections of normal mitotic phenomena. Additional evidence for this hypothesis was produced by electron microscope examination after direct fixation in chrom-osmium. The double membrane around the chromosomes of the I-M cell was indistinguishable from the normal NE. The results obtained by varying the pH of the medium containing the fused cells provide an indication that disruption or formation of the NE of Don cells depends on the balance reached between disruptive and formative processes.

INTRODUCTION

Fusion of interphase cells with metaphase cells of a variety of species by UV-inactivated Sendai virus usually leads to a phenomenon called "premature chromosome condensation" by Johnson and Rao (9, 16) to which we have applied the term "prophasing" (12) and which includes the induction of prophase and loss of the nuclear envelope (NE)¹ in the interphase nucleus of the resulting

binucleate cells (13). Another event was observed in our laboratory (7) in experiments with the Chinese hamster cell line Don, when the metaphase cells were exposed to Colcemid for a relatively protracted period of time before fusion. When such cells were fused with interphase cells, the interphase nucleus remained intact in a substantial fraction of the binucleate cell population. In this fraction, within 30 min after fusion, the metaphase chromosomes became enclosed in a membrane, yielding a telophase-like nucleus designated TLN. In the electron microscope this

¹ *Abbreviations used in this paper:* HAU, hemagglutinating units; I-M, interphase-metaphase; NE, nuclear envelope; TLN, telophase-like nucleus.

membrane was almost indistinguishable from the normal NE of the interphase nucleus. An additional finding was made (7) when multinucleate-fused cells, as distinguished from the binucleate cells, were examined. Irrespective of the time of exposure to Colcemid, it was found that the larger was the ratio of interphase nuclei to chromosome sets in the fused Don cells, the greater was the probability that TLN would form; conversely, the larger the ratio of chromosome sets to interphase nuclei the greater was the probability that prophase would occur. On the basis of these statistics, we proposed the hypothesis that the state of the NE is the outcome of a balance between disruptive agents detectable in metaphase cells and formative agents present in interphase cells (7).

The general hypothesis constructed from all the observations on the fused interphase-metaphase (I-M) cells is that prophase and membrane formation in TLN are structural changes that are normal, in the sense that they are almost indistinguishable from the normal mitotic events in mononucleate cells. In the fused cells these changes become independent of the events that are ordinarily antecedent to them in the normal mononucleate cell cycle (9, 20).

We now report results that strengthen these hypotheses. Paul (15), Rubin (17, 18), and Caccarini and Eagle (3) observed that the growth rates of mammalian cells in culture changed markedly as the pH of the medium is varied between pH 6.2 and 8.3. In particular, Sissen and Kinoshita (21) noted a delay in the telophase plus G₁ times of mammalian cells when the pH was dropped from 7.8 to 7.1. The possibility arose that prophase and TLN formation, representative of the G₂ to mitosis and mitosis to G₁ progressions of the normal cell cycle, respectively, might respond quite differently to pH changes in the medium and also parallel the effects of pH change on these progressions in the mononucleate cells.

MATERIALS AND METHODS

Cells and Medium

The aneuploid Chinese hamster cell line Don with the modal chromosome number of 23 was used throughout the experiments. The line was grown in RPMI 1640 culture medium (14) supplemented with 10% fetal calf serum either in monolayer or in suspension cultures. This line was used

in the earlier experiments from this laboratory (7, 13).

Adjustment of the pH of the medium before growth or suspension of cells was made with 1.0 N HCl or 1.0 N NaOH, using a Corning model 12 pH meter fitted with a Corning semi-micro combination electrode (Corning Glass Works, Science Products Div., Corning, N. Y.).

Monolayer cultures growing at pH 7.4 in log phase were exposed to Colcemid at a concentration of 0.08 $\mu\text{g}/\text{ml}$ for varying periods of time up to 5 h to obtain a cell population which included a sufficient number of mitotic cells. The medium was discarded and the cells were exposed to trypsin (Associated Biomedic Systems, Inc., Buffalo, N. Y., 0.2%) without Colcemid for 5 min at 37°C with gentle shaking. The obtained suspension of freed cells was diluted with an equal volume of Colcemid-free RPMI 1640 medium, and the cells were harvested by low-speed centrifugation for 3 min. The cells were washed once with pH-adjusted, prewarmed fresh medium containing 0.08 $\mu\text{g}/\text{ml}$ of Colcemid and then suspended at a cell concentration of 10⁷/ml in additional fresh, pH-adjusted prewarmed medium.

The method for preparation of a metaphase single cell population was essentially that of Stubblefield and Klevecz (22) as applied in this laboratory (13). Log phase monolayer cultures were exposed to 0.08 $\mu\text{g}/\text{ml}$ of Colcemid for 5 h at 37°C. After the cultures were shaken gently to detach the metaphase cells from the culture bottle, the obtained metaphase cell suspension was passed once through folded gauze to remove clumped cells. The cells were collected by centrifugation at about 1,000 rpm for 5 min. In the present experiments metaphases accounted for over 85% of the cells in such a suspension. The details of treatment of these cells appear in the individual experimental protocols.

Cell Fusion

UV-inactivated Sendai virus, containing 20,000 hemagglutinating units (HAU/ml) in glucose-free Hanks' balanced saline as a stock virus solution, was used for cell fusion experiments. The strain of the virus, the methods for its inactivation, and the technique used for preparation of the virus stock were the same as those described in a previous paper (10). Procedures for cell fusion and for slide preparation were essentially the same as those described earlier (6, 7). Cell suspensions were supplemented with virus at a final concentration of 2,000 HAU/ml. The mixture was allowed to stand for 10 min at about 1°C. After gentle shaking of the cell-virus suspension at 37°C for 10 min, the sample was diluted sixfold in prewarmed medium of the same pH and the diluted sample was incubated at 37°C

for 20 min. Occasionally, the time of incubation was extended beyond 30 min. The cells were then collected by brief centrifugation at room temperature, treated with 15 mM sodium citrate in 0.5 ml suspension for 10 min at room temperature, and fixed by addition of the same volume of acetic acid:methanol, 1:3. After centrifugation the supernate was discarded and the cells were resuspended in 0.3–0.5 ml of the fixative and air-dried on slides without flaming. The air-dried cells were stained with Giemsa's.

The criteria for scoring of prophasing or membrane formation, i.e., TLN, were described earlier (7, 12). 100 I-M binucleate cells were examined at random at each pH after fusion or after any particular time of exposure to Colcemid, and the frequency of prophasing or TLN formation was recorded.

Electron Microscope Procedures

DOUBLE FIXATION: Glutaraldehyde fixation and postfixation with chrom-osmium were performed as described previously (7, 12), before embedding, sectioning, and staining with uranyl acetate and lead citrate.

DIRECT FIXATION WITH CHROM-OSMIUM: Samples of cells were pelleted by centrifugation and the culture fluid was decanted. 1 ml of 1% chrom-osmium (4) was layered over the pellet for 2 h in the cold or at room temperature. The cell pellet was washed with 2% uranyl acetate in 0.54% sucrose and allowed to stand in this solution for at least 2 h. The specimens were processed in graded ethyl alcohol 50, 70, 95, and 100%, respectively, further dehydrated with propylene oxide, and infiltrated with Epon-Araldite mixture. Embedding was made with the same mixture of Epon-Araldite. Polymerization was carried out at 60°C for 18 h and then at 80°C for 30 h. Thin sections were cut with a diamond knife, using an LKB ultramicrotome. The sections were mounted on copper grids and first stained with 2% aqueous uranyl acetate for 10 min, followed by lead citrate for 10 min. The specimens were examined with Hitachi 11A and JEM 7 electron microscopes and photographs taken at accelerating voltages of 75 and 80 kV, respectively, and instrumental magnification of 3,000–20,000 diameters.

RESULTS

The Effect of pH on Prophasing and TLN Formation

In Figs. 1 and 2 appear typical examples of prophasing and TLN, respectively. These are the I-M binucleate cell types that were scored 30 min after fusion was complete. At pH 7.2, the results previously reported (7) were readily

repeated (Fig. 3); as the time of exposure to Colcemid increased, the frequency of TLN formation increased while that of prophasing decreased. In the present study, however, it was demonstrated that if the pH was 6.6 during 30 min of fusion, 70–80% of the I-M cells exhibited prophasing; if the pH was 8.0, about 80% of the cells exhibited TLN. These results are shown in Fig. 4. The yields were independent of the time of exposure to Colcemid.

The results obtained during fusion with a given log phase culture after 5 h of exposure to Colcemid as a function of pH during and after fusion appear in Fig. 5 and substantiate the results obtained at pH 8.0, individually. Similar results were obtained if metaphase cells, obtained separately (22) from a culture arrested for 5 h with Colcemid, were fused with interphase cells from a separate culture. For example, in one experiment where fusion occurred at pH 6.6, prophasing in I-M cells was 65% while TLN formation was 28%; at pH 8.0 the percentages were 7 and 86%, respectively. Thus, relatively high pH favors TLN formation regardless of the time of exposure to Colcemid.

Electron Microscope Studies of TLN

Two methods of fixation for visualizing nuclear membranes are being used in our laboratory. The first involves fixation in glutaraldehyde followed by osmium tetroxide, i.e., double fixation; the second employs direct fixation in chrom-osmium (see Materials and Methods). The original electron microscope studies of the TLN employed double fixation (7). The present study is an extension in which direct fixation has been used.

Fig. 6 typifies the appearance of telophase (2, 5) of mononucleate Don cells before cytokinesis as seen after direct fixation. Fig. 7 *a* and *b* give examples of the TLN viewed after double and direct fixation, respectively. Fig. 8 is an enlargement of a section of Fig. 7 *b*.

It is difficult to distinguish the envelope of the TLN from the NE of mononucleate telophase or from the NE of the interphase nucleus of the binucleate cell in which the TLN resides. At the magnifications used, there seems to be no difference between the normal NE at pH 7.2 and the envelope of the interphase nucleus of TLN at pH 8.0. These observations reinforce the earlier conclusion (7) that the double membrane of the TLN is probably normal NE.

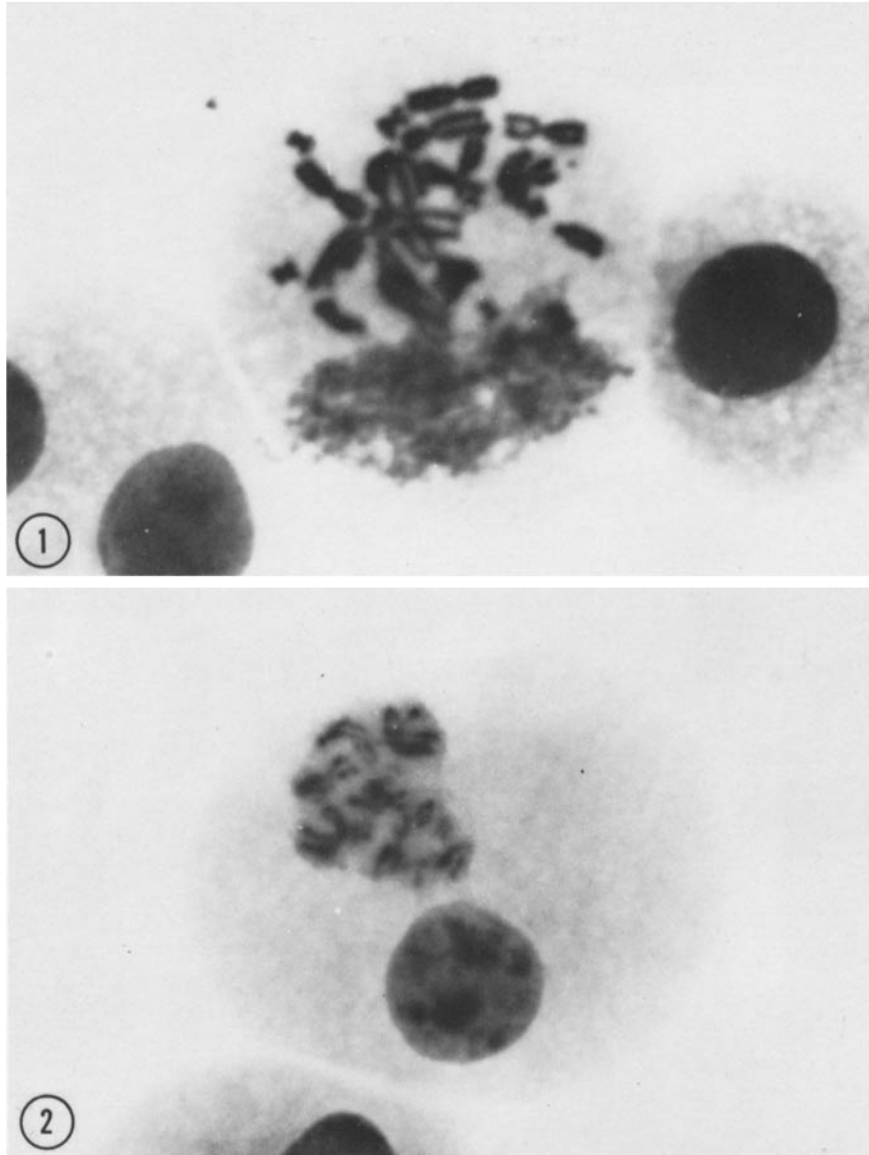


FIGURE 1 Prophasing in an I-M cell. 5 h after exposure of a log phase culture (pH 7.4) to Colcemid at 37°C, the monolayer was washed with fresh medium, treated with trypsin (Colcemid absent), and the cells were fused at pH 6.6 (Colcemid present). Fixing and staining was applied after the 30 min incubation period at 37°C (see Materials and Methods).

FIGURE 2 TLN formation in an I-M cell. The procedure was similar to that for Fig. 1 except that fusion and subsequent incubation occurred at pH 8.0.

Nevertheless, there are at least two differences between the TLN and the early new nucleus of telophase: the shape of the TLN is much more regular, and dense intrachromatin inclusions that are seen in telophase are absent from TLN (compare Fig. 6 with Fig. 7 *b*).

Effects of pH of the Medium on the Rate of Progression from Metaphase to G₁ and from G₂ to Metaphase

The effect of pH of the medium on the rate of progression of mononucleate cells from metaphase

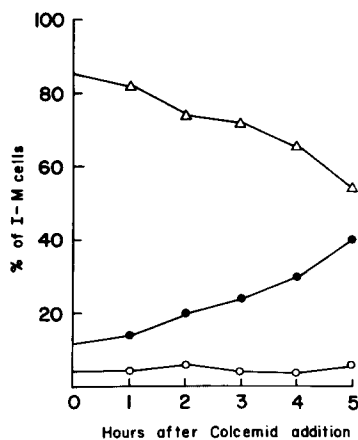


FIGURE 3 Effect of the period of exposure to Colcemid on prophasing and TLN formation at pH 7.2. Approximately 15 h after seeding T-60 flasks with 10^5 cells, individual log phase cultures were supplemented with $0.08 \mu\text{g}/\text{ml}$ of Colcemid. After the indicated times of incubation at 37°C , the medium was removed, and the cells recovered by trypsinization in the absence of Colcemid. Further treatment appears in Materials and Methods. Fusion occurred at pH 7.2 with Colcemid present and the observation was made 30 min after incubation at 37°C . Δ — Δ , prophasing; \bullet — \bullet , TLN; \circ — \circ , I-M cells showing neither prophasing nor TLN.

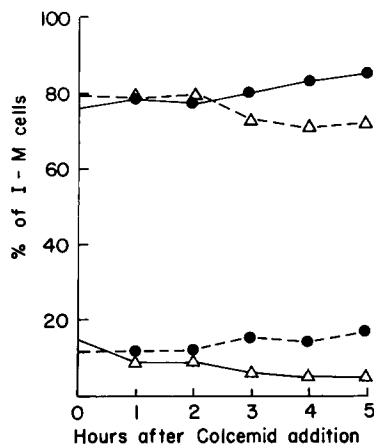


FIGURE 4 Prophasing and TLN formation during fusion at relatively low or relatively high pH with and without previous exposure to Colcemid. Trypsinization of log phase cells before fusion was the same as for the experiment described in Fig. 3. Fusion with Colcemid present in all cases occurred at pH 6.6, dashed lines, or pH 8.0, solid lines. Open triangles, prophasing; closed circles, TLN formation.

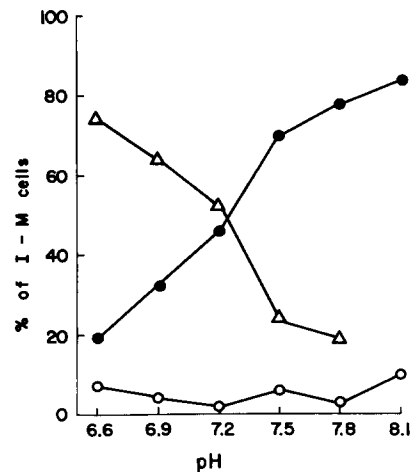


FIGURE 5 Prophasing and TLN formation as a function of the pH during fusion after a constant time of exposure, 5 h, of a log phase culture to Colcemid. Δ — Δ , prophasing; \bullet — \bullet , TLN formation; \circ — \circ , I-M cells from which prophasing and TLN formation were both absent.

to G_1 was examined because of the following reasoning. Let it be assumed that formation of a NE around the metaphase chromatin of the TLN is a normal process, but that it is out of its proper order in mitosis, since it occurs without anaphase. Because the frequency of occurrence of this normal process is strongly pH dependent, it might be expected that the transition of normal mononucleate Don cells from metaphase to G_1 would also show the same kind of dependence, since NE formation is a major event in that transition.

Accordingly, metaphase cells were shaken from arrested monolayers after 5 h and washed to remove Colcemid. They were transferred to media preadjusted to pH 6.6, 7.3, or 8.0 at 37°C , and the fall in the mitotic index as a function of time was recorded. The results are shown in Fig. 9. When the pH was 6.6, only about one-third of the cells left metaphase in 60 min; when the pH was 8.0, at least 90% of the cells left metaphase. The rate at pH 8.0 was considerably faster than at pH 7.3. In separate confirmatory experiments at pH 8.0, the cell concentration increased to about 90% of the expected doubling after 60 min as determined by hemocytometer counting (Table I). In separate experiments, the effect of the number of washings to remove Colcemid before incubation at pH 6.6 or 8.0 was examined. Identical results were obtained, regardless of whether the cells were washed twice or five times. It is likely that

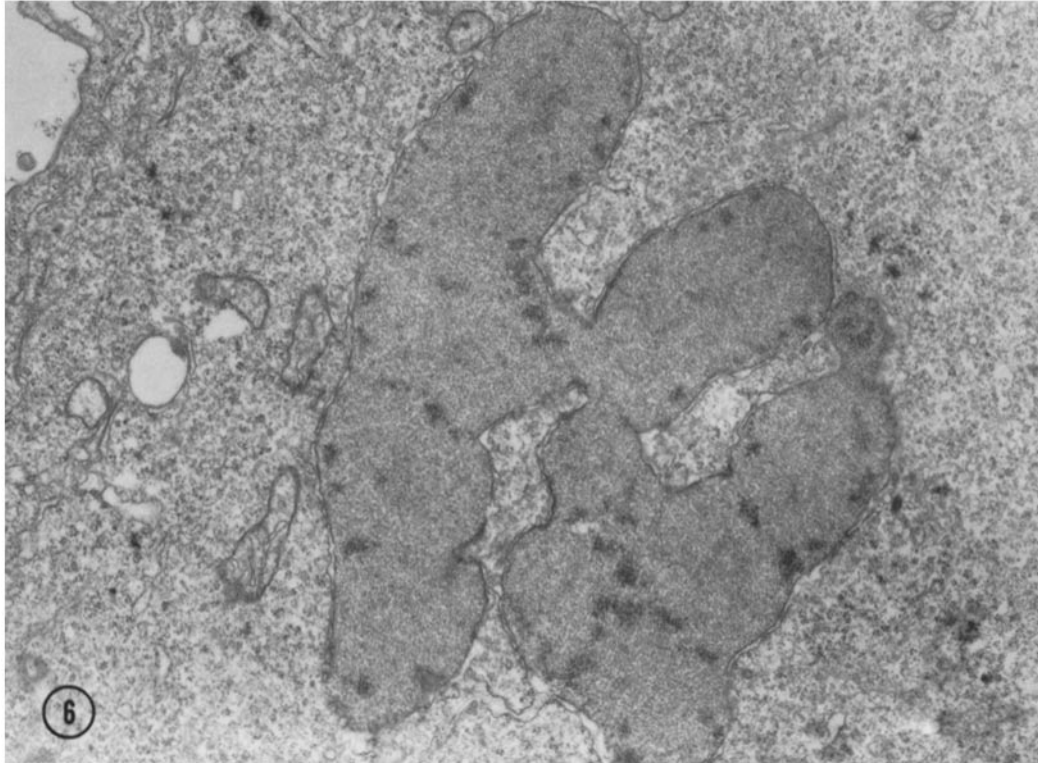


FIGURE 6 Appearance of chromosomes with newly formed NE in telophase. Metaphase cells were obtained after exposing a log phase monolayer to Colcemid for 2 h. They were washed three times in Colcemid-free medium and incubated in fresh medium, pH 7.2, for 30 min. Cells were subjected to direct fixation for electron microscopy as described in Materials and Methods. $\times 15,000$.

the pH effects are not due to differences in washing out of Colcemid during the subsequent incubations. Thus, high pH favors the metaphase to G_1 progression in which NE formation is a prominent process.

Similarly, the effect of pH on the progression of mononucleate Don cells from G_2 to metaphase was examined. The G_2 period of the Don line is 2.2 h at pH 7.4 and 37°C (11). Log phase cells were exposed to Colcemid at pH 6.6 or 8.0, and the mitotic index was measured from time to time during 3 h of incubation at 37°C . The results are shown in Fig. 10. The rate of the G_2 to metaphase progression at pH 6.6 was about twice the rate at pH 8.0. Thus, the pH that favors prophase, i.e., induction of a mitotic event in fused Don cells, also favors the mononucleate G_2 to metaphase progression.

DISCUSSION

Prophasing appears to reflect a normal mitotic event on structural (8, 9, 13, 19) and biochemical

(1, 11, 13) grounds. Structural similarity is the basis for considering the membranes of the TLN to be normal NE; this was the earlier conclusion (7) and it is supported by the present results in which direct fixation for electron microscope observation was used.

Additional evidence that both prophase and membrane formation in the I-M pair are representative of normal mitotic events is provided by the effects of pH adjustment during fusion and shortly thereafter. Using the limits of pH 6.6 and 8.0, prophase was predominant at low pH. At pH 6.6 the rate of the G_2 to metaphase transition in mononucleate cells was appreciably faster than at pH 8.0. At alkaline pH, membrane formation was appreciably faster than at pH 6.6. That is to say, the pH that favors the part of the normal cell cycle which includes prophase also favors prophase in I-M cells; the pH that favors the part of the cycle which includes telophase and normal NE formation also favors membrane formation in I-M cells.

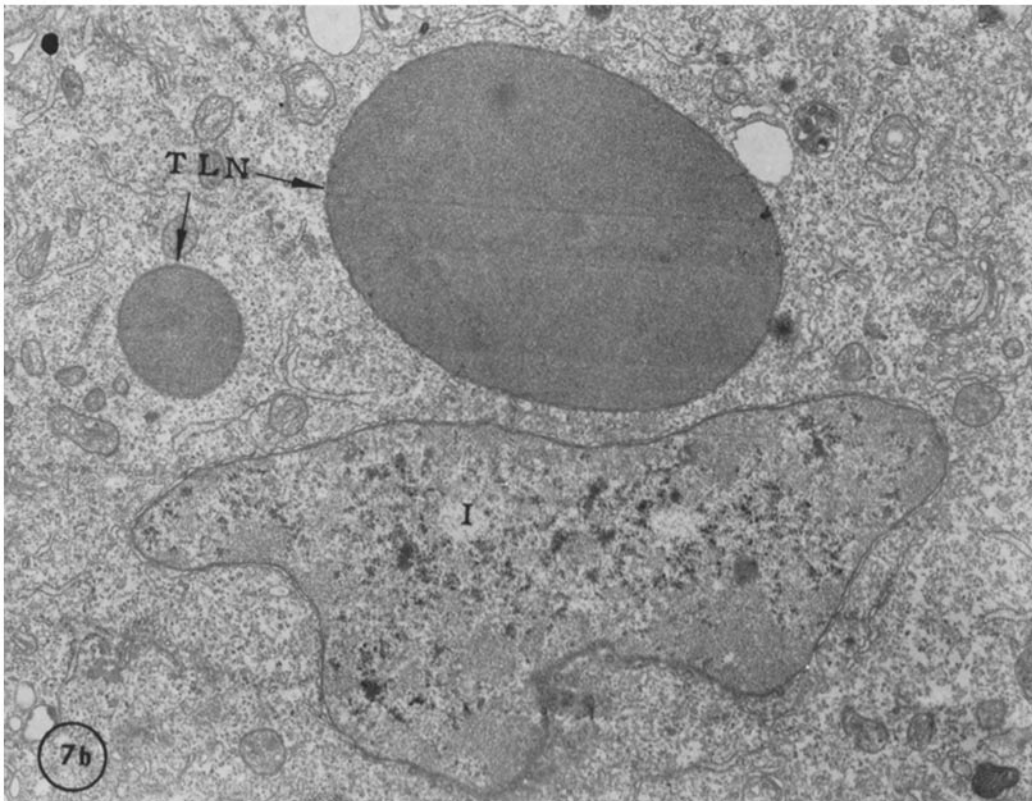
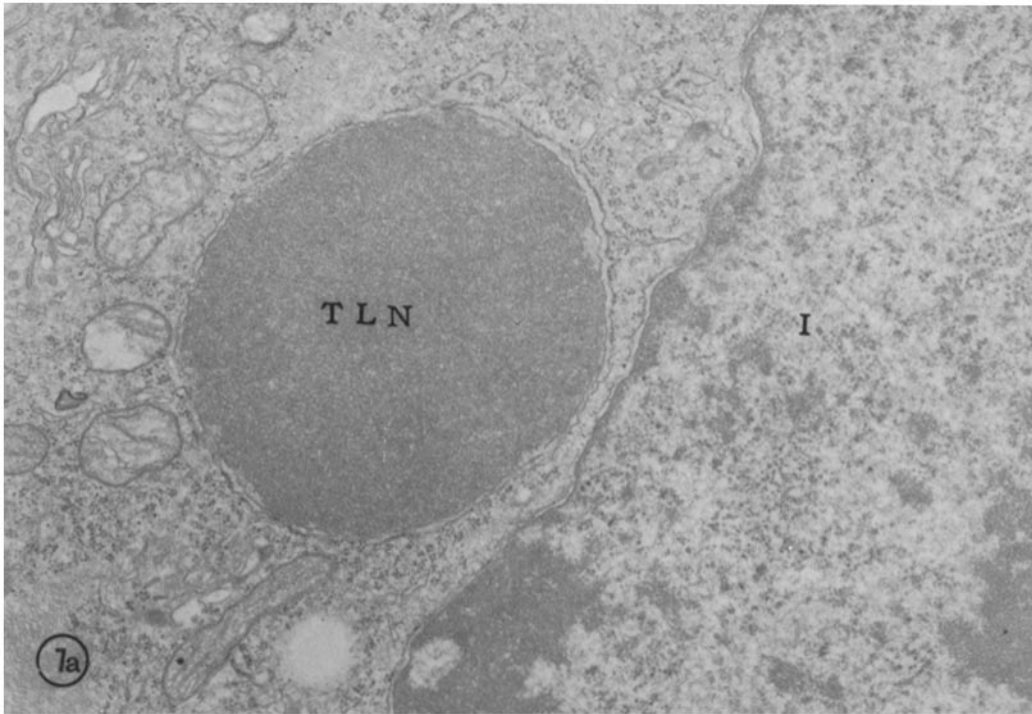


FIGURE 7 Appearance of TLN formed at pH 8.0 (a) after double fixation (b) after direct fixation. I, interphase nucleus. (a), $\times 18,000$; (b), $\times 9,500$.

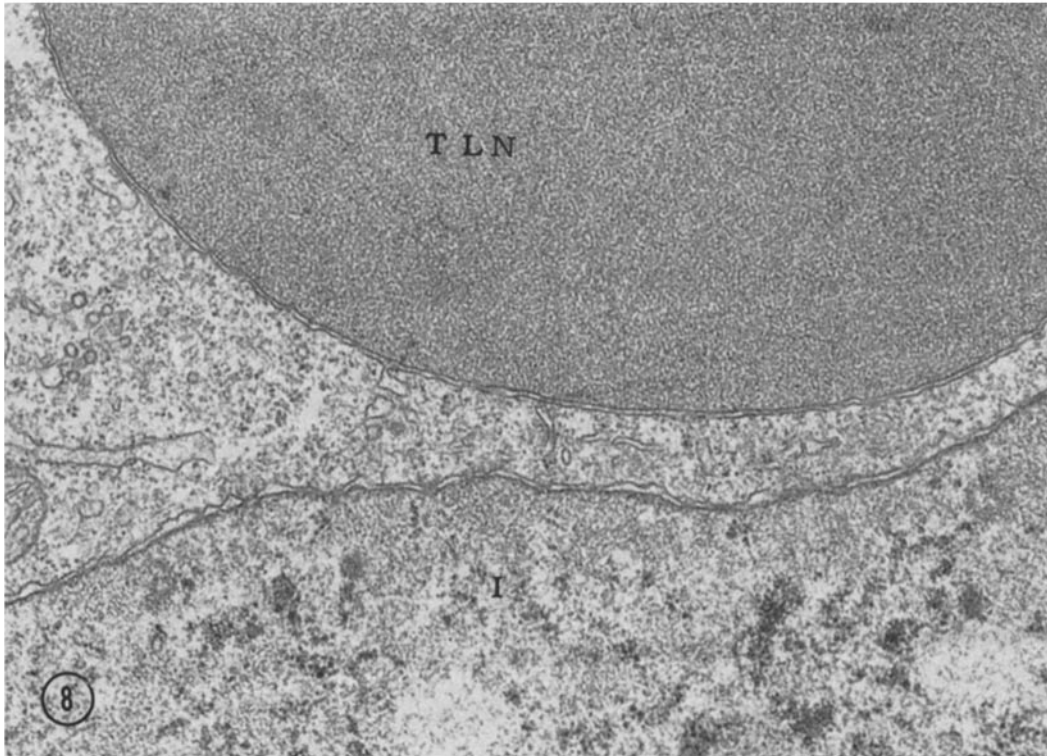


FIGURE 8 Enlargement of a region of Fig. 7 *b* showing portions of the interphase nucleus (*I*) and TLN. $\times 23,500$.

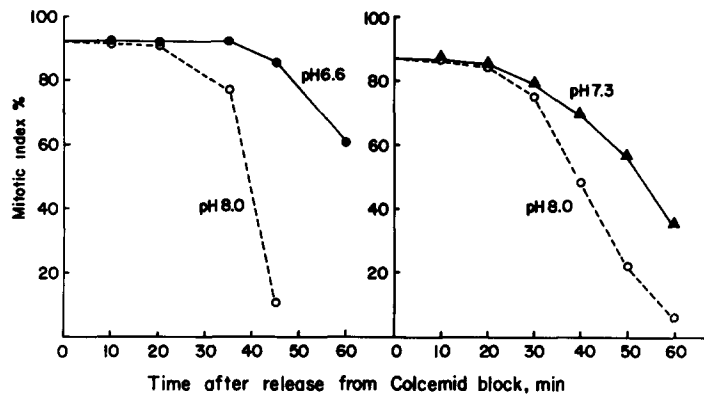


FIGURE 9 The effect of pH on the rate of progression from metaphase to G_1 . 5 h after exposure of log phase cultures to Colcemid, metaphase cells were obtained as described in Materials and Methods. After centrifugation, the cells were washed three times with cold Colcemid-free RPMI 1640 medium adjusted to pH 6.6, 7.3, or 8.0. The cells were then suspended in fresh prewarmed medium at the desired pH. From time to time during incubation at 37°C , aliquots were removed, and the cells were centrifuged, fixed, and stained with Giemsa's. Counts were made on at least 300 cells. The data of the left and right sections are from separate experiments and are representative of three separate experiments each.

It was proposed in the original study of the TLN (7) that whether formation of the NE of the Don cell takes place depends on the balance between disruptive and formative agents, one set being present in the metaphase cell, the other being

TABLE I
Metaphase to G₁ Progression after Release from Colcemid and Increase in Cell Number at pH 8.0

Experiment no.	Initial values		Values after 60 min	
	Mitotic index	Cells/ml	Mitotic index	Cells/ml
	%	($\times 10^{-6}$)	%	$\times 10^{-5}$
1	88.3	4.0	3.3	7.0
2	90.7	2.0	5.0	3.5
3	85.3	1.5	4.0	2.7

After log phase monolayer cultures were treated with Colcemid (0.08 $\mu\text{g}/\text{ml}$) at 37°C for 5 h, metaphase cells were collected by gentle shaking of the culture flasks. They were washed with cold fresh medium twice at pH 7.3 followed by one wash at pH 8.0. The cells were resuspended in fresh prewarmed medium at pH 8.0 and immediately sampled for mitotic index and cell density determinations. After 60 min of incubation at 37°C with gentle intermittent shaking, the mitotic indices and cell densities were again determined.

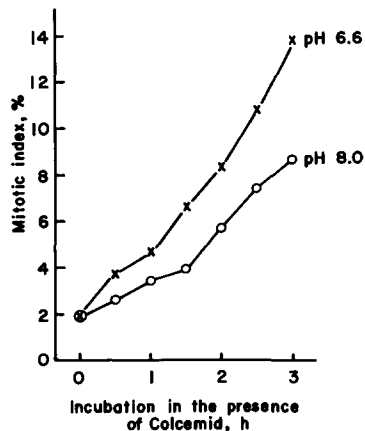


FIGURE 10 Rate of progression from G₂ to metaphase at pH 6.6 and at 8.0. Log phase monolayers were trypsinized for 3 min at 37°C, and the cells were centrifuged, washed in cold fresh medium, pH 7.2, centrifuged, and resuspended in prewarmed medium at pH 6.6 or 8.0 containing 0.08 μg of Colcemid/ml. From time to time during incubation at 37°C, aliquots of cells were recovered, fixed, stained, and counted as in Fig. 9.

present in the interphase cell. This concept receives support from the pH studies. About midway between the extremes of pH 6.6 and 8.1, characterized by the predominance of prophase and of TLN formation, respectively, there is about an equal probability that among the population of I-M cells, either prophase or TLN formation will occur in a given binucleate cell. The chance that both events will take place in the same I-M pair is nearly zero as evidenced by the present and previous (7) observations. A shift toward the acid or alkaline extreme favors only one of the mutually exclusive events. This points to the existence of at least one rate-limiting process having a relatively low pH optimum that is essential for prophase in which the NE disappears; and to at least one having a relatively high pH optimum that is essential for TLN formation. Since the pH extremes also characterize the more rapid rates of the G₂ to metaphase and metaphase to G₁ progressions, respectively, the fate of the NE in the mononucleate cell would depend on the competition between the two sets of reactions of which each rate-limiting process is a part.

The structural events seen in TLN formation are very much fewer than those seen in prophase. In the former instance, there seems to be a single predominant event, namely formation of an NE around metaphase chromatin. We propose that the fused I-M Don cell observed at pH 8.0 provides a tool for studying the parameters that regulate biosynthesis and organization of the NE as a separate and distinct mitotic event isolated temporally from much of its antecedent events in the metaphase to G₁ progression.

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