INTRODUCTION

Shifts in intracellular ion distributions have often been causally implicated in mitosis although the available data are not compelling. Heilbrunn postulated a release of Ca++ from the cell cortex to the endoplasm as the stimulus for spindle formation, but gave little direct evidence to support this view (1). Whitfield and coworkers have reported that the slight increase in total osmolarity effected by raising medium NaCl from 120 to 150 mM reduces postirradiation mitotic delay (2, 3). Increasing extracellular Ca++ from 0.12 mM to 2 mM caused a more rapid entry of thymocytes into mitosis (4), while increasing extracellular Ca++ to 60 mM leads to visible chromosome aggregation at the light microscope level (5). High NaCl also appears to increase the rate at which rat bone marrow cells enter mitosis, and although this effect is not observed in thymocytes (6), thymus nuclei show marked inhibition of protein, RNA, and DNA synthesis at high cation levels (6a).
Preliminary data of Gaulden and Jones (7) indicated that hypertonic solutions cause chromosome aggregation at the light microscope level.

Although elevated salt concentrations may induce chromosome aggregation, it is unlikely that a generalized increase in intracellular ion concentration occurs in mitosis, since this would presumably stimulate a compensatory influx of water with rapid dilution of the hypertonicity. However, cellular controls may exist for varying macromolecular charge density (e.g. by varying pH), and this, in turn, may exert control over the activities of electrolytes in compartmentalized regions of the cell without producing an osmotic effect: the lower the macromolecular charge density, the fewer bound ions there would be and the higher their effective local concentration would be. The converse would also hold. Intracellular compartmentalization of small ions has, in fact, been demonstrated in the nucleus where Na⁺ may be as high as 0.3 M (8, 9). It is conceivable that the degree to which this Na⁺ is bound at different stages of the cell cycle has a profound influence on the state of aggregation of nuclear structures.

Since we cannot experimentally induce localized intracellular ion sinks, we have extended Gaulden’s approach by examining the ultrastructural and biochemical effects of the generalized increase in intracellular ion concentrations that must ensue when cells are transferred to a hypertonic medium. While our results emphasize certain similarities between the transitions occurring during mitosis and those induced with hypertonic medium, there are also very significant differences. Our main purpose in presenting these data, therefore, is not to expand on a theory of mitosis but rather to explore one of several possible control mechanisms that may come into play as the cell enters prophase.

MATERIALS AND METHODS

Cells

All experiments were carried out with HeLa cells (S2 strain) which were routinely maintained either as monolayers or in suspension culture in Eagle’s medium supplemented with 7% fetal calf serum (10). In some experiments, cells were synchronized by selective detachment of cells in mitosis from monolayers propagated in low Ca²⁺⁺ medium (11).

Effects of Hypertonic Solutions on Cell Morphology

Hypertonic medium was prepared by adding crystalline NaCl, MgCl₂, or KCl to Eagle’s medium or to Earle’s salt solution to give final salt concentrations that ranged from 1.6 to 2.8 times isotonicity.

Approximately 10⁶ cells were pelleted at 100 g and resuspended in various hypertonic media for 2-15 min at 0°, 25°, or 37°C. Following treatment they were spun and resuspended in 2% glutaraldehyde made up in the same hypertonic solution under test. Subsequent fixation with OsO₄, embedding, and sectioning were done as previously described (12). Sections were routinely stained with aqueous uranyl acetate and lead citrate and examined in a Siemens Elmiskop IA microscope.

Effects of Hypertonicity on Macromolecular Synthesis

4 × 10⁶ cells were pelleted and then resuspended in 2 ml of various solutions of Eagle’s medium made hypertonic as above. After 2-15 min, they were pulse labeled for 15 min by adding thymidine-¹⁴C uridine-¹⁴C, reconstituted protein hydrolysate-¹⁴C for monitoring DNA, RNA, and protein synthesis, respectively. The final precursor concentration was 0.5 µc/ml of cell suspension. The cells were washed, made 5% with TCA, and the TCA-precipitable material was collected on Millipore filters. The filters were dissolved in Bray’s fluid and counted in a Beckman liquid scintillation counter.

Reversibility of the effects of hypertonic solutions on macromolecular synthesis was studied by pelleting the cells and returning them to isotonic growth medium for 10 min, after which they were pulse labeled as above.

The effects of hypertonic solutions on the synthesis of histones was also investigated. 2 × 10⁷ cells were suspended in 50 ml of Eagle’s medium minus lysine and tryptophan made 1.6 × isotonic with NaCl. After 10 min, one-half of the sample was returned to regular growth medium minus lysine and tryptophan and pulse labeled for 15 min with 5 µc of lysine-¹⁴C (New England Nuclear, Boston) plus 50 µc of tryptophan-¹⁴H and the second half was pulse labeled in the hypertonic medium. Cells were lysed by adding the detergent NP 40 (Shell Chemical Co., New York) to a final concentration of 0.5% (13), and nucleii were collected by centrifugation. Twice-washed nucleii were resuspended in 0.25 N HCl for 30 min at 4°C. The solubilized nucleoproteins, which include histones, were made 2% in SDS, dialyzed against 0.1% sodium dodecyl sulfate (SDS) and 0.01 M PO₄ buffer, pH 7.2, and run on polyacrylamide gels as described in detail elsewhere (14, 15).
Abbreviations

Chr, Chromosomes  N, Nucleolus
C, Centriole  NE, Nuclear envelope
MVB, Multivesicular body  P, Polyribosomes
MB, Midbody  R, Ribosomes
MT, Microtubules  G, Golgi complex

Figure 1a  Untreated interphase HeLa cell showing characteristic morphology of nucleus and cytoplasm. Chromatin is diffuse and fibrous; nucleolus contains both granular and fibrous components. Contour of nuclear envelope is relatively unruffled. Numerous polyribosomes, mitochondria, etc. are scattered through the cytoplasm. × 10,000.
FIGURE 1 b  Interphase cell treated for 2 min with 1.6 × isotonic medium. Chromatin is condensed preferentially along inner aspect of nuclear envelope and about nucleolus. Nuclear envelope is ruffled. Nucleolus shows diffuse granularity and absence of fibrous component. Polyribosomes are intact. At arrow, the condensed intranucleolar chromatin is continuous with the chromatin in the nucleoplasm proper. × 10,000.

Phoretic patterns obtained were compared with those from untreated samples.

Effects of Hypertonic Solutions on Polyribosomes

$10^7$ cells were pelleted, resuspended in Eagle's medium, made 1.6 × isotonic with appropriate additions of NaCl, KCl, or MgCl$_2$, and allowed to stand at 37°C for 10 min. They were pelleted again so as to remove the hypertonic medium and lysed by adding NP 40 (0.5%) in either isotonic or hypotonic buffer (4°C). After the nuclei were removed by centrifugation, the cytoplasm was layered on 15-30% linear sucrose gradients and centrifuged for 2½ hr at 24,000
rpm in the Spinco SW 27 rotor. The gradients were tapped through a Gilford recording spectrophotometer and the optical density (OD) was monitored at 260 mµ. In some experiments, cells were returned to isotonic medium before lysis and the reversibility of the effects induced by hypotonic treatment was studied with and without actinomycin D.

Isolated polyribosomes disaggregate in 0.5 M NaCl solutions unless 0.05 M Mg²⁺ is present (S. Penman, personal communication). Although the cell membrane is not freely permeable to Mg²⁺, we have observed that raising the extracellular Mg²⁺ concentration does cause a limited increase in intracellular Mg²⁺ levels (E. Robbins and T. Pederson, unpublished observations). We, therefore, examined the sparing effects of 0.05 M extracellular Mg²⁺ on polyribosomes in cells subjected to hypertonic treatment. 2 × 10⁷ cells were placed in isotonic Eagle’s medium containing 0.05 M MgCl₂ and an osmotically equivalent reduction in NaCl. After 1 hr, the medium was removed and the cells were resuspended in medium made 1.6 X isotonic with NaCl. After 10 min at 37°C, polyribosomes were prepared and analyzed on sucrose gradients as above. In addition, the sparing effects of 0.05 M MgCl₂ on the disaggregation of polyribosomes that occur normally during mitosis were followed:

**Figure 1c** Untreated prophase cell shown for comparison with Fig. 1 b. Distribution of condensed chromatin is similar; however, both chromatin and nucleolus display a more fibrous appearance than they do following hypertonic treatment. × 7,000.
2½ hr before the expected wave of mitosis, monolayers of thymidine-synchronized cells were placed in Eagle's medium containing 0.05 M MgCl₂ as above, plus 0.1 µg/ml of colchicine. After 2½ hr, cells arrested in metaphase were collected by selective detachment. They were washed in isotonic Earle's

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Figure 3 a  Interphase cell treated for 10 min with 2.8 X isotonic medium. Nuclear envelope is not visible along most of nuclear-cytoplasmic boundary. Mitochondrial and lysosomal membranes remain discernible although higher magnification (Fig. 3 b) indicates that they too are affected by the high salt. Paired arrows indicate nuclear-cytoplasmic boundary. 3 a, X 14,000; 3 b, X 56,000.
salt solution, lysed in 0.5% NP 40 in isotonic buffer, and the polyribosomes were then analyzed on sucrose gradients as above. Control cultures treated with colchicine, but not with 0.05 M MgCl₂ were run in parallel.

RESULTS
Morphological Effects of Hypertonic Medium

Most of the results to be described pertain to the effects of Eagle's medium made 1.6 or 2.8 X isotonic with NaCl; the effects of MgCl₂ and KCl were similar after short exposures (2-4 min), and longer treatments were not studied in detail.

The most striking ultrastructural effects of increasing extracellular NaCl are noted within 2 min and are confined to the nucleus. As reported at the light microscope level by Gaulden and Jones (7), the normally fibrillar chromatin of interphase (Fig. 1 a) is transformed into scattered condensed masses which are preferentially disposed about the nucleolus and just within the inner membrane of the nuclear envelope (Fig. 1 b). The over-all appearance of the chromatin is prophase-like, as is evident by a comparison with the normal prophase cell in Fig. 1 c; however, unlike that of the prophase cell, the nucleolus loses its fibrous element and appears diffusely granular. The fortuitous section in Fig. 1 b also illustrates that condensed intranucleolar chromatin is continuous with the chromatin in the nucleoplasm proper (arrow). This phenomenon, observed several times, may be a general one indicating that nucleolar DNA is not independently encapsulated within the nucleolus.

The nuclear envelope shows a marked degree of irregularity which presumably results from water loss, but which is exaggerated beyond the boundary irregularities noted in other organelles or in the cell surface. It is of interest that during normal prophase the nuclear envelope also exhibits a marked undulation which is, however, confined to that region of the envelope bordering the centrosome (16).

A significant difference between normal prophase and the effects of hypertonic NaCl lies in the wider variation in size and shape of the condensed chromatin masses produced by the high salt. In addition, mitotic condensation usually yields a more fibrillar and a more discrete chromosome than is seen in Fig. 1 b, although this is variable.

The cytoplasmic changes induced by 1.6 X isotonic medium are less pronounced than the changes in the nucleus, the most evident being a
dispersion of polyribosomes. After 2 min in the hypertonc solution, the polyribosomes still retain their morphological integrity (Fig. 1 b), but by 4 min (Fig. 2) the majority of characteristic clusters have dispersed as occurs during metaphase (17). Organelles that undergo significant transformations during mitosis, including Golgi complex, lysosomes, centrioles, and spindle tubules
FIGURE 4b  Interphase cell treated for 10 min with 2.8 X isotonic medium followed by isotonic medium for 10 min. The nuclear envelope is again visible and intact. The nucleolus has regained its fibrous component. This, in conjunction with the decondensing chromatin, gives the nucleus a telophase-like appearance. Few polyribosomes are seen. X 13,000.
FIGURE 5 Electron micrograph of nucleus isolated in hypotonic Tris buffer, and then exposed to 1.6 × isotonic medium. Morphology is not significantly different from that of nucleus in untreated interphase cell. × 11,000.
Figure 6  Electron micrograph of nuclei isolated in hypotonic Tris buffer and then exposed to 0.1 M CaCl$_2$. Chromatin is grossly precipitated. $\times$ 17,000.
(16), do not manifest any obvious morphological alterations in response to 1.6 × isotonicity; however, the nuclear envelope does appear to be differentially sensitive to still higher salt concentrations. Fig. 3 a shows a cell that has been exposed to 2.8 × isotonic medium for 10 min. At this concentration of salt, the nuclear envelope is undetectable along most of the nucleocytoplasmic boundary. This, in conjunction with the scattered, condensed chromatin, produces an image similar to that seen in colchicine-induced metaphase arrest. While the nuclear envelope is singularly sensitive to high salt concentrations, membranes bounding other organelles are also affected, albeit to a lesser degree. Mitochondrial membranes, for example, show the effects seen in Fig. 3 b. The unit membrane architecture is indistinct, and the light space between the two electron-opaque lines is grossly narrowed.

Cells placed in 2.8 × isotonic medium for 10 min and then returned to 1 × isotonic medium go through a rapid recovery, with complete reversal of the induced changes we have described. In the early phases of this reversal, the nucleus bears a close resemblance to the untreated telophase nucleus as can be seen by comparing Figs. 4 a and b. As in the untreated telophase cell (Fig. 4 a), the chromosomes in Fig. 4 b have begun to unfold and the nucleolus has reassumed its interphase morphology. Occasional polyribosomes are seen, although their complete re-formation requires about 30 min.

**Effects of Hypertonic Solutions on Isolated Nuclei**

When isolated nuclei prepared by Dounce homogenization in either hypotonic or isotonic buffer are treated with hypertonic solutions, their response is strikingly different from that seen in the intact cell. No concentration of salt tested on isolated nuclei induced the transitions shown in Fig. 1 b. Gradually increasing the NaCl concentration up to 2.4 × isotonicity has little effect, and the isolated nuclei are indistinguishable from the nuclei seen in the untreated, intact interphase cell (Fig. 5). Raising the concentration to 2.8 × isotonicity leads to complete nuclear dissolution, and the DNA is released as a viscous aggregate. Substituting KCl, MgCl₂, or CaCl₂ altered the degree of chromatin clumping, but never produced a prophase-like nucleus at the electron microscope level. For example, Fig. 6 illustrates the effects of 0.1 M CaCl₂ which has been described as causing chromosome condensation (19). It is clear that the divalent cation has induced indiscriminate precipitation which is quite different in character from that seen in Figs. 1 b or c.

**Effects of Increased Tonicity on Macromolecular Synthesis**

Increasing salt concentration causes a significant but differential decrease in macromolecular synthesis, with amino acid incorporation being the most sensitive (Fig. 7). It is noted that between 1.3 and 2 × isotonicity protein synthesis decreases about 62% while RNA synthesis falls only 16%; however, at 2.8 × isotonicity—that concentration at which the nuclear envelope is dispersed (Fig. 3)—all macromolecular synthesis ceases. Fig. 7 in conjunction with Fig. 1 b demonstrates that DNA replication and RNA transcription can occur on markedly condensed chromatin for at least 15 min. In addition, Table I illustrates that the profound depression of macromolecular synthesis that occurs at 2.8 × isotonicity is rapidly reversed upon return of the cells to isotonic medium; 10
Cell suspensions were made 2.8 X isotonic by appropriate addition of NaCl. 10^6 cells were incubated for 10 min as described in Materials and Methods. A second set of samples was incubated similarly, returned to isotonic medium for 10 min, and labeled as above ("Reversed"). Incorporation was compared with that of untreated cells.

Table I
Effects of Hypertonic Treatment on Macromolecular Synthesis

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**Effects of Hypertonic Solutions on Polyribosomes**

The polyribosome profiles plotted in Fig. 8 extend the morphological data given in Fig. 2. Exposure of cells to 1.6 X isotonic medium causes almost total disaggregation of polyribosomes within 4 min as assessed by sucrose density gradient analysis. The rapid reversibility of the hypertonic effect is also evident in the figure; 30 min after the cells are returned to isotonic medium, the cytoplasmic OD260 profile is almost indistinguishable from the control pattern. This re-formation occurs similarly in the presence of actinomycin D at a concentration which inhibits

**Figure 8** Effects of hypertonic medium on polyribosomes. Three aliquots of 10^6 randomly growing cells were incubated for 10 min at 37°C in: isotonic medium (---); 1.6 X isotonic (- - -) medium; and 1.6 X isotonic medium followed by isotonic medium for 30 min (----). The cells were washed in Earle's salts and lysed at 4°C in isotonic buffer containing 0.5% NP 40 detergent (13). The nuclei were removed by centrifugation, and the cytoplasmic extracts were analyzed on 15-30% sucrose gradients as described in the text.

Following reversal, only protein synthesis remains below control levels. This is consistent with the morphological data showing a slow re-formation of polyribosomes after exposure to this high salt concentration.

**Figure 9** Effects of hypertonicity on histone synthesis. 2 X 10^6 cells synchronized in S were: (A) labeled for 15 min with 5 μCi of lysine-14C and 50 μCi of tryptophan-3H; or (B) suspended in 2.8 X isotonic medium for 10 min and then labeled, or (C) treated for 10 min with hypertonic medium and returned to isotonic medium containing label as in A. After washing and disruption by Dounce homogenization, the nucleohistones were extracted with 0.25 N HCl and analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate as described previously (15). The histones are bracketed. (-----) lysine-14C, (---) tryptophan-3H.

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RNA synthesis by 85–90% (2 μg/ml), emphasizing the resistance of messenger RNA to the hypertonic treatment. The reassociation of messenger RNA and ribosomes to form functional polyribosomes in the absence of new RNA synthesis has been reported in several other instances including the postmitotic (G1) phase of the cell life cycle (18).

The functional integrity of the polyribosomes was also reestablished as judged by their capacity to synthesize specific proteins. Nucleohistones are synthesized on small polyribosomes and identified on polyacrylamide gels by their high lysine and low tryptophan incorporation (15). When cells that had been treated and reversed for 10 min were pulse labeled and newly synthesized nucleohistones displayed on polyacrylamide gels, the general pattern of radioactivity in the various peaks was the same as that obtained from untreated cells (Fig. 9, panel A vs. panel C). However, the peak heights were reduced in the reversed sample since at the end of 15 min protein synthesis has not yet returned to control levels (see Table I). Cells that were pulsed in hypertonic medium show no significant incorporation (panel B).

This complete inhibition of histone synthesis occurs at salt concentrations that are only 40% inhibitory to DNA synthesis. We have noted that incorporation of thymidine-14C may continue as long as 1 1/2 hr in hypertonic medium, emphasizing that the syntheses of the nucleic acid and protein components of the chromosomes can be at least partially uncoupled even though they occur concomitantly in the untreated cell.

Whether the polyribosome breakdown observed in hypertonic solutions and that occurring during normal mitosis have the same basis is not established; however, it is of interest that in both cases breakdown may be partially prevented by high extracellular concentrations of Mg++. Fig. 10A shows polyribosome profiles from random interphase cells that were treated with Eagle's medium containing 0.05 M Mg++ for 1 1/2 hr followed by 1.6 X isotonic medium for 10 min; there is a significant sparing of polyribosomes, as compared to the preparation treated with only the 1.6 X isotonic medium. Similarly, synchronized cells treated with high Mg++ and then arrested in metaphase also show polyribosome sparing, as compared to cells that were allowed to enter meta-
phase arrest without prior exposure to high Mg\textsuperscript{++} (Fig. 10 B). The degree to which intracellular polyribosomes resist breakdown in high salt concentrations in the presence of high extracellular Mg\textsuperscript{++} is not as marked as in the case of isolated polyribosomes, presumably due to the limited permeability of the cell membrane to the Mg\textsuperscript{++} ions.

**DISCUSSION**

We have shown that profound but reversible intracellular effects may be induced by manipulating extracellular ion concentrations. Although there are important discrepancies, many of the morphological and biochemical responses bear comparison with mitotic phenomena, lending credence to the hypothesis that ions may play a crucial role in cell division. If the data of Langendorf et al. (8) and of Naora et al. (9), and others, with respect to high concentrations of bound intranuclear Na\textsuperscript{+} are correct, then the selective mobilization of this ion during mitosis could, in a controlled way, lead to localized regions of high Na\textsuperscript{+} concentration. Resultant effects might be grossly comparable to those we have induced with hypertonic medium, even though we have been necessarily indiscriminate by concentrating all intracellular ions.

We have already noted the nonspecific chromatin clumping caused by Ca\textsuperscript{++} (Fig. 6); likewise, ethionine (20) and mengovirus (21) have been reported to induce chromatin clumping; however, it is our impression that these effects are qualitatively quite different from those shown in Fig. 1 b and bear less resemblance to the phenomenon of unperturbed prophase.

Although we have induced a characteristic type of chromosome condensation by increasing intracellular ion concentrations, other factors besides ions must also play a role in condensation because increased ion density alone does not have the same morphological effect on isolated nuclei prepared by the most gentle methods available. The nuclear response in the intact cell may be partly dependent on unknown molecules inadvertently lost to the medium during separation of nuclei from cytoplasm. Unfortunately, however, this hypothesis is not easily testable.

While most of the morphological changes induced with hypertonic solutions are similar to those seen during mitosis, this resemblance is less pronounced with respect to biochemical events. Thus, although macromolecular synthesis is grossly depressed during mitosis as well as following hypertonic treatment, we have noted an important difference: the concentration of salt necessary to halt DNA and RNA syntheses also stops protein synthesis; in contrast, mitotic cells maintain amino acid incorporation at 20–30% of interphase levels even though DNA and RNA syntheses cease.

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