RNA SYNTHESIS IN HELA CELLS

Pattern in Hypertonic Medium and Its Similarity to Synthesis during G_2-Prophase

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ABSTRACT

Interphase HeLa cells manifest a stepwise shutoff of RNA synthesis when the tonicity of the extracellular medium is gradually increased. Synthesis of heterogeneous nuclear RNA is most sensitive and is selectively inhibited at 1.5 times isotonicity (450 milliosmols/liter), while 45S ribosomal RNA synthesis is not affected significantly below 2.0 times isotonicity. Transfer RNA synthesis is least sensitive to increased osmolarity and is not completely inhibited until the electrolyte concentration of the medium is elevated to 2.8 times isotonicity. Although the transcription and methylation of 45S ribosomal precursor is unaffected at 1.5 times isotonicity, there is pronounced impairment of its processing into 32S and 18S RNA. Using a refined cell synchronization technique, we have been able to compare these effects of hypertonicity with the shutoff of RNA synthesis which occurs during the G_2-prophase interval of the cell division cycle. In this case, as with random cells in hypertonic medium, a selective inhibition of heterogeneous nuclear RNA synthesis and slowed processing of 45S ribosomal RNA were found, whereas synthesis of 45S and transfer RNA continued unabated throughout G_2-prophase. While it is known that RNA synthesis essentially ceases during metaphase, we have noted that transfer RNA synthesis continues in metaphase at 10–15% of the interphase rate, which is of particular interest in view of the relative resistance of this species to hypertonicity. The close correlation between the patterns of cessation of RNA synthesis at mitosis and during exposure to hypertonic medium supports our earlier contention that alteration of intracellular electrolyte levels provides a useful model for studying the mechanism of mitosis.

INTRODUCTION

We have shown previously that exposure of interphase HeLa cells to a hypertonic medium elicits reversible mitotic-like transitions, including chromatin condensation, nuclear envelope dispersion, and polyribosome disaggregation with parallel decreases in macromolecular synthesis (1). In the present report we have compared the stepwise inhibition of RNA synthesis in cells exposed to hypertonic medium with the turnoff of RNA synthesis which accompanies the entry of synchronized populations into mitosis. A striking similarity between the two patterns of RNA synthesis is noted.

MATERIALS AND METHODS

Cells and Synchronization

Randomly growing HeLa cells (S_3 strain) were maintained in suspension culture as detailed previously (2); all cultures used were free of *Mycoplasma* contamination. Populations with a high degree of

synchrony in G_2 and prophase were obtained by slight modifications of previously described methods (3, 4): metaphase populations, selectively detached from partially confluent monolayers grown in low Ca^{++} medium, were placed in suspension culture with 2 mM thymidine, thus allowing daughter cells to proceed through G_1 and up to S, but no further (5). After 12 hr the thymidine was removed by resuspending the cells in fresh growth medium; thereafter they passed through S, G_2, and mitosis with a degree of synchrony that was unattainable with any other available technique. This method has a significant advantage over single or double thymidine blockades alone because no cells are in the S phase when the excess thymidine is added.

Pulse Labeling of RNA in Hypertonic and Mitotic Cells

Random interphase cells were concentrated to a cell density of 2×10^6/ml, adjusted to the desired tonicity by the addition of 5 M NaCl, and incubated with uridine-^{3}H (specific activity approximately 24 Ci/mmole) or, in some cases with methionine-^{14}C (methyl group labeled, specific activity 50 mCi/mmole) in methionine-free Eagle's medium containing 1% calf serum. Suspensions of synchronized G_2 or prophase cells were labeled similarly, except that the medium was isotonic.

Cell Fractionation and RNA Extraction

The following abbreviations are used:

RSB: 0.01 M NaCl, 0.0015 M $MgCl_2 \cdot 6H_2O$, 0.01 M Tris-HCl, pH 7.2
HSB: 0.8 M NaCl, 0.08 M $MgCl_2 \cdot 6H_2O$, 0.016 M Tris-HCl, pH 7.2
SDS: sodium dodecylsulfate
NETS: 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl, pH 7.2, 0.5% SDS
TCA: trichloroacetic acid
SUP: 1.0% SDS, 0.5 M urea, 0.01 M phosphate buffer, pH 7.0

Cells were washed twice in cold (4°C) Earle's salt solution, disrupted by Dounce homogenization in RSB (6), and separated into nuclei and cytoplasm. Detergent-rinsed nuclei (7) were lysed in HSB containing DNase, 100 μg/ml, and separated into nucleolar and nucleoplasmic fractions by zonal centrifugation in sucrose gradients (8). RNA was released from the pellet of nucleoli by resuspension in NETS buffer and was recovered from the supernatant nucleoplasmic fraction by precipitation with 2.5 volumes of 95% ethanol (1 hr at −20°C), after which the RNA was collected by centrifugation (10 min at 3000 rpm, International model PR-2, International Equipment Company, Needham Heights, Mass.) and dissolved in NETS. The RNA samples were centrifuged through linear 15–30% sucrose gradients in NETS buffer (see individual figure legends), and the TCA-precipitable incorporation in each fraction was assayed by Millipore filtration (Millipore Corporation, Bedford, Mass.). Nucleoli isolated from cells labeled with methionine-^{14}C were dissolved in NETS, and the RNA was extracted with phenol and chloroform at 55°C (7), precipitated with ethanol, redissolved in NETS, and centrifuged in gradients as above. Transfer RNA was prepared by centrifuging cytoplasmic extracts for 1 hr in the Spinco type 40 rotor (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) (39,500 rpm), adding SDS to the supernatant to 0.5%, and extracting this mixture with phenol-chloroform as above; after ethanol precipitation, the RNA was dissolved in SUP and electrophoresed in 12 cm of 7.5% polyacrylamide gels containing 0.1% SDS (9). A current of 7.5 ma/gel was applied until a bromphenol blue marker had migrated to the end of the gels, which were then fractionated mechanically into scintillation vials (9), mixed with Bray's fluid, and counted.

Isotopes were purchased from Schwarz Bio Research Inc., Orangeburg, N. Y. Electrophoretically purified DNase was obtained from Worthington Biochemical Corp., Freehold, N. J., and actinomycin D was a gift of Merck, Sharpe & Dohme, West Point, Pa.

RESULTS

RNA Synthesis in Interphase Cells Exposed to Hypertonic Medium

The effects of hypertonicity on the syntheses of nucleolar and nucleoplasmic RNA's in randomly growing cells are illustrated in Figs. 1 A and 1 B. A 20 min exposure to Eagle's medium made 1.5 times isotonic (450 milliosmols/liter) results in a rapid and selective inhibition of heterogeneous nuclear RNA synthesis (Fig. 1 B), while 45S ribosomal precursor synthesis in the nucleolus shows only a slight decrease (Fig. 1 A). When the osmolarity is elevated to 2.0 times isotonicity, no further reduction of heterogeneous RNA (HnRNA) synthesis occurs, while 45S RNA synthesis now ceases completely. When the osmolarity is raised still further to 2.8 times isotonicity, the remaining HnRNA synthesis, about 50% of the untreated sample, is reduced to zero (not shown).

The selective effect of increased extracellular tonicity on HnRNA is the same whether analyses are made on the nucleoplasmic fraction alone, as

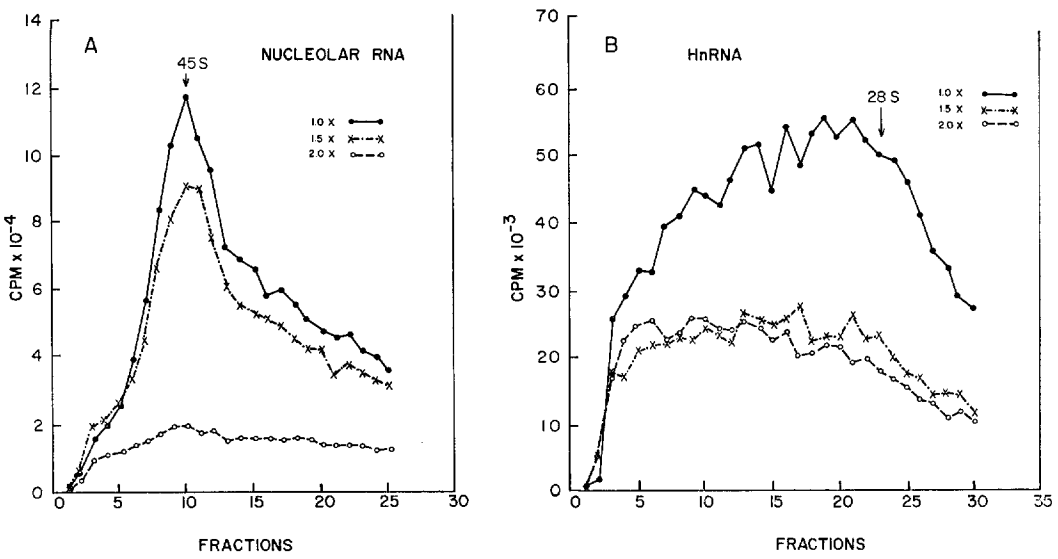


FIGURE 1 Effects of hypertonicity on synthesis of nucleolar and nucleoplasmic RNA. 33 ml suspensions of cells at a density of 2×10^6/ml were made 1.5 or 2.0 times isotonic by adding 0.5 ml or 1.0 ml of 5 M NaCl, respectively. After 10 min, 200 μCi of uridine-^{3}H was added to each and incorporation was allowed to proceed for an additional 10 min. Cells were fractionated into nucleoli and nucleoplasm, and the RNA was extracted as described in Materials and Methods; the RNA samples were layered onto 36 ml 15–30% sucrose-SDS gradients, and, after centrifugation in the Spinco SW 27 rotor (Spinco Division, Beckman Instruments, Inc. Palo Alto, Calif.) for 12 hr at 20,000 rpm (21°C), the TCA-precipitable incorporation into each fraction was measured. Direction of sedimentation is from right to left. Panel A, nucleolar RNA; B, nucleoplasmic RNA. The OD 260 profiles (not shown) of the three gradients in A were identical, as were those in B.

in Fig. 1 B, or on a mixture of nucleoplasm and cytoplasm, obtained by lysing nuclei in the whole Dounce homogenate and removing the nucleoli by zonal centrifugation. This rules out the possibility of HnRNA leakage from nuclei of cells exposed to hypertonicity. In addition, we have determined that prelabeled HnRNA decays in actinomycin with the same kinetics in untreated cells and in cells treated with hypertonic medium, so that the results illustrated in Fig. 1 B are not due to increased HnRNA turnover during exposure to hypertonic medium.

Fig. 2 illustrates the response of transfer RNA (tRNA) synthesis to increased extracellular osmolarity, which resembles the response of nucleolar RNA in that there is only a slight inhibition at 1.5 times isotonicity, but about 60% inhibition at 2.0 times isotonicity; however, whereas 45S synthesis is totally shut off at 2.0 times isotonicity (Fig. 1 A), cessation of tRNA synthesis is not complete until 2.8 times isotonicity. The pulse-labeled RNA in Fig. 2 migrates more slowly than the equilibrium-labeled 4S tRNA marker, indicated at fraction 34; this reflects the preponderance of uridine-^{3}H label in tRNA precursor molecules after a 10 min pulse, rather than in the smaller 4S transfer RNA (10).

The selective effect of 1.5 times isotonic medium on HnRNA synthesis is in agreement with data on total uridine-^{3}H incorporation. Thus, after 20 min of exposure to 1.5 times isotonic medium, total cellular RNA synthesis is reduced by about 25%; since HnRNA molecules comprise half of the radioactive RNA after a 20 min pulse, the 25% inhibition of total uridine-^{3}H uptake can be entirely accounted for by the demonstrated 50% inhibition of HnRNA synthesis (Fig. 1 B), emphasizing the lack of an effect on the other major RNA species.

Although synthesis of 45S ribosomal precursor RNA remains relatively unaffected for at least 30 min in 1.5 times isotonic medium, hypertonicity has a pronounced effect on processing of the precursor into 32S and 18S ribosomal RNA. In the following experiments methionine-^{14}C, labeled exclusively in the S-methyl group, was used rather than uridine, so that any contaminating heterogeneous nuclear RNA, which is not appreciably

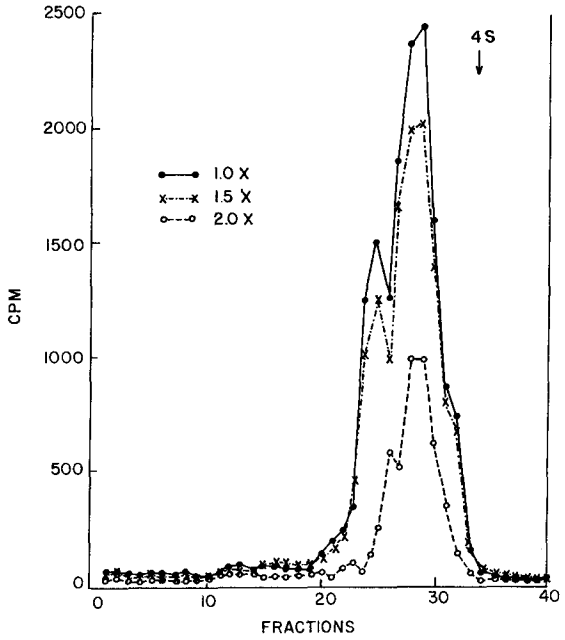


FIGURE 2 Effects of hypertonicity on transfer RNA synthesis. The cytoplasmic extracts from the experiment described in Fig. 1 were centrifuged at 100,000 *g* for 1 hr and the supernatants were made 0.5% in SDS. RNA was extracted with phenol and chloroform, precipitated with ethanol, dissolved in SUP, and electrophoresed in 12 cm of 7.5% polyacrylamide gels containing 0.1% SDS. Electrophoresis was performed at 7.5 ma/gel until a marker of bromphenol blue dye had migrated to the end of the gels (about 10 hr at 21°C). Direction of migration is from left to right.

methylated (11), would not contribute radioactivity. Fig. 3 depicts sucrose gradient analyses of nucleolar RNA extracted from control cells and from cells exposed to 1.5 times isotonic medium for 30 min of continuous methionine-^{14}C labeling. In addition to the peak of incorporation at 45S seen with shorter labeling times, radioactivity is now noted also in the 32S intermediate (12–14). The ratio of 45S to 32S radioactivity is a reflection of the processing rate, and, while this is about 1.1 (peak fraction) in untreated cells (panel A), exposure to hypertonicity results in a 45S/32S peak ratio of 2.6, indicating a markedly reduced rate of conversion. This effect is illustrated more clearly by the following: equal numbers of cells were suspended in either isotonic or 1.5 times isotonic medium and labeled for 25 min with methionine-^{14}C. Aliquots were removed and RNA synthesis was inhibited in the remaining portions by addition of actinomycin D; samples were taken at 12 and 25 min after drug addition to monitor the flow of radioactivity from prelabeled 45S precursor into 32S molecules. Fig. 4 illustrates that after 12 min about 75% of the radioactivity initially present as 45S RNA in the control sample has moved into the 32S peak, while in the hypertonic cells even after 25 min relatively little 32S intermediate has become labeled, indicating a pronounced impairment of ribosomal RNA processing. The slowed cleavage of 45S precursor into 32S and 18S RNA in cells exposed to hypertonicity is also evident from analysis of the labeling kinetics of cytoplasmic ribosomal RNA (Fig. 5). After 30 min of continuous labeling, newly synthesized 18S ribosomal RNA is found in the cytoplasm of untreated cells but not in that of cells exposed to 1.5 times isotonic medium.

Since processing of 45S ribosomal precursor is significantly slowed in hypertonic cells, while its rate of synthesis is relatively unaffected, the total nucleolar content of 45S RNA should increase during exposure of cells to hypertonic medium. It can be seen from the optical density tracings in Fig. 6 that the steady-state level of 45S RNA in the nucleolus actually does increase by about 25% during 25 min of hypertonic treatment.

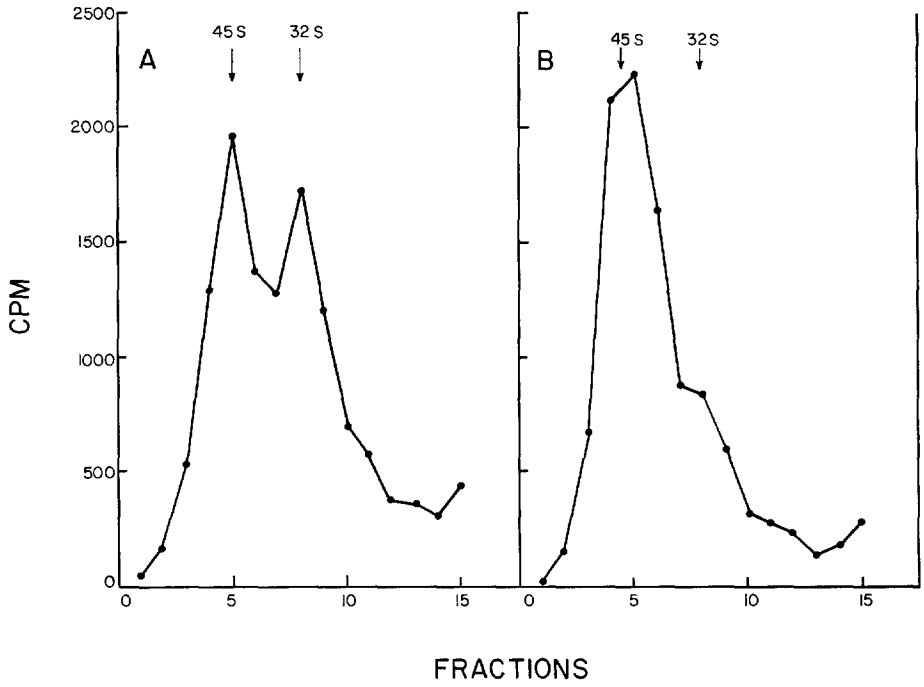


FIGURE 3 Effect of 1.5 times isotonic medium on ribosomal RNA processing. A suspension of 10^6 cells/ml in 33 ml of methionine-free Eagle's medium (1% calf serum) was adjusted to 1.5 times isotonicity by adding 0.5 ml of 5 M NaCl, while a second culture was left untreated; both were labeled for 30 min with 10 μCi of methionine-^{14}C (methyl group labeled, specific activity 51 mCi/mmole). Nucleoli were isolated, dissolved in NETS, and the RNA was purified by phenol-chloroform extraction and ethanol precipitation. Samples were layered onto 16 ml of 15–30% sucrose-SDS gradients and centrifuged for 14 hr at 22,000 rpm in the SW 25.3 rotor (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) (21°C). A, control; B, hypertonic-treated cells.

Sequential Inhibition of RNA Synthesis during Mitosis

To study in detail the shutoff of RNA synthesis in cells entering mitosis, it was necessary to obtain cell populations with a high degree of synchrony in G_2 and prophase. Fig. 7 depicts the time course of DNA synthesis and mitosis in cell populations collected in metaphase, cultured for 12 hr with 2 mM thymidine, and then resuspended in fresh medium (0 hr in Fig. 7). The cells immediately enter the S phase following thymidine removal and display the characteristic DNA synthesis curve; however, unlike synchronization by the simple addition of excess thymidine to random cultures, the population shown in Fig. 7 displays an interval, from 8 to 9½ hr after inhibitor removal, when the rate of DNA synthesis has decreased to a minimum, but the mitotic index has not yet begun to rise. Such populations thus have a distinct G_2 phase of at least 1½ hr whereas if cells are synchronized by double thymidine blockade of random cultures or by selective detachment of metaphases alone, the mitotic index begins to rise before the DNA curve has attained its minimum (T. Pederson and E. Robbins, manuscript in preparation).

It is of considerable interest that RNA synthesis in cells entering mitosis follows a pattern similar to that found in interphase cells exposed to hypertonic medium. Aliquots from a synchronized population such as that in Fig. 7 were pulsed for 10 min with uridine-^{3}H during early G_2 (No. 1), late G_2 (No. 2), and prophase (No. 3). Synthesis of nucleolar and nucleoplasmic RNA at these times is illustrated in Fig. 8 A–C. 45S ribosomal precursor synthesis (panel A) is unaffected even when 50% of the cells have reached prophase (curve 3), whereas the synthesis of heterogeneous nuclear RNA (panel B) declines gradually over a 1½ hr period, displaying a pattern reminiscent of that observed in inter-

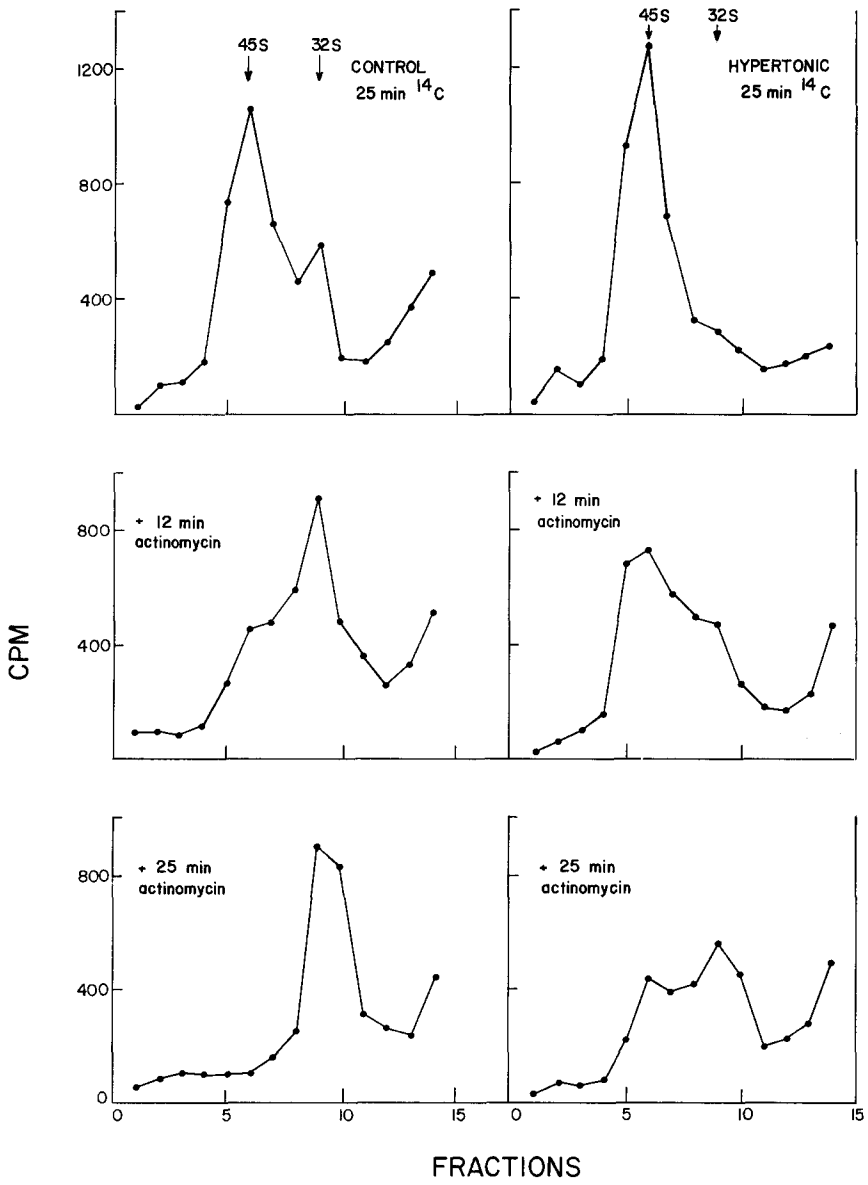


FIGURE 4 "Actinomycin chase" experiment in isotonic and hypertonic medium. Two cultures of cells at 3.5 × 10^6/ml were prepared in methionine-free medium, and one was adjusted to 1.5 times isotonicity with NaCl. Both were immediately labeled for 25 min with 10 μCi of methionine-^{14}C, after which aliquots were removed and actinomycin D was added to the remainder of each suspension at 5 μg/ml; additional samples were removed at 12 and 25 min after adding the drug. Nucleolar RNA was purified as in Fig. 3 and centrifuged in 15–30% sucrose-SDS gradients for 11 hr at 24,000 rpm in the SW 25.3 rotor (21°C).

phase cells exposed to gradually increasing hypertonic media (Fig. 1 B). However, in G_2-prophase cells the decreased synthesis occurs mainly among the lighter, more slowly sedimenting HnRNA molecules (fractions 15–40); this has been noted consistently in populations entering mitosis, but not in cells exposed to hypertonic medium. Comparisons of actinomycin decay times for HnRNA in S, G_2, and prophase have indicated equal stabilities, so that the progressive decrease in HnRNA

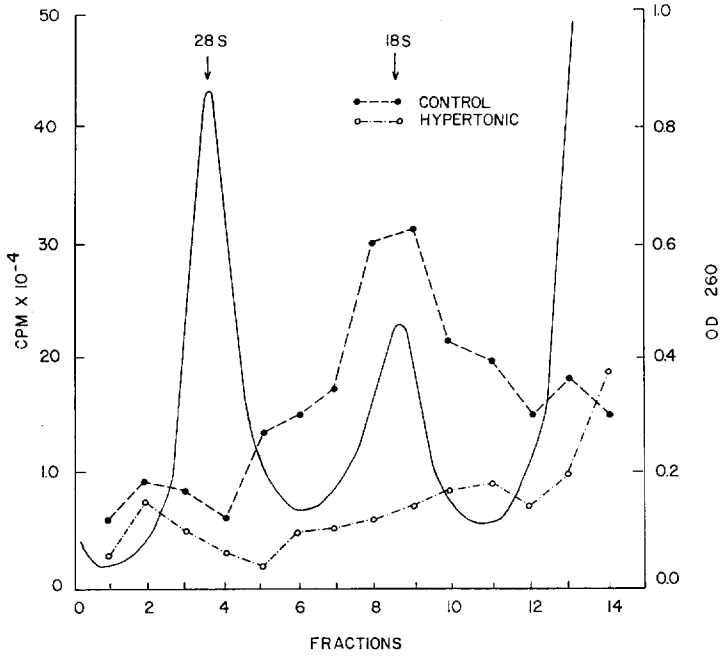


FIGURE 5 Labeling of cytoplasmic ribosomal RNA in isotonic and hypertonic medium. 33 ml of cell suspension at 3×10^6/ml was made 1.5 times isotonic, and another was left untreated; both were labeled for 30 min with 200 μCi of uridine-^{3}H. Cytoplasmic extracts were prepared by Dounce homogenization, adjusted to 0.5% SDS, layered onto 15–30% sucrose-SDS gradients, and centrifuged at 24,000 rpm for 16 hr in the SW 25.3 rotor (21°C). O---O: control; O-·-O: hypertonic; — OD 260 profile of control gradient. Direction of sedimentation is from right to left.

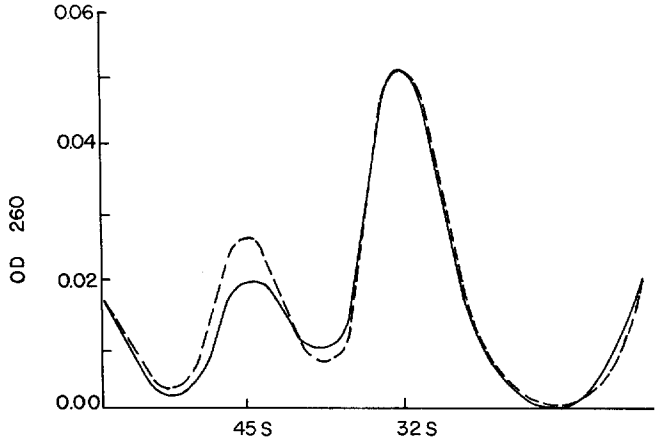


FIGURE 6 Effect of hypertonicity on nucleolar content of 45S RNA. The curves are the OD 260 profiles of the 25-min samples illustrated in Fig. 4. —: control; ---: hypertonic.

labeling during G_2 and prophase would not seem to be due to an increased rate of turnover. Like 45S ribosomal precursor, the synthesis of transfer RNA (panel C) continues unabated throughout G_2-prophase, and, even in pure metaphase populations obtained by selective detachment (3), tRNA is synthesized at 10–15% of the interphase rate (not shown). The reduction of HnRNA synthesis

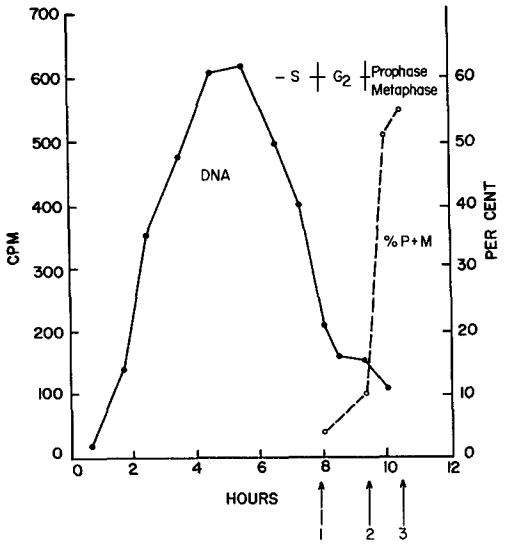


FIGURE 7 DNA synthesis and mitosis in synchronized cells. A population of pure metaphase cells was obtained by selective detachment from partially confluent monolayers (3, 4) and immediately placed in suspension culture for 12 hr with 2 mM thymidine. The cells were then resuspended in fresh medium and the rate of DNA synthesis was monitored at hourly intervals by incubating 1.0 ml of cell suspension with 0.5 μCi of thymidine-^{14}C (specific activity 30 mCi/mmole) for 15 min at 37.5°C, and measuring the incorporation into 10% TCA-precipitable material. Colchicine, 0.2 μg/ml, was added to the culture at 6 hr to arrest cells dividing subsequently in metaphase. •——•: thymidine-^{14}C incorporation as cmp; o- - - - -o: % of cells in prophase or metaphase.

during G_2 and prophase is the only case where a selective inhibition of a single class of RNA has been observed during the cell cycle (15–19).

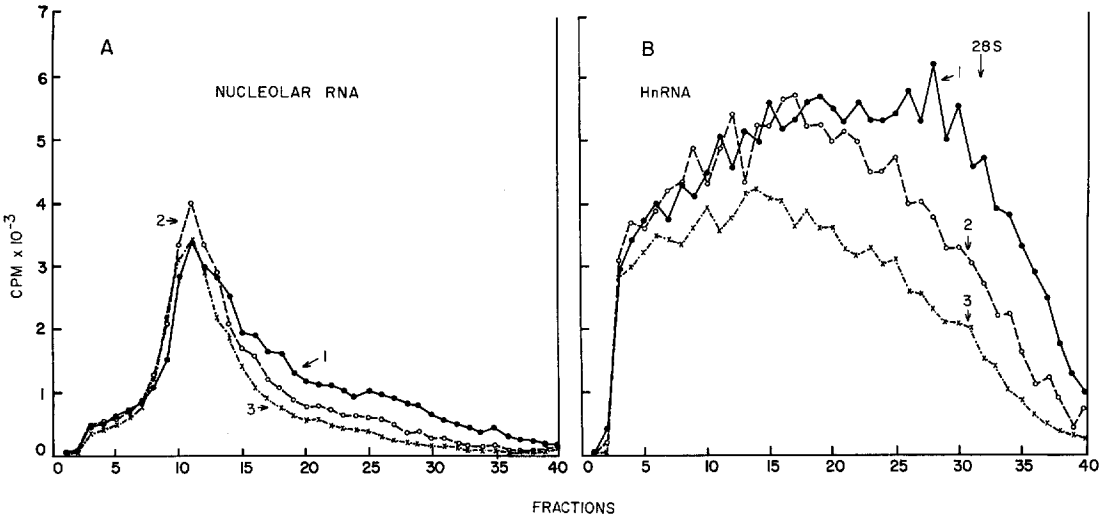
It was also of interest to determine whether the reduced rate of 45S RNA processing noted in hypertonic cells occurs in populations entering mitosis as well. When synchronized cells in either late S or G_2 are labeled for 30 min with methionine-^{14}C, and the purified nucleolar RNA is analyzed on sucrose gradients, the results are as shown in Fig. 9. In the S phase sample the ratio of counts in the 45S peak (fractions 6–8) to those in the 32S peak (fractions 10–12) is 1.1, as in randomly growing cells, while in G_2 this ratio is 1.4 indicating a slowed rate of processing like that observed after hypertonic treatment. Although this effect in G_2 is less pronounced than it is with hypertonicity, it is consistently observed, and, in combination with the early shutoff of HnRNA synthesis, further emphasizes the similarity between the cellular responses during G_2 prophase and those induced in interphase cells by increased extracellular tonicity.

DISCUSSION

Hypertonicity is thus far unique as an experimental condition which elicits selective inhibition of heterogeneous nuclear RNA synthesis. This is in contrast to low doses of actinomycin D (8, 20, 21), mitomycin C,[1] cycloheximide (22), cordycepin (23), or infection with poliovirus (24), all of which inhibit 45S RNA synthesis most profoundly. The inhibition of HnRNA synthesis by hypertonicity may well be related to the marked chromatin condensation which occurs within 2 min at 1.5 times isotonicity (1). Although increased nucleolar granularity also accompanies this ionic change (1), the present results demonstrate that this does not affect 45S RNA transcription, and suggest that the granularity might manifest an accumulating population of 80S nascent ribosomal particles (25) commensurate with impaired 45S RNA processing. It is also possible that the differential effect of hypertonicity on nucleolar and nucleoplasmic RNA synthesis is related to the existence of distinct nucleolar and chromosomal RNA polymerases as previously described in nuclei from rat liver (26–29) and sea urchin embryos (30). The two enzymes have separate ionic requirements, which might account for the selective effect of 1.5 times isotonic medium on HnRNA synthesis. The inhibition of 45S RNA synthesis in 2.0 times isotonic medium would be due to intranuclear salt attaining a level inhibitory to both enzymes, and a similar argument would apply to the sequential inhibition of the two RNA species during G_2 and prophase. The fact that transfer RNA synthesis is even less sensitive to increased tonicity than either 45S or HnRNA might indicate the existence of a third polymerase.

The decreased rate of 45S RNA processing in hypertonic cells and in those entering mitosis, without diminution of the precursor's rate of synthesis, is consistent with the fact that ribosome synthesis in HeLa cells is, in several instances, controlled at the level of processing rather than at the level of 45S RNA transcription. For example, in puromycin-treated cells 45S RNA molecules are degraded, while their rate of synthesis is unaffected (31); methionine deprivation results in 45S pre-

[1] Laing, R., and S. Penman, unpublished data cited in reference 8.



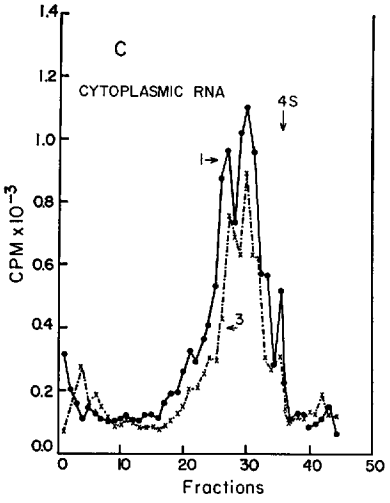


FIGURE 8 Synthesis of nucleolar, nucleoplasmic, and transfer RNA during G_2 and prophase. At the times indicated by the arrows in Fig. 7. equal portions of the synchronized population were concentrated to 2×10^6 cells/ml in 25 ml and labeled for 10 min with 200 μCi of uridine-^{3}H. Nucleolar and nucleoplasmic RNA's were centrifuged through sucrose-SDS gradients in the SW 27 rotor (17,000 rpm for 16 hr at 21°C), and transfer RNA samples were electrophoresed in 12 cm of 7.5% polyacrylamide gels as detailed in Fig. 2. The three gradients in panel A contained the same amount of OD 260 at 45S and 32S, while those in B had equal 28S OD peaks.

cursor being undermethylated and thus improperly processed, but again, its rate of synthesis is relatively unchanged (32). However, in the cases of valine deprivation (33) or cycloheximide treatment (23), both 45S transcription and processing are affected. Although we cannot definitively state whether the effect of hypertonicity on 45S processing is a direct consequence of increased intracellular electrolyte levels or is secondary to the 30% inhibition of protein synthesis which occurs in 1.5 times isotonic medium (1), the former appears more likely, since 6 hr of methionine deprivation, or 15 hr of valine depletion, reduce protein synthesis 70–80% (32, 33), and yet the effect on processing in these instances is not nearly so pronounced as in hypertonic medium.

The present experiments demonstrate that a pattern of RNA synthesis characteristic of G_2 and prophase HeLa cells can be induced in interphase cell populations by exposure to hypertonic growth medium. This effect, together with our previous demonstration of other mitotic-like biochemical and morphological responses to hypertonicity (1), further emphasizes that changes in intracellular electrolytes provide a useful model system for studying the physiological and structural transitions of mitosis.

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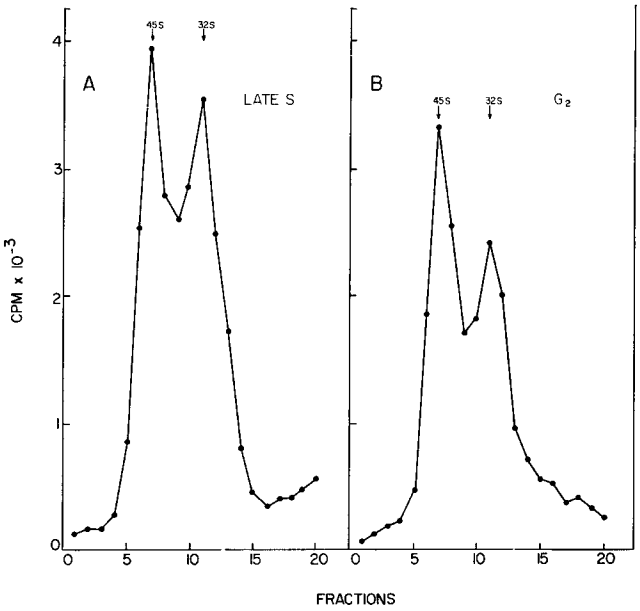

FIGURE 9 Nucleolar RNA processing in S and G_2 cells. Populations synchronized as in Fig. 7 were concentrated to 2×10^6 cells/ml in 20 ml of methionine-free medium in either late S (6 hr after thymidine removal or G_2 (9 hr after thymidine removal), and labeled for 30 min with 10 μCi of methionine-^{14}C. RNA was extracted from the isolated nucleoli as in Fig. 3 and centrifuged through sucrose-SDS gradients for 12 hr at 22,000 rpm in the 25.3 rotor (21°C).

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