

Effects of Zinc on Stationary-Phase Phenotype and Macromolecular Synthesis Accompanying Outgrowth of *Candida albicans*

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When cultures of *Candida albicans* which had entered stationary phase due to the depletion of zinc (zinc-limiting conditions) were compared with cultures which had entered stationary phase due to the depletion of another growth-limiting component (zinc-excess conditions), at least two cellular characteristics were found to differ: (i) zinc-limited cells appeared more homogeneous and larger on the average than zinc-excess cells, and (ii) zinc-limited cells evaginated on the average 40 min later than zinc-excess cells. In the present study, it is demonstrated that (i) the distribution of volumes for a stationary-phase culture of zinc-excess cells is skewed towards very small volumes, but even the smallest cells contain nuclei; in contrast, the volumes of zinc-limited cells are evenly distributed around a much larger mean value; (ii) the evagination kinetics of zinc-excess cells released into fresh medium are far less synchronous than are those of zinc-limited cells, and the smaller cells in the population take much longer to evaginate than do the larger cells; (iii) the onset of net RNA accumulation and achievement of a maximum rate of [³H]uridine incorporation occur significantly earlier in zinc-excess cells than in zinc-limited cells released into fresh medium; and (iv) the onset of net protein accumulation and [³H]leucine incorporation occur significantly earlier in zinc-excess cells than in zinc-limited cells released into fresh medium. These results indicate that although zinc-excess cells are extremely heterogeneous in volume, they may still be homogeneously blocked in the nuclear division cycle, and that the later average evagination time of released zinc-limited cells may be due to a delay in the onset of protein synthesis, which in turn may be due to the time necessary to reaccumulate zinc to levels sufficient for the reinitiation of RNA synthesis.

When budding cells of the infectious yeast *Candida albicans* enter stationary phase due to zinc depletion (2, 15; D. R. Soll, in M. R. McGinnis, ed. *Current Topics in Medical Mycology*, vol. 1, in press), they accumulate as unbudded singlets in the G1 phase of the cell cycle (13). When these cells enter stationary phase, they become pluripotent (5, 8, 9, 11, 13, 16), capable of forming either buds or mycelia depending on the pH of the medium into which they are released (4). When released into fresh growth medium at 37°C and pH 4.5, they synchronously and exclusively form buds after an average period of ca. 135 min, and when released into fresh medium at 37°C and pH 6.7, they synchronously and exclusively form mycelia after the same average period of 135 min. A recent analysis of protein synthesis during the pre- and postevagination periods uncovered a very discrete program which was similar in budding and mycelium-forming populations (3; R. Finney, C. Langtimm, and D. R. Soll, manuscript in preparation). During an initial 50-min period, virtually no protein synthesis was detectable either by isotope labeling or by direct measurement of total cell protein (3). Between 50 min and roughly the time of evagination (135 min), protein was synthesized at a low but constant rate. At roughly the time of evagination, the rate of protein synthesis increased dramatically (3). It was further demonstrated that the major proteins synthesized before evagination were also synthesized after evagination and that at the time of evagination an additional set of major proteins began to be synthesized (3; Finney et al., manuscript in preparation).

In contrast, cells which enter stationary phase in excess zinc, due to the depletion of another growth-limiting component (e.g., glucose or another trace ion, see reference 15), are also pluripotent, capable of forming buds or mycelia when released into fresh nutrient medium at low or high pH,

respectively (2). These zinc-excess cells have been found to differ from zinc-limited cells in at least two respects (2, 15). First, they appear to be less homogeneous in volume and to contain a higher proportion of budded cells, leading to the suggestion that they may be asynchronously blocked throughout the cell cycle (2). Second, when released into fresh medium, these cells evaginate on the average 40 min earlier than zinc-limited cells, leading to the suggestion that zinc-limited cells take longer to reinitiate protein synthesis because they must reaccumulate zinc (15; Soll, in press) to levels necessary to reinitiate RNA synthesis (6, 7). In the present report, we have investigated the above suggestions. Experiments are first described which demonstrate that although the distribution of cell volumes in zinc-excess cultures includes a far greater number of smaller spheres, almost all contain nuclei, indicating that zinc-excess cells may also be synchronously blocked in the nuclear division cycle. When released into fresh medium to reinitiate growth, zinc-excess cells evaginate earlier than zinc-limited cells but evaginate far more asynchronously due to the increased proportion of small cells which must grow for a longer period than the large cells in the population before evaginating. It is further demonstrated that macromolecular synthesis begins earlier in populations of released zinc-excess cells than in released zinc-limited cells, thus supporting the suggestion that the zinc content of stationary-phase cells may dictate the subsequent timing of RNA synthesis, and in turn protein synthesis and evagination.

MATERIALS AND METHODS

Growth of stock cultures. Stock cultures of strain 3153A were maintained on nutrient agar by methods previously described (13). For experimental purposes, cells were grown in 25 ml of defined medium (8) supplemented with arginine

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(14) in 125-ml plastic Erlenmeyer flasks rotated at 200 rpm at 25°C. Cells were cloned once a month to ensure genotypic and phenotypic homogeneity. Care was taken to ensure a minimum of zinc contamination by methods described in a previous report (15).

For zinc-limiting conditions, in which cells stop multiplying at stationary phase due to the depletion of zinc from the medium (15), culture medium was supplemented with zinc sulfate to 0.1 μ M. For zinc-excess conditions, in which cells stop multiplying due to the depletion of another growth-limiting component (15), culture medium was supplemented to either 2.5 or 9 μ M zinc sulfate. Cells were considered in stationary phase when sphere concentration had plateaued and remained stable for roughly 25 h. Cells which entered stationary phase due to the depletion of zinc will be referred to as zinc-limited cells, and cells which entered stationary phase in excess zinc will be referred to as zinc-excess cells.

Initiation of outgrowth. Stationary-phase cells were lightly sonicated and resuspended in 25 ml of defined medium (8), pH 4.5, supplemented with arginine (14) in 125-ml plastic Erlenmeyer flasks. The cell concentration ranged between 1×10^6 and 4×10^6 spheres per ml. These cultures were then rotated at 200 rpm at 37°C. Samples were removed at time intervals and suspended in 3% formaldehyde, and 100 cells were scored at each time point as previously described (10).

Measurement of cell volumes. Stationary-phase cells were placed on a glass slide and examined with an oil immersion objective ($\times 100$). A video recording was made of the cells by methods previously described (16). Measurements of mother cells and buds were made from the video tape on a calibrated video screen; linear measurements were converted to volume (V) by using the formula for an ellipsoid ($V = [\pi/6]lw^2$, where l is length and w is width).

Nuclear staining. Nuclei were stained with acridine orange by a method previously described in detail (18). In brief, cells were heat fixed on glass slides, treated with RNase, dehydrated in ethanol, and then stained in a solution of acridine orange. Preparations were air dried. To examine for staining, a droplet of sucrose solution was placed on the preparation, a cover slip was mounted, and then the preparation was viewed under a Leitz fluorescence microscope, filter module H, with a halogen light source.

Incorporation of [3 H]uridine into RNA. Stationary-phase cells were sonicated and placed at a final concentration of 5×10^6 spheres per ml in fresh medium at 37°C (pH 4.5) containing 0.8 μ Ci of [5,6- 3 H]uridine (Amersham Corp.; 40 Ci/mmol; 162 mCi/mg) per ml. Duplicate 1-ml samples were pipetted at intervals into tubes containing 1 ml of an ice-cold solution of 16% trichloroacetic acid containing 100 μ g of unlabeled uridine per ml. The precipitated samples were collected on glass-fiber filters presoaked in carrier uridine and washed with 20 ml of ice-cold 8% trichloroacetic acid containing 100 μ g of unlabeled uridine per ml, followed by 20 ml of ice-cold 95% ethanol. The filters were dried and then counted in a toluene-based scintillation solution.

Measurements of total RNA. Stationary-phase cells were sonicated and placed in fresh medium at 37°C (pH 4.5) at a final sphere concentration of 10^7 /ml. At intervals, duplicate 10-ml samples were removed, pelleted, and washed twice in 5 ml of ice-cold distilled water. The final pellet was suspended in 1 ml of ice-cold water and diluted with 1 ml of ice-cold 0.5 M HClO₄. The mixture was incubated on ice for 15 min with occasional mixing and then pelleted. The pellet was resuspended in 0.25 M HClO₄ and incubated for an additional 15 min on ice. The sample was pelleted again and resuspended in 1 ml of 0.5 M HClO₄, followed by incubation

for 2 h at 37°C. Then the sample was spun, and the pellet was washed in 0.5 M HClO₄; both supernatants were pooled, and the final volume was brought to 2 ml with 0.5 M HClO₄. To 1 ml of each sample was added 3 ml of orcinol reagent (1 volume 1% orcinol, 4 volumes 0.09% FeCl₃ · 6H₂O in concentrated HCl), and the mixture was placed in a boiling-water bath for 20 min. Volumes were readjusted to 4 ml and read at 670 nm (Gilford 240 spectrophotometer). A standard curve was generated with yeast RNA (type III; Sigma Chemical Co.) that had been incubated at 37°C for 2 h in 0.5 M HClO₄.

Incorporation of [3 H]leucine into protein. Stationary-phase cells were treated as described under incorporation of [3 H]uridine into RNA, except that medium was prepared without leucine and contained 0.5 μ Ci of L-[4,5- 3 H]leucine (Amersham Corp.; 145 Ci/mmol; 103 mCi/mg) per ml. Duplicate 1-ml samples were pipetted at intervals into tubes containing 1 ml of an ice-cold solution of 16% trichloroacetic acid containing 100 μ g of unlabeled leucine per ml. Samples were immediately placed in a boiling water bath for 15 min and then in an ice bath for at least 1 h. Each sample was collected and washed as described under [3 H]uridine incorporation, except that the unlabeled carrier was 100 μ g of leucine per ml. Dried filters were counted in toluene-based scintillation solution.

Measurements of total cell protein. Stationary-phase cells were treated as described under measurement of total RNA. At intervals, duplicate 11-ml samples were removed, pelleted, and washed twice in 5 ml of ice-cold distilled water. The final pellet was resuspended in 1 ml of 0.5 N KOH, placed in a boiling water bath for 15 min, and then spun at 1,000 rpm in a Beckman TJ-6 centrifuge. For the protein assay, 0.1 ml of the sample was added to 0.7 ml of distilled water, followed by 0.2 ml of Bio-Rad dye concentrate (Bio-Rad Laboratories). After 5 min, samples were read at 595 nm in a Beckman DU-8 spectrophotometer. A standard curve was generated with bovine albumin (fraction V; Sigma Chemical Co.) that had been incubated at 100°C for 15 min in 0.5 N KOH. Lowry determinations gave protein readings that were 1.5 times those of parallel Bio-Rad determinations. To preserve consistency with previous protein determinations (3, 14), we have multiplied all Bio-Rad determinations by a factor of 1.5.

Sedimentation analysis of labeled RNA. Stationary-phase cells were sonicated, pelleted, and resuspended in 50 ml of fresh medium at 37°C (pH 4.5) at a final sphere concentration of 2×10^7 /ml in 125-ml Erlenmeyer flasks. At 70 or 170 min, 25 ml of cell suspension was placed in a 50-ml Erlenmeyer flask containing 50 μ Ci of [5,6- 3 H]uridine (Amersham Corp.; 40 Ci/mmol). After 10 min at 37°C, cells were pelleted and stored at -70°C. Droplets of cell suspension were removed from the original flasks to monitor evagination kinetics.

For sedimentation analysis, each pellet was resuspended in extraction buffer (0.05 M NaOAc, 0.05 M NaCl [pH 7.5]), and 5.5 g of acid-washed glass beads (diameter, 0.45 to 0.50 mm) were added, in a 50-ml screw-capped polypropylene centrifuge tube. Cell samples were vortexed for 1 min at room temperature, resulting in more than 95% cell breakage. To each sample, 500 μ l of 20% sodium dodecyl sulfate was added, and the mixture was vortexed. An additional 9 ml of extraction buffer was added, and the solution was mixed. Then 10 ml of phenol solution (550 ml of phenol distilled over zinc, 70 ml *m*-cresol, 0.5 g of 8-hydroxyquinoline [water saturated]) was added, and the mixture was shaken in an ice bath and then spun at 5,000 rpm for 10 min. The aqueous phase was removed, the phenol phase was washed with 5 ml

of extraction buffer, and the aqueous phase and wash were combined. The last preparation was ethanol precipitated, air dried, and dissolved in 1 ml NETS (0.2 g of sodium dodecyl sulfate, 0.12 g of Na₂EDTA, 0.12 g of Tris-hydrochloride, 0.58 g of NaCl; in 100 ml of water [pH 6.8]). A 500- μ l sample of each preparation was overlaid on a 10 to 25% sucrose gradient and centrifuged in a SW40 rotor at 20°C for 18 h at 26,000 rpm. Fractions of 0.4 ml each were collected by inserting a long needle through the gradient to the tube bottom. Each fraction was measured for absorbance at 260 nm. From each fraction, 100 μ l was removed, mixed with toluene-based scintillation fluid, and counted.

RESULTS

Stationary-phase phenotype of zinc-limited and zinc-excess cells. When cells entered stationary phase due to the depletion of zinc, the final concentration was below 1.5×10^8 /ml, and when they entered stationary phase in excess zinc, the final concentration was above 4.5×10^8 /ml. The volume distributions are presented for zinc-limited and zinc-excess cells, respectively, after 24 h in stationary phase (Fig. 1A and B). The zinc-limited population exhibited a relatively symmetrical distribution around the mean volume of 27 μm^3 (standard deviation, 13.2). The average cell in this population was in the volume range of 26 to 30 μm^3 . In contrast, the latter population exhibited an asymmetric distribution weighted towards the lower range of volumes. The mean volume in this population was 18.8 μm^3 (standard deviation, 12), and the average cell was in the volume range of 16 to 20 μm^3 .

As is evident in the stationary-phase population of zinc-excess cells (Fig. 1B), many of the spheres were extremely small, indeed no bigger than the expected volume of the nucleus of the cell. We therefore tested whether small cells contained nuclei. Populations of cells which had entered stationary phase under zinc-limiting and zinc-excess conditions were heat fixed, treated with RNase, and stained with acridine orange (18). In the former case, 400 spheres were scored for nuclear content, and in the latter case, 800 spheres were scored. In the stationary-phase population of zinc-limited cells, 100% of the spheres contained nuclei; in the stationary-phase population of zinc-excess cells, 98% contained nuclei. Therefore, even spheres with volumes of 5 μm^3 or less contained nuclei. In these cases, the nucleus filled virtually the entire cell space.

Outgrowth kinetics. In previous reports, it was demonstrated that zinc-limited cells synchronously formed evaginations after an average period of 135 min, but zinc-excess cells semisynchronously formed evaginations after an average period of ca. 90 min. In these original studies (2, 15), evagination kinetics were scored for cells which were not separated by sonication. Therefore, cells which had not cell separated were scored as a single unit, giving an incomplete picture of evagination. To obtain a more complete picture of outgrowth kinetics, stationary-phase populations of zinc-limited and zinc-excess cells were lightly sonicated. This separated the population into roughly 85% unbudded singlets and 15% doublets. These cells were inoculated into fresh medium at 37°C (pH 4.5) and scored for evagination with time. In a parallel culture of released zinc-limited cells, the first new evaginations appeared at ca. 90 min (Fig. 2). At 100 min, 10% of the population had evaginated; at 135 min, 50% had evaginated; and at 170 min, 90% had evaginated (Fig. 2). In contrast, the evagination kinetics of released zinc-excess cells were far less synchronous. Cells began to evaginate after 20 min, reaching roughly 50% by 90 min and 90% by 160

min (Fig. 2). Zinc-limited cells evaginated at a maximum rate of ca. 11% per 10 min; in contrast, zinc-excess cells evaginated at a maximum rate of ca. 5.5% per 10 min (Fig. 2).

The decrease in synchrony in released cultures of zinc-excess cells may be the result of the heterogeneity in cell volumes, in particular the increase in the proportion of very small cells. Chaffin and Sogin (5) presented evidence that larger cells evaginated earlier than small cells in a population

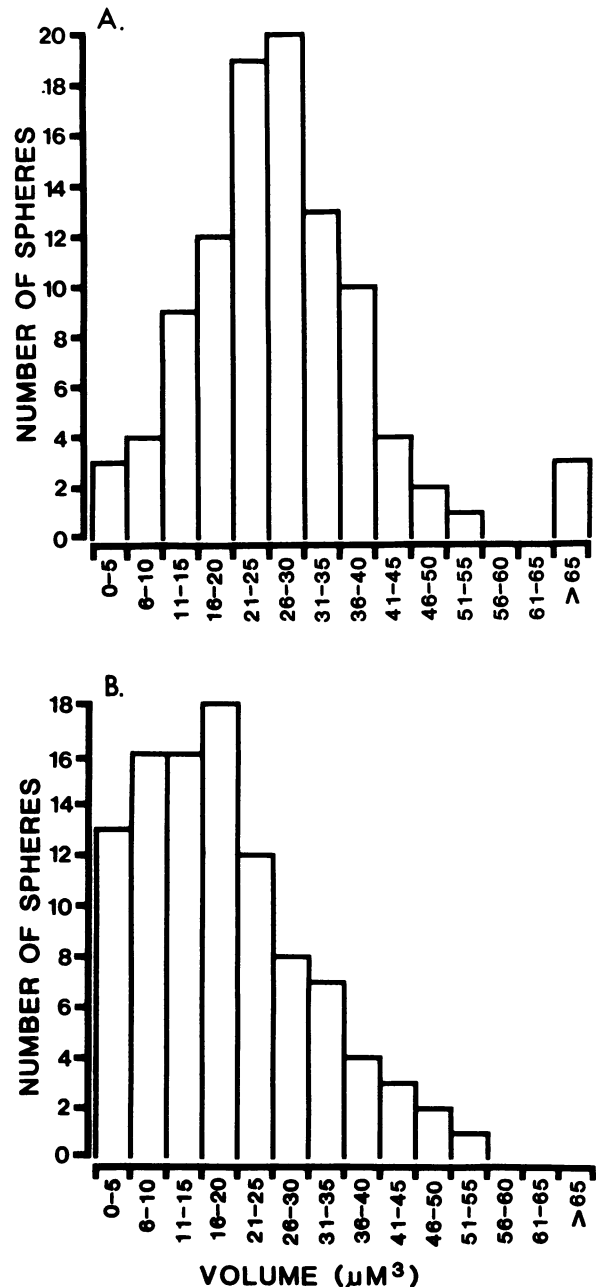


FIG. 1. Volume distributions of stationary-phase populations of zinc-limited (A) and zinc-excess (B) cells. Cell populations in stationary phase for roughly 24 h were lightly sonicated. Individual spheres were then measured. For zinc-limited cells, the average cell exhibited a volume of 26 to 30 μm^3 and the mean volume was calculated to be 27 μm^3 (standard deviation, 13.2). For zinc-excess cells, the average cell exhibited a volume of 16 to 20 μm^3 and the mean volume was calculated to be 18.8 μm^3 (standard deviation, 12).

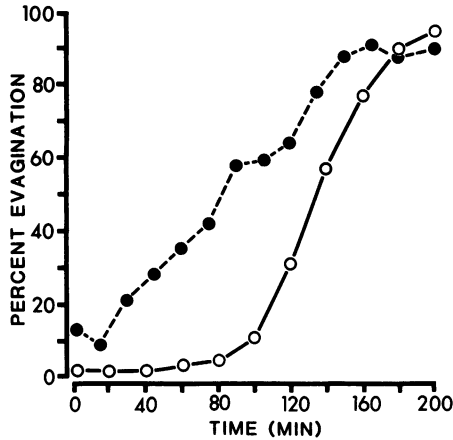


FIG. 2. Evagination kinetics of zinc-limited (○) and zinc-excess (●) cell populations. Cell populations in stationary phase for roughly 24 h were pelleted, resuspended in fresh medium at 37°C, and rotated at 200 rpm. The T_{50} of zinc-limited cells was 135 min, and the T_{50} of zinc-excess cells was 90 min. Note the extreme difference in the synchrony of evagination between the two populations.

of stationary-phase cells released into fresh medium. We tested this possibility for zinc-excess cells. We compared the distributions of volumes for cells which had evaginated and for cells which had not evaginated in a population 105 min after release into fresh medium, when 60 to 65% of the entire population had evaginated (Fig. 3). Unbudded cells exhibited a volume distribution between 6 and 35 μm^3 , and the average unbudded cell was within the volume range of 16 to 20 μm^3 (Fig. 3A). Budded cells exhibited a volume distribution between 11 and 65 μm^3 , and the average budded cell was within the volume range of 31 to 35 μm^3 (Fig. 3B), almost twice the volume of the average unbudded cell.

RNA synthesis. Cells which enter stationary phase under zinc-limiting conditions may require more time to reacquire the levels of cytoplasmic zinc which are necessary for the resumption of macromolecular synthesis (2, 6, 7) after they are released into fresh medium. If this is the case, as has been suggested (15; Soll, in press), then one would expect the resumption of RNA synthesis to occur much later in zinc-limited cells than in zinc-excess cells released into fresh medium. To test this possibility, we monitored the kinetics of RNA synthesis in the alternate populations, first by incorporation of [^3H]uridine into RNA and second by direct measurements of RNA by the Orcinol method (19).

[^3H]uridine incorporation. Both zinc-limited and zinc-excess populations began to incorporate [^3H]uridine into RNA ca. 10 min after release into fresh medium at 37°C (pH 4.5) (Fig. 4). However, the rate of initial incorporation in the population of zinc-limited cells (between 10 and 70 min) was far below the rate of incorporation in the population of zinc-excess cells. In repeat experiments, the initial rate of incorporation in zinc-limited cultures was one-third to one-fourth the rate observed in zinc-excess cells. For zinc-limited cells, it is clear that the rate of [^3H]uridine incorporation into RNA abruptly changed from the relatively low but constant level during the initial period (between 10 and 70 min) to a much higher level after 80 min (Fig. 4A). In contrast, for zinc-excess cells [^3H]uridine incorporation into RNA rapidly achieved a maximum rate between 10 and 20 min; the rate then remained constant. Therefore, zinc-limited and zinc-excess cells released into fresh medium achieve a maximum rate of RNA synthesis at roughly 60 and 20 min, respectively, a difference of 40 min. This is roughly the same difference

as that observed for the average times of evagination (Fig. 2). Interestingly, the final rates of [^3H]uridine incorporation into RNA in the two populations were comparable (cf. Fig. 4A and B). Repeat experiments gave similar results (data not shown).

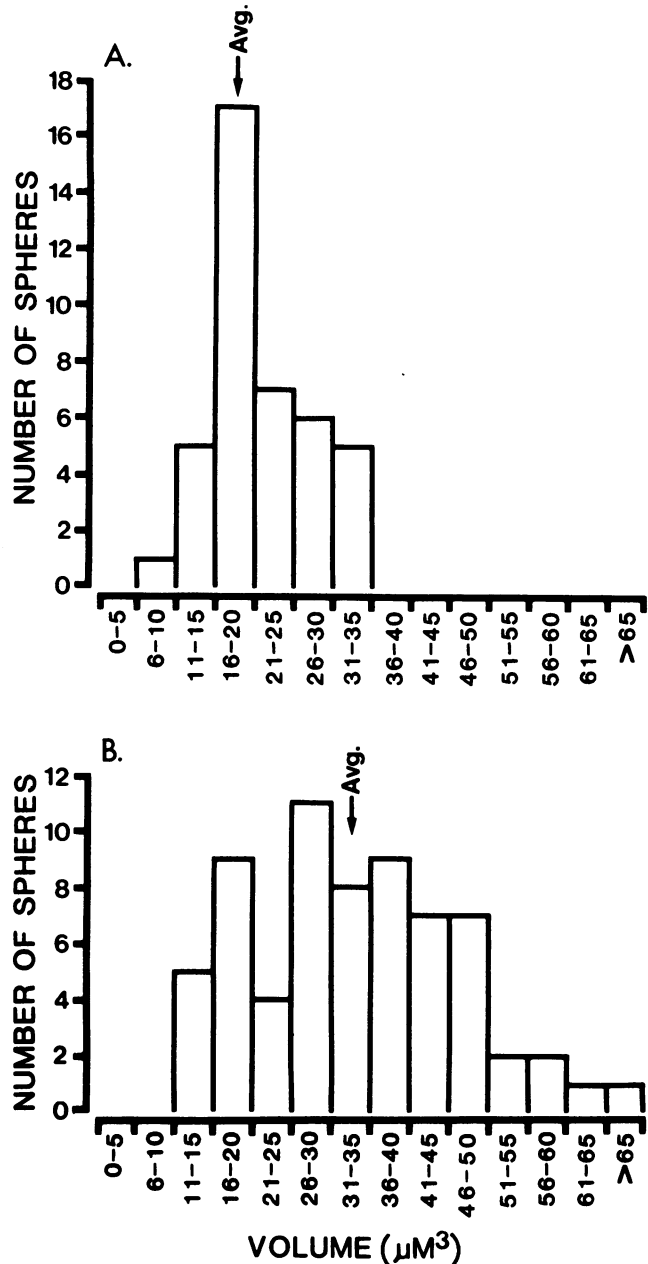


FIG. 3. Volume distributions of unbudded (A) and budded (B) cells in a partially evaginated population of zinc-excess cells. A population of zinc-excess cells in stationary phase for roughly 24 h was pelleted, resuspended in fresh medium, and rotated for 105 min. At this time, roughly 60 to 65% of the population had evaginated. Cells then were removed, measured for volume, and scored for evagination. The average unbudded cell was in the volume range of 16 to 20 μm^3 , and the average budded cell was in the volume range of 31 to 35 μm^3 . Note that if combined, the volume distribution of the entire population shifted to far higher values than those observed in the original stationary-phase population (Fig. 1B), indicating that the original cells in the population have increased their volumes dramatically.

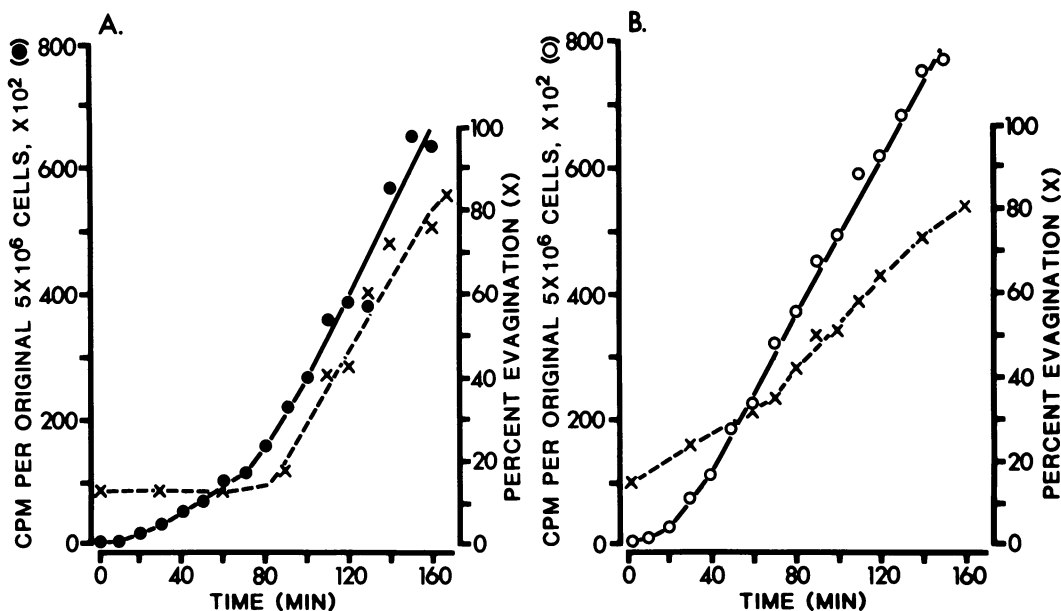


FIG. 4. Kinetics of [³H]uridine incorporation into RNA in evaginating populations of zinc-limited (A) and zinc-excess (B) cells. The kinetics of evagination (x) are presented for both populations.

Total RNA. Since the kinetics of [³H]uridine incorporation may not accurately reflect the rate of RNA synthesis due to changes in accessibility and pool expansion during outgrowth (see below for a more detailed treatment of this question), we also monitored total cellular RNA during outgrowth in populations of zinc-limited and zinc-excess cells by the Orcinol method (19) (Fig. 5A and 5B, respectively). In released cultures of zinc-limited cells, total RNA remained relatively stable for ca. 110 min. It then began to accumulate at a relatively constant rate. Interestingly, the first cells with new evaginations were also observed at

roughly 100 min in the particular experiment presented in Fig. 5A. In contrast, total RNA remained stable for only the first 30 min in released cultures of zinc-excess cells. RNA then began to increase at a low but constant rate between 30 and 90 min. At 90 min, the rate of accumulation increased dramatically and then remained constant for at least the subsequent 90-min period. To demonstrate the reproducibility of these complex kinetics, two sets of data acquired in independent experiments, and for cells grown at different concentrations of excess zinc (2.5 and 9.0 μM), are presented (Fig. 5B). Again, it should be noted that the initial onset of

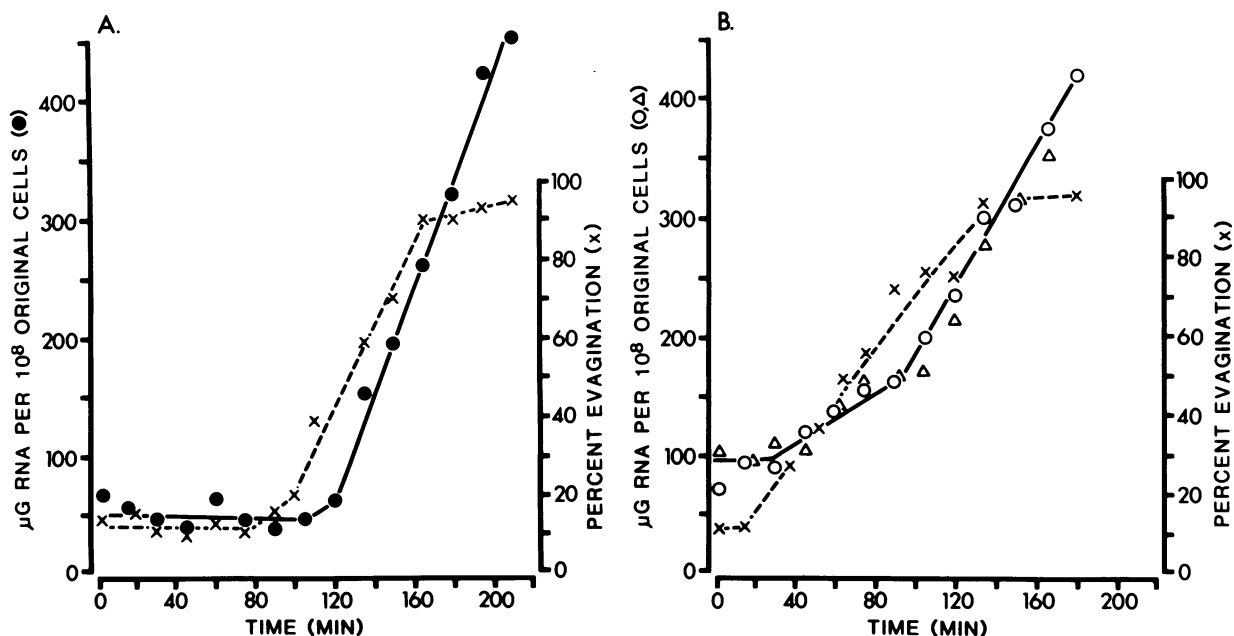


FIG. 5. Measurements of total RNA in evaginating populations of zinc-limited (A) and zinc-excess (B) cells. The kinetics of evagination (x) are presented for both populations. For zinc-excess cells, sets of data for two independent experiments (○, Δ) are presented to dramatize the two transition points at roughly 30 and 90 min, respectively.

net RNA accumulation occurred very close to the time that evaginations first appeared in the population (Fig. 5B). The more complex data of zinc-excess cells may reflect the degree of heterogeneity in the evaginating population.

Sedimentation profiles of newly synthesized RNA. To test which species of RNA represent the major transcription products in released cultures of zinc-limited and zinc-excess cells during the pre- and postevagination periods, cultures were labeled between 70 and 80 min and between 170 and 180 min with [^3H]uridine. Total RNA was extracted and separated in a sucrose gradient (see above for details). The profiles of radioactive RNA are presented for zinc-limited cells pulsed between 70 and 80 min and between 170 and 180 min, respectively (Fig. 6A and B); the profiles of radioactive RNA are presented for zinc-excess cells pulsed during the same respective periods (Fig. 6C and D). The results demonstrate that for both zinc-limited and zinc-excess cells, the RNA synthesized during the pre- and postevagination periods was predominantly ribosomal.

Protein synthesis. If protein synthesis is coupled to RNA synthesis, then the onset of protein synthesis should occur earlier in zinc-excess cells. This possibility was investigated first by following the continuous kinetics of [^3H]leucine incorporation into protein and second by measuring total cell protein with time.

[^3H]leucine incorporation. Populations of zinc-limited cells began to incorporate [^3H]leucine into protein roughly 60 min after they were diluted into fresh medium (Fig. 7A). Cells incorporated [^3H]leucine at a relatively constant rate for the subsequent 70 min. After 130 min, the rate of incorporation increased dramatically (Fig. 7A). In contrast, zinc-excess cells began to incorporate [^3H]leucine into protein roughly 20 min after they were diluted into fresh medium (Fig. 7B), 40 min earlier than zinc-limited cells. In addition, an apparent increase in rate occurred at roughly 100 min (Fig. 7B), 30 min earlier than a comparable increase in zinc-limited cells.

Total protein. Total protein of zinc-limited cells remained stable for roughly 60 min (Fig. 8A). Between 60 and 135 min, total cell protein increased at a low but relatively constant rate. After 135 min, the rate increased dramatically. In contrast, total protein of zinc-excess cells remained stable for roughly 30 to 40 min in repeat experiments (Fig. 8B). At this time, total cell protein began to increase at a relatively constant rate. Between 110 and 140 min in repeat experiments, the rate increased dramatically. Therefore, net protein accumulation began between 20 and 30 min earlier in zinc-excess cultures than in zinc-limited cultures.

DISCUSSION

Stationary-phase phenotype. The role of zinc in the growth and dimorphism of the infectious yeast *Candida albicans* has become a renewed topic of interest (Soll, in press), primarily because of its effects on the phenotype of stationary-phase cells (2, 15, 20, 21; Soll, in press). When cells enter stationary phase due to the depletion of zinc, they are predominantly unbudded and relatively homogeneous in volume. It has been demonstrated by inhibitor studies that when these cells are released into fresh medium, they must progress through a phase of DNA replication before nuclear division (1, 12, 18; D. R. Soll, in P. J. Szanislo, ed., *Fungal Dimorphism: With Emphasis on Fungi Pathogenic to Humans*, in press), indicating that the stationary-phase cell population is homogeneously blocked in the G1 phase (12). Direct measurements of nuclear DNA support this conclusion (14). In contrast, when cells enter stationary phase in excess zinc, the distribution of cell volumes is more heterogeneous and

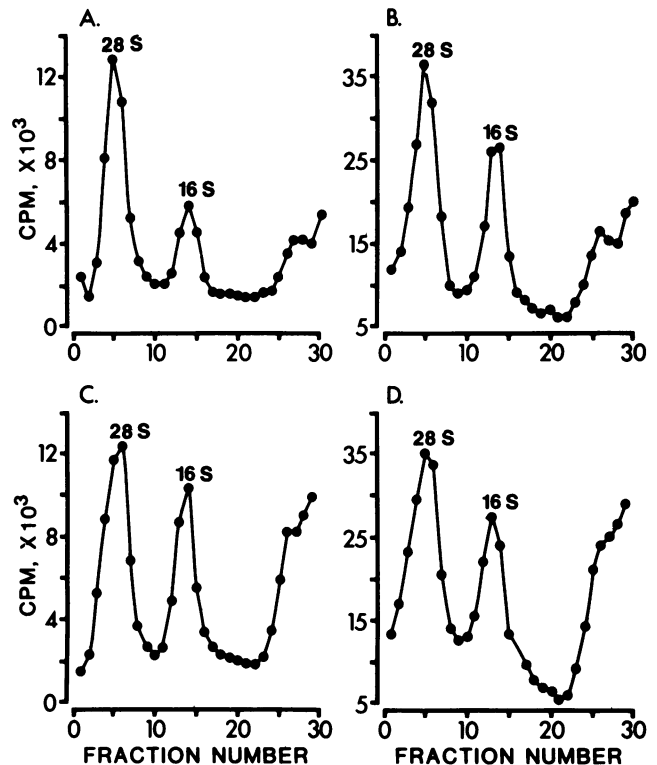


FIG. 6. Sucrose gradient profiles of the RNAs labeled during the pre- and postevagination periods of zinc-limited cells and during the more asynchronous evagination period of zinc-excess cells. Zinc-limited cells were labeled with [^3H]uridine between 70 and 80 min (A) during the preevagination period and between 170 and 180 min (B) during the postevagination period. Zinc-excess cells were labeled with [^3H]uridine between 70 and 80 min (C) and between 170 and 180 min (D) as well. The predominance of rRNA synthesis in both populations is most noteworthy. Sedimentation coefficients for ribosomal RNAs are taken from reference 22.

skewed in the direction of lower values. Thirty percent exhibit volumes of less than $10\ \mu\text{m}^3$, far below the mean volume of $27\ \mu\text{m}^3$ for zinc-limited cells. We have demonstrated that even these small cells contain nuclei. Therefore, unlike zinc-limited cells, zinc-excess cells appear to be heterogeneously blocked in the bud growth cycle, but like zinc-limited cells, they appear to be homogeneously blocked in the nuclear division cycle. Direct measurements of nuclear DNA and the inhibition of nuclear division by hydroxyurea indicate that this is indeed the case (M. Herman and D. R. Soll, unpublished observations).

We have presented evidence in this report that when zinc-excess cells are released into fresh medium, larger cells evaginate earlier than smaller ones, an observation similar to one made by Chaffin and Sogin (5) on presumably zinc-limited cells. Recently, it was demonstrated that zinc-limited cells released into fresh medium grow in volume during the entire preevagination period (M. Herman and D. R. Soll, *J. Gen. Microbiol.*, in press), indicating that cells must grow either to a particular threshold volume or for a particular period of time before evagination can occur. Analysis of volume distributions of populations of zinc-excess cells after release into fresh medium also indicates an increase in volume before evagination (D. R. Soll, unpublished data). More interestingly, it is clear that the smaller cells in the population grow for a longer period than larger cells before evagination occurs (Fig. 3). Therefore, it is probable that the

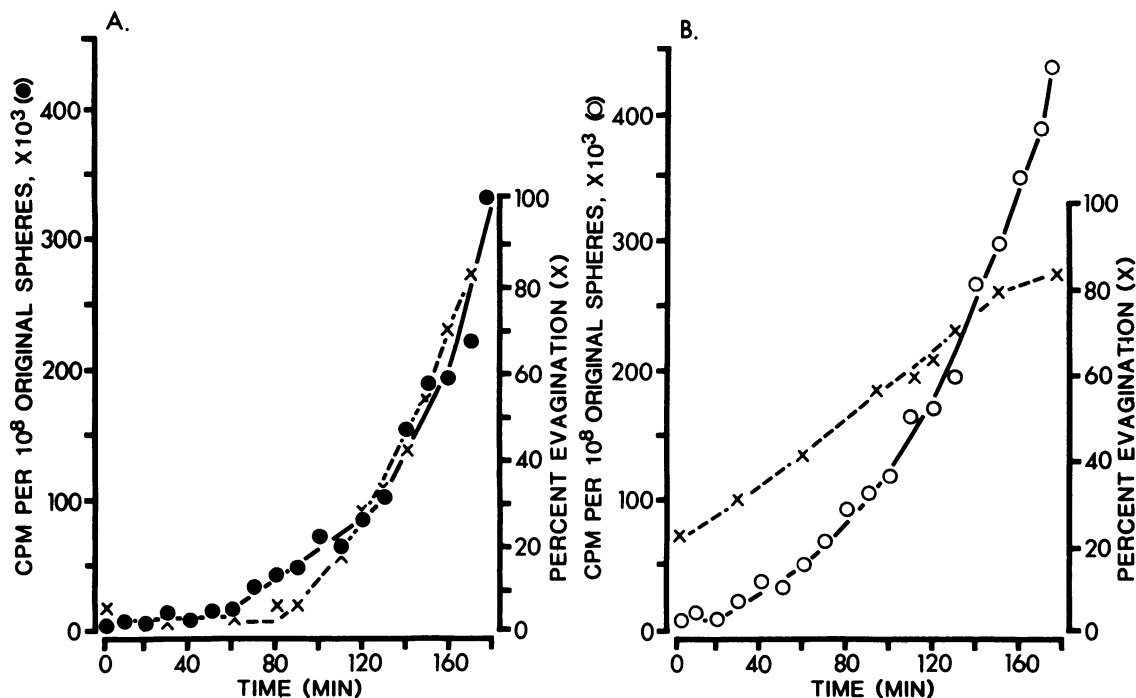


FIG. 7. Kinetics of [³H]leucine incorporation into protein in evaginating populations of zinc-limited (A) and zinc-excess (B) cells. The kinetics (x) of evagination are presented for both populations. Most noteworthy is the 40-min difference in the onset of incorporation between the alternate populations.

decrease in the synchrony of evagination in zinc-excess cultures, observed in the present study, is due to the increased proportion of very small cells and the greater heterogeneity of original cell volumes.

RNA synthesis. Both zinc-limited and zinc-excess cells begin to incorporate [³H]uridine into RNA roughly 10 min after the cells are released into fresh nutrient medium (see synopsis in Fig. 9). Zinc-excess cells rapidly achieve a

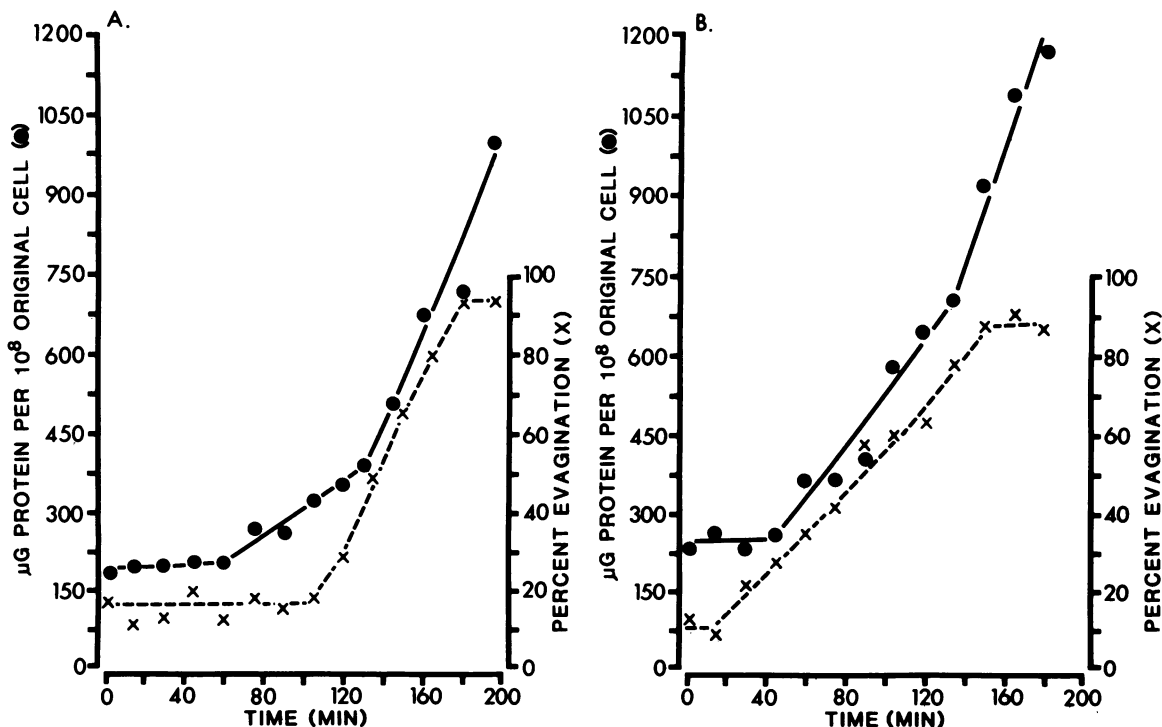


FIG. 8. Measurements of total protein in evaginating populations of zinc-limited (A) and zinc-excess (B) cells. The kinetics of evagination (x) are presented for both populations. Again, the difference in the onset of net protein accumulation between the alternate populations should be noted.

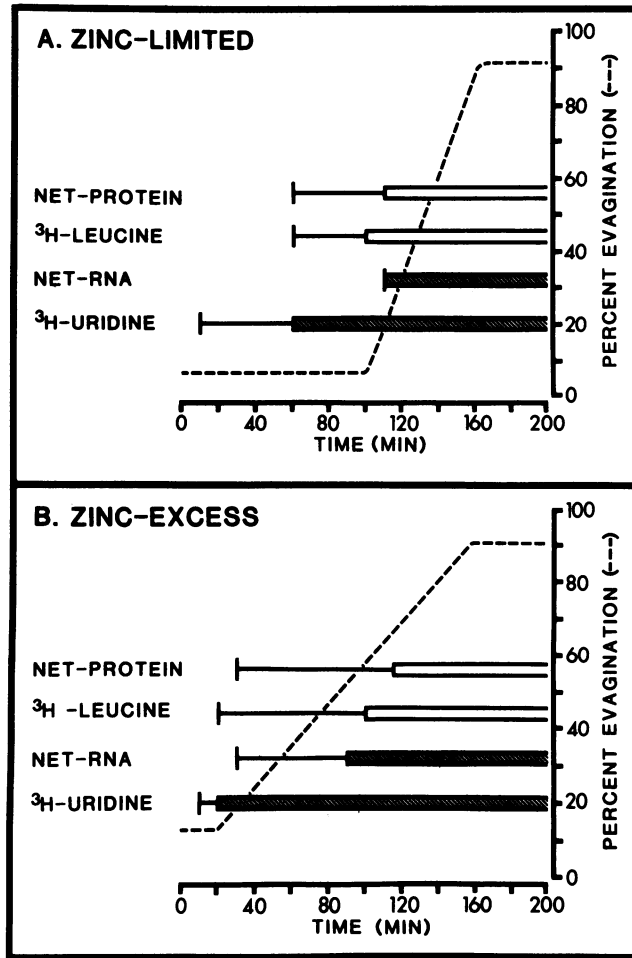


FIG. 9. Summary of the kinetics of [^3H]uridine incorporation into RNA, net RNA accumulation, [^3H]leucine incorporation into protein, and net protein accumulation during evagination of released populations of zinc-limited (A) and zinc-excess (B) cells. The initial vertical bar for each parameter represents onset. The thin horizontal bar represents incorporation or net accumulation at a low rate, and the wider horizontal bar represents incorporation or net accumulation at a higher rate. Representative kinetics of evagination are presented as dashed lines. This summary depicts the detailed results presented in Fig. 4 to 8.

maximum rate of incorporation by 20 min, but zinc-limited cells incorporate [^3H]uridine into RNA at a low but constant rate for 60 subsequent min and then shift to a maximum rate between 70 and 80 min. The difference in the time at which a maximum rate is achieved in the alternate populations is 50 min, roughly the same difference observed between the average times (T_{50} s) of evagination. However, caution must be exerted in comparing the results obtained in the alternate populations because of the difference in evagination synchrony. It is clear that the population of zinc-excess cells reaches a maximum rate of incorporation at roughly the time that the first evaginations appear in the population (Fig. 9). However, if these kinetics reflect the rates of RNA synthesis in all cells, then one must conclude that the rate of RNA synthesis reaches a maximum level far before a cell evaginates, on the average. This is supported by the incorporation kinetics in the more synchronous cultures of zinc-limited cells. In these cultures, the rate of [^3H]uridine incorporation reaches a maximum rate 20 to 30 min before the appearance

of the first evaginations in the population and roughly 60 min before the time of average evagination (Fig. 9). Together, these results indicate that the onset of RNA synthesis may precede evagination in an individual cell by as much as 1 h. This is not a surprising result since, as previously mentioned, cells do grow significantly in volume during the preevagination period (Herman and Soll, in press). However, caution must again be exerted in comparing the kinetics of precursor incorporation since rates can easily be influenced by changes in uptake rate as well as by expansion of the internal pools. These concerns may be especially relevant in cases in which growth is reinitiated.

To help us assess the observed differences in [^3H]uridine incorporation, we have monitored total RNA with time. In zinc-limited cultures, total cellular RNA remains stable up to the time of evagination (roughly 110 min) and then begins to increase at a constant rate. Surprisingly, net RNA accumulation begins roughly 50 min after cells begin to incorporate [^3H]uridine at the maximum rate. Therefore, incorporation kinetics in this case do not appear to accurately reflect the rates of RNA synthesis, although turnover rates of RNA will have to be measured before this can be firmly concluded. It is probably safe to suggest that the time at which cells begin to incorporate [^3H]uridine at the maximum rate may reflect the true initiation point for RNA synthesis, but the onset of net RNA accumulation may be a more accurate indicator of the time at which the maximum rate of RNA synthesis begins. In contrast to zinc-limited cultures, net RNA accumulation in zinc-excess cultures begins at roughly 30 min, very soon after evaginations first appear in the population. Net RNA increases at a constant rate for roughly 50 subsequent min. The rate then increases to a maximum rate. What is perhaps most notable about the results for both zinc-limited and zinc-excess cells is the correlation between the onset of net RNA accumulation and appearance of the first evaginations in both populations. Again, the kinetics of net accumulation are difficult to compare between the two populations because of the difference in evagination synchrony. Indeed, the complexity of the accumulation plot for zinc-excess cells (Fig. 5B) indicates more heterogeneity in this population than is apparent in the simple evagination plots.

Protein synthesis. In contrast to the results obtained for RNA synthesis, the results obtained by [^3H]leucine incorporation and direct protein measurements are consistent (Fig. 9). Both methods demonstrate that in zinc-limited cultures, protein synthesis begins at 60 min, the same time that [^3H]uridine incorporation increases to a maximum rate. In addition, both methods indicate that a dramatic increase in protein synthesis accompanies evagination and the onset of net RNA accumulation. Both methods also demonstrate that in zinc-excess cultures, protein synthesis begins between 20 and 30 min and increases to a maximum rate between 100 and 115 min. Again, the onset of protein synthesis correlates with the achievement of a maximum rate of [^3H]uridine incorporation. Perhaps the most noteworthy aspect of these results is the difference in the onset of protein synthesis between zinc-limited and zinc-excess cultures. In the latter population, protein synthesis begins roughly 30 to 40 min earlier than in the former, roughly the same differences observed for the T_{50} or evagination and for the achievement of a maximum rate of [^3H]uridine incorporation into RNA.

Zinc and the regulation of macromolecular synthesis during outgrowth. It is clear from the results presented in this study that the zinc history of stationary-phase cells affects not only average cell size and subsequent evagination kinetics but

also the timing of macromolecular synthesis after release from stationary phase. The results are consistent with the suggestion previously made (17; Soll, in press) that the difference in the average time of evagination between zinc-limited and zinc-excess cells may be due to roughly a 30- to 40-min delay in the onset of RNA and protein synthesis in the former cells. Although direct evidence has not been obtained, it is likely that the difference in the onset of macromolecular synthesis is due to the zinc content of the two cell populations. The increase in the time preceding the onset of macromolecular synthesis in zinc-limited cells may reflect the time necessary for these cells to reaccumulate zinc to levels sufficient to support renewed RNA and in turn protein synthesis (7, 15; Soll, in press).

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