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Regulation of Protein Synthesis During Energy Limitation of *Saccharomyces cerevisiae*

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Arsenate, a competitive inhibitor with phosphate in phosphorylation reactions, has been used to lower adenine and guanine nucleotide levels in *Saccharomyces cerevisiae* to study nucleotide effects on protein synthesis. By measuring polysome levels, we have shown that initiation of protein synthesis is much more sensitive than elongation or termination to inhibition when the ATP/ADP, GTP/GDP ratios are low. When the arsenate-phosphate molar ratio was 0.27, protein synthesis was inhibited by about 85% and the kinetics of polysome decay was similar to that observed with the initiation inhibitor, verrucarin-76, or with the protein synthesis initiation mutant, ts187, at the restrictive temperature. With this level of arsenate, the adenylate energy charge dropped from 0.9 to 0.7 and the ATP/ADP and GTP/GDP ratios dropped from 6 to 2. The observed correlations between nucleotide ratio changes and inhibition of protein synthesis suggest that the former may be a control signal for the latter. The significance of these in vivo correlations will have to be tested with an in vitro protein synthesizing system. Higher arsenate levels resulted in even lower ATP/ADP, GTP/GDP ratios and in a slower decay of polysomes, implying that, eventually, elongation (in addition to initiation) was being inhibited.

Protein synthesis is an expensive process for a cell. For each polypeptide bond formed, at least four phosphoanhydride bonds are hydrolyzed and four ATP molecules are required to reform these phosphoanhydride bonds. The ATP pool in a cell, if not replenished, can support protein synthesis for only a very short time. For example, in yeast growing on glucose with about a 2-h doubling time, the ATP pool of 4 μmol per g of dry weight (45) would be exhausted in about 6 s if protein synthesis continued at its normal rate of 0.16 μmol of amino acids polymerized per s per g of dry weight (calculated from data of Waldron and Lacroute, 40). Clearly, the cell must have regulatory mechanisms that rapidly sense an energy-limiting situation and inhibit the energy-utilizing processes of the cell, especially those processes such as protein synthesis, which are such a heavy drain on a cell's energy supply.

In this paper, we have used arsenate to energy limit *Saccharomyces cerevisiae*. Arsenate (AsO_4^{-3}) can be used in cellular reactions in place of phosphate (PO_4^{-3}). Arsenate enters yeast cells by the phosphate transport system (28) and is used by enzymes which normally use phosphate, such as phosphoglyceraldehyde dehydrogenase and glycogen phosphorylase (31). Arsenate uncouples oxidative phosphorylation presumably by being used in place of phosphate (34). Arsenate esters and anhydrides are very labile, and abortive hydrolysis products can occur (18, 31). Thus, in the presence of arsenate, the phosphorylation of ADP to ATP is inhibited and cells become energy limited. Under these conditions, we find that protein synthesis is inhibited at an initiation step. The GTP/GDP and ATP/ADP ratios and adenylate energy charge all dropped significantly. These nucleotide changes may be a sufficient signal for the observed inhibition in protein synthesis, although future in vitro experiments will be necessary to rigorously test this point.

MATERIALS AND METHODS

Organism and culture conditions. *S. cerevisiae* strain A364A *lys2 tyr1 his1 gal1 ade1 ade2 ura1* and a mutant of A364A, ts187, which has a temperature-sensitive defect in initiation of protein synthesis (13), were used in this study.

Cells were grown at room temperature with rotatory shaking. The glucose medium contained per liter: 6.7 g of yeast nirogen base (Difco Laboratories) 20 g of glucose,10 g of succinic acid (used as a buffer, pH 5.8 to 6.0), 10 mg of adenine, 10 mg of uracil, 40 mg of lysine, 40 mg of tyrosine, and 40 mg of histidine. Yeast-nitrogen base contains per liter: 5 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.1 g of NaCl, 0.1 g of CaCl, plus vitamins and trace elements (7).

Spheroplasts (14) were prepared by incubating 1×10^9 to 3.5×10^9 cells suspended in 10 ml of 1 M sorbitol with 0.2 ml of glusulase (Endo Laboratories) for 30 min at room temperature. Spheroplasts were collected by centrifugation (3,000 × *g*, 5 min) and suspended in glucose minimal medium stabilized with either 1 M sorbitol or 0.5 M $MgSO_4$ and regrown (at a cell concentration of about 5×10^7 cells/ml) at room temperature with rotatory shaking for at least 2.5 h before the experiment was started.

Polyribosome analysis. A 25-ml portion of the spheroplast culture (approximately 1.3×10^8 cells) was harvested by adding cycloheximide (1 mM final concentration), chilling, and centrifuging at 3,000 × *g* for 5 min. The pellets were stored at −70°C. To extract the polyribosomes (12), the spheroplast pellets were suspended in lysis buffer which contained 0.1 M NaCl, 0.03 M $MgCl_2$, and 0.01 M Tris-Chloride, pH 7.4. Deoxycholate (0.02 ml of 5% solution) was added and samples were held on ice for 5 min. Then, 0.03 ml of 5% Brij-58 (Atlas Powder Co., Wilmington, Del.) was added. Cell debris was removed by centrifugaton at 12,000 × *g* for 5 min. The supernatant was layered on a 10 to 40% linear sucrose gradient in lysis buffer. Gradients were centrifuged in a Spinco SW56 rotor at 55K for 25 min at 4°C, stopping without the brake. Gradients were collected by pumping 70% sucrose to the bottom of the centrifuge tube, thus pushing the gradient out the top into a flow-through cell in a Beckman DB-G spectrophotometer, and the absorbancy at 260 nm was recorded. The areas under the monosome and polysome regions were estimated by use of a planimeter or by cutting out and weighing tracings. The baseline of absorbancy at 260 nm for each gradient was assumed to be that of a blank (unloaded) sucrose gradient. No correction was made for trailing of the monosome peak into the polysome region.

Protein synthesis measurements. The rate of protein synthesis in spheroplasts was measured by following the rate of [*U*-^{14}C]lysine incorporation into acid-precipitable material. Lysine concentration in the growth medium was reduced to 8 μg/ml (1/5 normal) for these incorporation experiments.

Nucleotide measurements. When spheroplasts were suspended in regrowth medium (stabilized with sorbitol), [8-^{14}C]adenine was added for the regrowth period (2.5 h) at 10 μCi/ml, for a final specific activity of 45 μCi/mol. Both adenine and guanine nucleotides are labeled by exogeneously added [8-^{14}C]adenine since the yeast strain used is genetically blocked early in the common purine pathway. It required about 30 min to saturate the ATP pool of yeast spheroplasts with exogeneously added [8-^{14}C]adenine (25). Also, we found that the GTP pool is saturated with respect to the ATP pool by the end of our regrowth period.

Nucleotides were extracted from spheroplasts by adding 0.5 ml of culture to 0.1 ml of 35% $HClO_4$ acid and holding on ice for 30 min. Samples then were centrifuged 12,000 × *g* for 1.5 min in an Eppendorf Microfuge to remove precipitated proteins. The supernatant (0.5 ml) was neutralized with about 0.16 ml of 0.30 N KOH containing 0.058 M $KHCO_3$. After standing about 15 min, the $KClO_4$ salt was removed by centrifugation. Supernatants were stored at −70°C.

Two-dimensional thin-layer chromatography on PEI-cellulose plates (Cel 300 PEI from Macherey-Nagel and Co., distributed by Brinkmann Instrument, Inc.) was used to resolve the adenine and guanine nucleotides. Plates were spotted with 80 μl of sample and with 5 nmol of each nucleotide for visual standards. Plates were soaked for 30 min in a tank of water to remove the [8-^{14}C]adenine and dried. In the first dimension, LiCl was used as described by Randerath and Randerath (27). The plates were developed in 0.2 M LiCl for 2 min, in 1.0 M LiCl for 6 min, and in 1.6 M LiCl to 15 cm above the origin. Plates were rinsed in methanol for 15 min (27) to remove the LiCl and dried. The second dimension was developed in 0.75 M KH_2PO_4, pH 3.4, with 1 mM EDTA (Sprague, 1977, [32] used this solvent for one-dimensional development) to 15 cm above the origin.

After autoradiography, the appropriate spots of the PEI plate were cut out and counted in toluene-2,5-diphenyloxazole scintillation fluid.

RESULTS

Arsenate inhibition of protein synthesis. The rate of protein synthesis in yeast cells has been plotted as a function of the arsenate concentration (Fig. 1). At 2 mM arsenate, which represents an arsenate-phosphate ratio of 0.27, the rate of protein synthesis was inhibited by about 85%. The effect of this level of arsenate on plyribosomes and nucleotide levels was studied.

Initiation of protein synthesis is inhibited. Inhibition of protein synthesis at an initiation step can be distinguished from inhibition at an elongation-termination step by measurement of polyribosome levels in the cells. An inhibition at initiation leads to decay of polysomes, whereas an inhibition at elongation or termination conserves polysomes (11–14, 20, 24, 25, 33, 45). For normal yeast spheroplasts, polysomes represent about 90% of the total ribosomal material. After arsenate addition, this polysome

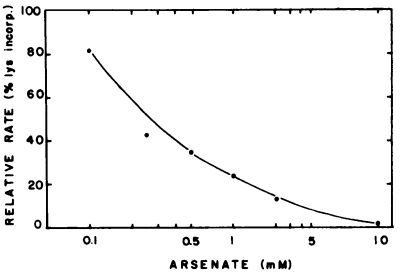

FIG. 1. *Effect of arsenate concentration on the rate of protein synthesis in yeast spheroplasts. The rate of [U-^{14}C]lysine incorporation into trichloroacetic acid-precipitable material over a 30-min period was determined for yeast spheroplasts incubated in medium with various concentrations of arsenate. The phosphate concentration in the medium was 7.5 mM.*

pattern changed dramatically (Fig. 2). Polysomes had decayed and monosomes had accumulated. The time course of polysome decay was followed; as shown in Fig. 3, the percentage of total ribosomes in polysomes declined in about 4 min from 90% in the control to 30 to 35% with arsenate. Thus, initiation of protein synthesis appears to be inhibited.

The residual polysome level of 30% is higher than expected with a residual [^{14}C]lysine incorporation rate of 15%. However, when polysome levels are low, the large monosome peak trails significantly into the polysome region and no correction has been made for this trailing. Thus, it is probable that the observations are consistent.

The kinetics of polysome decay in the presence of arsenate was compared to that found for a known initiation inhibitor, verrucarin-76 (21)

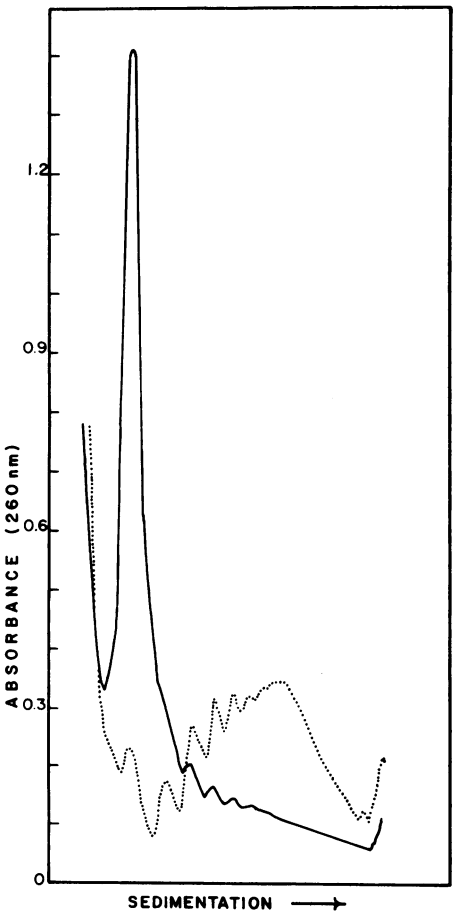

FIG. 2. *Sucrose gradients of polyribosomes from yeast spheroplasts treated with arsenate. Control spheroplasts (· · ·); spheroplasts incubated with 2 mM arsenate for 6 min (——).*

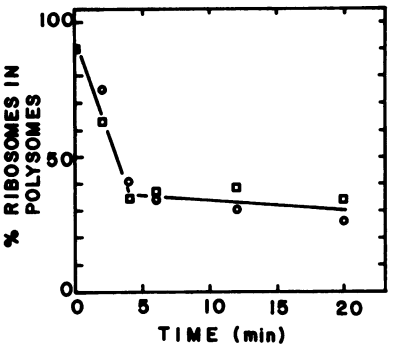

FIG. 3. *Effect of 2 mM arsenate on the polyribosome content of yeast spheroplasts. Arsenate was added to the spheroplast culture at zero time. Data from two separate experiments are plotted.*

and for a temperature-sensitive initiation mutant (13). As shown in Fig. 4, the kinetics of polysome decay for verrucarin-76 and for ts187, at the restrictive temperature, were the same as for the decay with 2 mM arsenate. Similar polysome decay rates were observed with 1 mM and 0.5 mM arsenate (data not shown). Thus, low arsenate levels appear to block protein synthesis preferentially at an initiation step.

Adenine and guanine nucleotide levels. The concentraions of ATP, ADP, AMP, GTP, GDP, and GMP were followed after the addition of 2 mM arsenate. As shown in Fig. 5, the ATP concentration had begun dropping by 1 min and, after 3 min, stabilized at 20% of the control level. There were only small changes in the ADP and AMP levels. The sum concentration of ATP + ADP + AMP dropped to about 25% of the control. The ATP/ADP ratio dropped from 6 to 2, and the adenylate energy charge (1) dropped from 0.92 to about 0.70. When the luciferase assay was used to measure adenine nucleotide levels (6) instead of radioactive labeling and thin-layer separation, identical results were obtained.

The changes in the GTP/GDP ratio and guanylate energy charge were similar to those for adenine nucleotides (Fig. 6). The main difference among the adenine nucleotides and guanine nucleotides was that the sum concentration, GTP + GDP + GMP, remained almost constant, unlike the adenine nucleotide sum. Thus, the GDP and GMP levels rose in a fashion reciprocal to the decrease in the GTP level. The nucleotide changes occurred rapidly enough so that they could be the signal for inhibition of protein synthesis.

Excess phosphate prevents arsenate effects. If it is the competition of arsenate with phosphate in metabolic reactions which causes

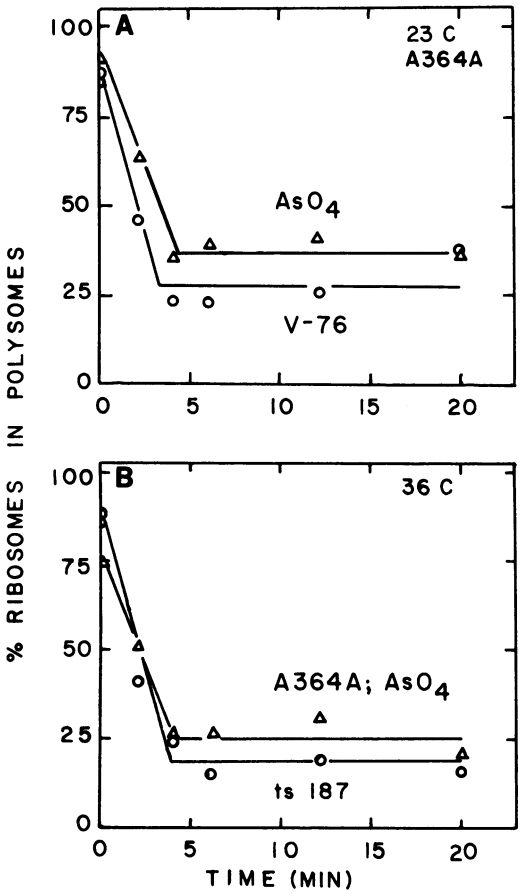

FIG. 4. *Polyribosome levels in A364A spheroplasts treated with verrucarin-76 and in ts187 spheroplasts at the restrictive temperature. (A) A364A (23°C). Verrucarin-76 (20 μg/ml)* (○); *arsenate (2 mM)* (△). *(B) At zero time cultures were shifted to 36°C (for rapid temperature shift, flasks were swirled in 55°C bath for 1 min, then transferred to 36°C shaking water bath). ts187* (○); *A364A with 2 mM arsenate added at zero time* (△).

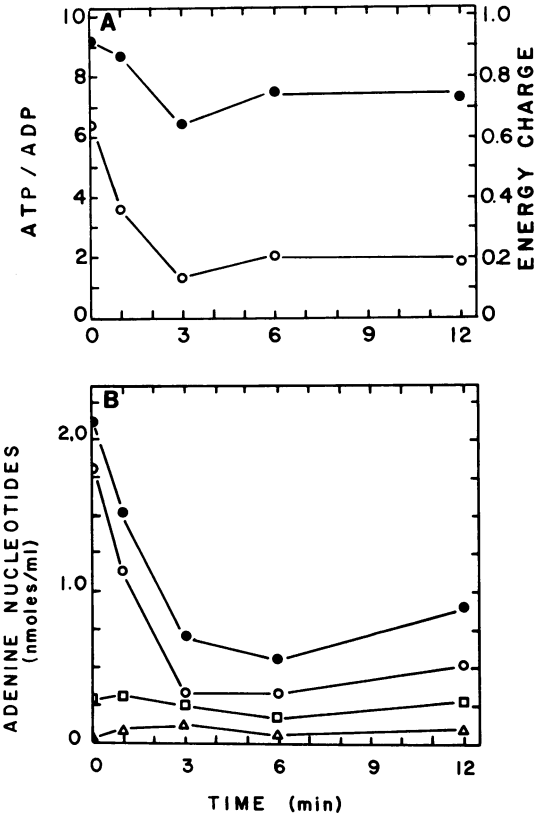

FIG. 5. *Effect of 2 mM arsenate on adenine nucleotide concentrations, ATP/ADP ratio, and adenylate energy charge in yeast spheroplasts. Spheroplasts were labeled with [^{14}C]adenine as described in the text. (A) ATP/ADP* (○); *adenylate energy charge, [ATP + ½(ADP)]/[ATP + ADP + AMP]* (●). *(B) ATP + ADP + AMP* (●); *ATP* (○); *ADP* (□); *AMP* (△).

the observed changes in nucleotide levels and polysome content, then adding excess phosphate with the arsenate should prevent these changes. To determine if this is the case, we added 2 mM arsenate and 100 mM phosphate together to a culture. There was no drop in the ATP/ADP or GTP/GDP ratio, or in the energy charge values and very little change in polysome content (Fig. 7). The small drop in polysomes from 85 to 75% after 12 min corresponds with the slight inhibition of [^{14}C]lysine incorporation into trichloroacetic acid-insoluble material, which was observed in some experiments. Though arsenate may have a slight effect on protein synthesis, which is not relieved by excess phosphate, it is clear that the rapid and substantial drops in polysome levels and in nucleotide concentration ratios are prevented when excess phosphate is present. Thus, arsenate apparently produces these effects via its action as a phosphate analog.

High arsenate levels can slow elongation rate. The studies described so far have been done with 2 mM arsenate (arsenate-phosphate ratio is 0.27), which inhibited lysine incorporation by about 85%. At 10 mM arsenate, lysine incorporation was inhibited by about 98%. In the presence of this higher arsenate concentration, the polysome content of spheroplasts also decayed, but at a slower rate (Fig. 8). About 12 min are required for the polysome decay compared to 4 min for the lower arsenate concentration. This slower polysome decay suggests that there is partial inhibition at an elongation step in addition to an essentially complete inhibition at initiation. This suggested elongation inhibition was confirmed by the findings that (i) ad-

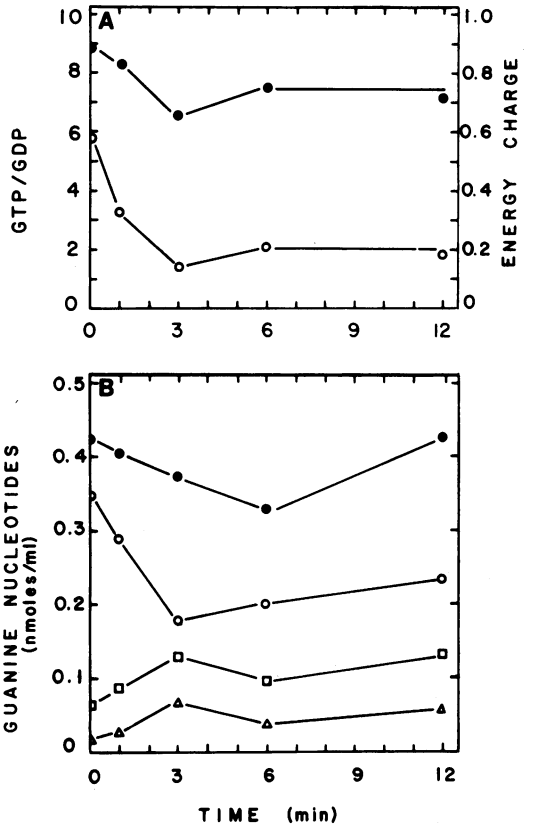

FIG. 6. *Effect of 2 mM arsenate on guanine nucleotide levels, GTP/GDP ratio, and guanylate energy charge in yeast spheroplasts. (A) GTP/GDP (○); guanylate energy charge, [GTP + ½(GDP)]/[GTP + GDP + GMP] (●). (B) GTP + GDP + GMP (●); GTP (○); GDP (□); GMP (△).*

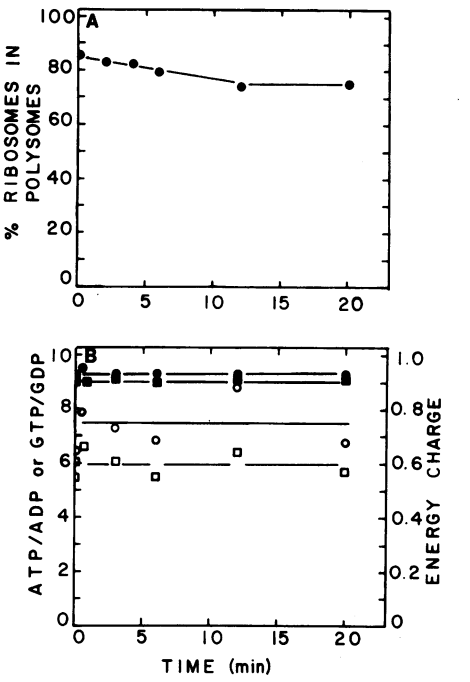

FIG. 7. *Effect of simultaneous addition of arsenate (2 mM) and phosphate (100 mM) on polyribosome levels and nucleotide concentration ratios. (A) Polyribosome content in spheroplasts. (B) ATP/ADP (○); GTP/GDP (□); adenylate energy charge (●); guanylate energy charge (■).*

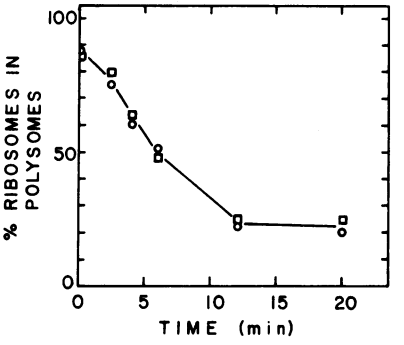

FIG. 8. *Effect of 10 mM arsenate on the polyribosome content of yeast spheroplasts. Arsenate was added to the culture at zero time. Data from two separate experiments are plotted.*

dition of 10 mM arsenate to an A364A culture treated with verrucarin-76 slowed the decay of polysomes (Fig. 9A), and (ii) the addition of 10 mM arsenate to a culture of the mutant ts187 slowed the decay of polysomes at the restrictive temperature (Fig. 9B).

In the presence of 10 mM arsenate, the adenine and guanine nucleotide concentration changes occurred more rapidly and were somewhat more extreme than with the lower arsenate level (Fig. 10). By 1 min, the ATP/ADP and GTP/GDP ratios were 1 or less and the adenylate and guanylate energy charges were about 0.5 (Fig. 11). The partial elongation inhibition may be due to the even lower nucleotide ratios.

DISCUSSION

Preferenial inhibition at initiation. Examination of relative polysome-monosome levels in cells is an established method for determining where in the ribosome cycle an inhibitor acts (24). The decay of polysomes observed when cells were energy limited by arsenate addition indicates that an initiation step has been inhibited. At low arsenate levels that produce 85% inhibition of protein synthesis (or less), the polysome level decayed within about 4 min, i.e., at about the rate observed with known inhibitors

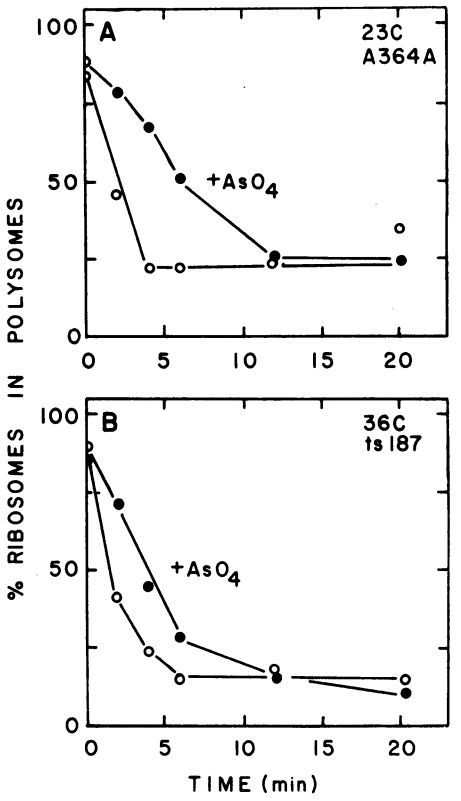

FIG. 9. *Polyribosome levels in A364A spheroplasts treated with verrucarin-76 and 10 mM arsenate and in Ts187 spheroplasts treated with 10 mM arsenate at the restrictive temperature. (A) A364A (23°C): verrucarin-76 (20 μg/ml)* (○); *verrucarin-76 (20 μg/ml), and 10 mM arsenate added 15 s later* (●). *(B) ts187: Cultures transferred to 36°C at zero time (for rapid temperature shift, culture flask swirled in 55°C bath for 1 min, then transferred to 36°C shaking bath). 10 mM arsenate added at zero time* (●); *no addition* (○).

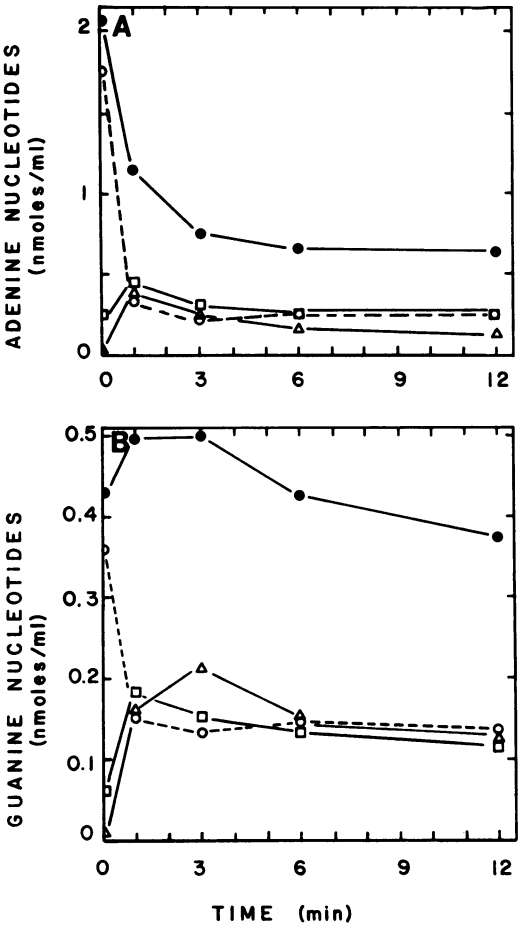

FIG. 10. *Effect of 10 mM arsenate on adenine and guanine nucleotide concentrations in yeast spheroplasts. (A) ATP* (○); *ADP* (□); *AMP* (△); *ATP + ADP + AMP* (●). *(B) GTP* (○); *GDP* (□); *GMP* (△); *GTP + GDP + GMP* (●).

of initiation. At a higher arsenate concentration that inhibited protein synthesis by about 98%, polysomes decayed, but more slowly, requiring about 12 min. This slower polysome decay rate indicates a partial inhibition of an elongation step(s) in addition to the inhibition of an initiation step(s). These polysome studies indicate that during energy limitation there is preferential control of polypeptide synthesis at an initiation step and perhaps additional inhibition at an elongation step with very severe energy limitation. Inhibition of mRNA transcription in yeast would not give the rapid polysome decay observed in these experiments. The half-life of mRNA in yeast is about 20 min (26, 36). The decay of polysomes to equivalent levels requires about 60 min in the temperature-sensitive *rna*-1 mutant, which is defective in cytoplasmic mRNA production at the restrictive temperature (11, 15).

Initiation inhibition, sometimes accompanied by elongation inhibition, has been reported for energy limitation of ascites tumor cells (38, 39), thymic lymphocytes (22), reticulocytes (10, 19, 29), *Escherichia coli* (8, 9, 16), and liver (23). It also should be noted that a few reports show inhibition preferentially at an elongation step during energy limitation in liver (3), moss (4), and *E. coli* (17).

Nucleotide changes and protein synthesis. After the addition of 2 mM arsenate to cells, the ATP/ADP and GTP/GDP ratios dropped threefold from 6 to 2. The adenylate and guanylate energy charges dropped from 0.90 to

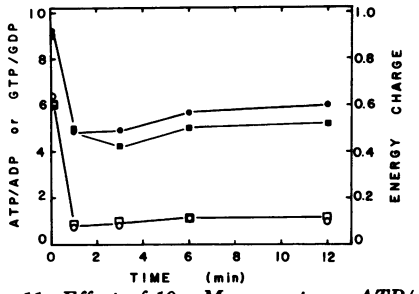

FIG. 11. *Effect of 10 mM arsenate on ATP/ADP and GTP/GDP ratios and on adenylate and guanylate energy charge values in yeast spheroplasts. ATP/ADP (○); GTP/GDP (□); adenylate energy charge (●); guanylate energy charge (■).*

about 0.70. ATP and GTP both are required for protein synthesis. ATP is required for formation of aminoacyl-tRNA and perhaps for binding of mRNA to the 40S initiation complex (37). GTP is required in both initiation and elongation steps of protein synthesis and GDP is a product and competitive inhibitor of these reactions. Walton and Gill (41–43) have studied the effect of changes in the GTP/GDP ratio on ternary complex formation (GTP:factor:aminoacyl-tRNA) for purified initiation factor-2 and purified elongation factor-1 from rabbit reticulocytes. The former (eIF-2) is more sensitive to inhibition by changes in the GTP/GDP ratio than is the latter (EF-1). The basis for this differential effect is related to the affinities of the respective factors for GTP and GDP. Both factors have high affinities for the substrate GTP. However, the initiation factor has a much greater affinity for the product GDP than for GTP. Thus, the binding of GTP to eIF-2 is very dependent on the GTP/GDP ratio (2). In contrast, the elongation factor has a greater affinity for GTP than GDP, so binding of GTP to EF-1 is relatively insensitive to the GTP/GDP ratio. Our in vivo results in yeast, showing preferential inhibition at initiation, are consistent with their studies in vitro with purified reticulocyte factors. Such in vitro studies have not been done with yeast factors.

Adenine and guanine nucleotide interrelationships. It is interesting that the changes in the GTP/GDP and ATP/ADP ratios parallel each other. Although we would expect both ratios to drop under an energy-limiting condition, it was not obvious that such parallel changes would occur. After all, GTP and ATP are used as energy sources in different metabolic reactions, and the sum of the rate at which each is used must be quite different. However, there is one metabolic reaction which provides an interrelation between the adenine and guanine nucleotide concentrations. This is ATP + GDP $\rightleftarrows$ ADP + GTP, catalyzed by the enzyme, nucleotide diphosphokinase. The observation that the ratio [(ADP)(GTP)]/[(ATP)(GDP)] remains about 1 suggests that this reaction is close to equilibrium in vivo. Changes in the adenylate and guanylate energy charge values parallel each other also. This result implies that the triphosphate-to-monophosphate ratios, in addition to the triphosphate-to-diphosphate ratios, are the same for the adenine and guanine nucleotides in vivo. Apparently, the product-substrate ratio for the reaction catalyzed by GMP kinase, $(GDP)^2$/[(GTP)(GMP)], is maintained in vivo nearly identical to the product-substrate ratio for the reaction catalyzed by adenylate kinase, $(ADP)^2$/[(ATP)(AMP)]. We see no evidence in these experiments for a cascade effect, i.e., a small change in the adeylate energy charge results in a large change in guanylate energy charge (35); instead, the variations are almost identical. The one difference between changes in adenine nucleotides and changes in guanine nucleotides in these experiments is that the sum concentration (ATP + ADP + AMP) decreased 70 to 75%, whereas the sum concentration (GTP + GDP + GMP) was nearly constant. It has been observed in liver that, under conditions which caused a drop in the energy charge, the total adenine nucleotide pool also dropped and IMP accumulated (5). It has been proposed that the conversion of AMP to IMP by AMP deaminase may be a mechanism by which the cell dampens a potentially very large energy charge drop (5). IMP does not appear to accumulate in our cells. Conversion of AMP to adenine also has been proposed as a mechanism to stabilize the energy charge (30). We cannot test this with our labeling procedure. At present, we do not fully understand the mechanism for the decrease in the adenine nucleotide pool. But under these conditions, where the total adenylate pool size changes and the total guanylate pool size is constant, it is especially interesting that the ATP/ADP and GTP/GDP ratio changes completely parallel each other.

The known involvement of GTP and ATP in protein biosynthesis and the correlations shown in this paper between the drop in the GTP/GDP, ATP/ADP ratios and inhibition of initiation of protein synthesis are suggestive evidence that these nucleotide changes have a regulatory role. To prove that changes in the adenine and guanine nucleotide ratios are one of the regulatory signals that set the rate of protein synthesis, equivalent studies must be undertaken with an in vitro protein-synthesizing system. The data in this paper demonstrate the range of nucleotide changes which occur in vivo under energy limitation conditions.

ACKNOWLEDGMENTS

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