Metabolically Induced Shifts in the Translational Efficiency of Yeast Spheroplast Polysomes*

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ABSTRACT: This paper presents a study into the *in vivo* translational efficiency of yeast polysomes as their level passes through a cycle of buildup, constancy, and subsequent decline during the incubation of freshly protoplasted cells in growth medium. The *in vivo* rate of protein synthesis was determined from amino acid incorporation data as corrected by two independent methods for the specific radioactivity of a precursor, *viz.*, the cellular pool of free amino acids and nascent protein. During the initial phase of polysome buildup the translational efficiency is at first very high but declines to a lower value before the maximum level of cellular polysomes is attained. Two explanations are offered to account for the described phenomena: (1) occurrence of a metabolic "overshoot" during the readjustment of the multistep system for protein synthesis while the cells recover from the starvation condition of protoplasting, and (2) the existence of a specific translational control mechanism which may involve an unstable repressor that is resynthesized during the recovery period.

The significance of our results is discussed with reference to indirect assays of messenger ribonucleic acid *via* the kinetics of protein synthesis.

Cytoplasmic levels of mRNA in eucaryotic cells are generally estimated by methods which are restricted to the assay of bulk mRNA or, at best, to relatively large heterogeneous populations of messengers. With certain restrictions, however, it is possible to experimentally determine the relative changes in the cellular amounts of a given mRNA species from the kinetics of synthesis of the respective protein. This indirect method is based on the assumption that at any given time the rate of synthesis of a protein (dP/dt) is proportional to the amount of its functional mRNA (M), as expressed by the equation

$$dP/dt = k_{sp}(M) \quad (1)$$

where k_{sp} is the rate constant for protein synthesis. Thus, if further mRNA synthesis is inhibited, the remaining population of mRNA molecules (M_0) will decay with presumably first-order kinetics and in the process sustain the synthesis of a limited amount of protein (P) which can be computed from the integrated form of eq 1, *i.e.*

$$P = k_{sp}/k_{dm} \times M_0(1 - e^{-k_{dm}t}) \quad (2)$$

where k_{dm} is the rate constant for mRNA decay and t the time elapsed after the inhibition of mRNA synthesis. As t approaches effective infinity the amount of mRNA at time zero can be computed from the reduced form of eq 2, *i.e.*

$$M_0 = P(k_{dm}/k_{sp}) \quad (3)$$

The validity of this model rests on the condition that k_{sp}, as defined by eq 1, is indeed a rate constant and as such does not vary over the course of the experiment. This condition would be realized if the translational efficiency of the protein synthesizing system remains unchanged.

Experimental support for this model, as applied to a specific protein–messenger system, comes mainly from work with inducible enzymes in bacteria (Kepes, 1963; Hartwell and Magasanik, 1963). Work with eucaryotic cells has not yet provided similar experimental support for the model with regard to individual messenger species. However, its applicability can be tested, although in a less rigorous form, by analyzing the relationship between bulk messenger and the rate of total protein synthesis. Since presumably all functional messenger of a cell exists in the form of polysomes, the invariance of the overall efficiency of polysomal peptide synthesis during changes in the polysome levels should reflect the constancy of the mean k_{sp}.

The literature already contains a few reports specifically relating changes in the polysome level to the *in vivo* rates of protein synthesis. During the actinomycin D induced breakdown of polysomes in rat liver, the decrease in the *in vivo* rate of leucine incorporation, corrected for the specific radioactivity of the free leucine pool of the tissue, was found to be roughly parallel to the decreasing polysome level (Wilson and Hoagland, 1967). Similar experiments with mammary carcinoma cells did not reveal such a correlation (Trakatellis *et al.*, 1965b). However, the specific radioactivity of the amino acid pool was not determined in this study. Variations in the efficiency of polysomes have been found during the maturation of reticulocytes *in vitro* (Knopf and Lamfrom, 1965; Trakatellis *et al.*, 1965a), but not *in vivo* (Glowacki and Millette, 1965). Polysome efficiency was furthermore observed to vary during the *in vitro* recovery of reticulocytes after the dissociation of their polysomes by both NaF (Coconi *et al.*, 1966) and ethionine (Villa-Trevino *et al.*, 1964).

In view of the potential of the model with respect to the

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quantitation of individual mRNA species, we were interested in obtaining information concerning its applicability to a unicellular eucaryotic organism. Bakers' yeast (*Saccharomyces cerevisiae*) was chosen for the study since protoplasted cells (spheroplasts) offer the possibility to investigate the relation between polysomes and the rate of protein synthesis without the necessity for metabolic inhibitors.

Methods and Materials

All reported experiments were conducted with strain S2112D (aUr4) of *S. cerevisiae* kindly supplied by Dr. R. K. Mortimer (University of California). This strain is auxotrophic for histidine and uracil. Cells were grown aerobically at 30° in 1-l. batches of synthetic medium (Roman, 1956). Cells were harvested from logarithmic phase cultures (*ca.* 10×10^6 cells/ml) by filtration on Millipore filters and briefly washed on the filter with water.

The yeast spheroplast suspension was prepared by first photoplasting the cells in 1 M sorbitol containing 1% (v/v) glusulase (crude snail gut preparation obtained from Endo Laboratories, Garden City, N. Y.). The process involved a 90-min incubation in a 30° water bath with gentle shaking. The spheroplasts were then sedimented at 1500*g* for 10 min and resuspended to 2×10^8 cells/ml in the final incubation medium. This medium, referred to in this paper as SM-5, is the YM-5 medium described by Hartwell (1967) supplemented with 10 μg/ml each of uracil and histidine and made 1 M with respect to sorbitol. Further incubation of the spheroplasts was carried out at 30° with gentle agitation.

Polysomes were isolated by a procedure similar to that of Hutchison and Hartwell (1967). Briefly, an aliquot of the spheroplast suspension was mixed with sufficient cycloheximide stock (2 mg/ml) to yield a final drug concentration of 100 μg/ml and was then quickly chilled. The spheroplasts were sedimented and washed by resuspension in ice cold 1 M sorbitol containing 100 μg/ml of cycloheximide. The washed spheroplasts were again sedimented and resuspended in a small volume of lysing buffer (0.01 M Tris, pH 7.4, 0.1 M NaCl, 0.0015 M $MgCl_2$) containing 100 μg/ml of cycloheximide and 1% (v/v) Brij 58 (kindly supplied by Atlas Powder Co., Wilmington, Del.). The suspension was frozen and thawed once for efficient cell breakage. After a 15-min extraction at 1°, cell debris was removed by low speed centrifugation and 0.3 ml of the supernatant layered over a 10–30% sucrose gradient prepared in lysing buffer. The gradients were centrifuged for 60 min at 35,000 rpm in an SW-39 rotor (Beckman/Spinco) and polysome fractions collected from a hole pierced in the bottom of the centrifuge tube.

Polysomal components were obtained from the dialyzed (against 0.001 M potassium acetate) and lyophilized polysome fraction by treatment with EDTA buffer (5 volumes of 0.1 M potassium acetate to 1 volume of 0.1 M EDTA, pH 5.8). The components were then separated in a 5–25% sucrose gradient prepared in the EDTA buffer, by centrifugation for 4 hr at 45,000 rpm in an SW-50 rotor (Beckman/Spinco).

For the assay of radioisotope content of polysomes and polysomal components, 2-drop fractions from the sucrose gradients were collected on squares of Whatman No. 3MM filter paper. The papers were dried, washed with ice cold 5% trichloroacetic acid containing 3% casamino acids and then with 95% ethanol, and finally transferred to vials containing a 0.5% solution of 2,5-diphenyloxazole (Packard Instrument Co.) in toluene and counted in the Beckman Model 1650 liquid scintillation spectrometer.

In order to determine the amount of radioactivity incorporated into protein, 2-ml aliquots were taken from spheroplast suspensions labeled with [^{14}C]histidine and drained into 5 ml of cold 10% trichloroacetic acid. The resultant precipitate was collected by centrifugation and washed once with cold 10% trichloroacetic acid. The final pellet was suspended in 5 ml of 5% trichloroacetic acid, extracted for 20 min at 90°, collected, and resuspended in 0.5 ml of water. The material was then quantitatively transferred to squares of Whatman No. 3MM filter paper and counted as described above.

For the determination of the specific activity of the cellular histidine pool aliquots of 5 ml were withdrawn from the [^{14}C]histidine-labeled spheroplast suspension. The samples were quickly chilled and the cells sedimented and washed twice with ice cold 1 M sorbitol. The amino acids were extracted with 2 ml of ice cold 5% trichloroacetic acid and the trichloroacetic acid was subsequently removed with ethyl ether. The amino acid extract was then lyophilized and taken up in 1.5 ml of pH 2.2 citrate buffer (Beckman Instruction Manual AIM-2). From this solution a 0.2-ml aliquot was removed for total radioactivity determination using 2,5-bis-2-(5-*t*-butylbenzoxazolyl)thiophene (Packard Instrument Co.) scintillation mixture (Yall *et al.*, 1967). A 1-ml aliquot was taken from the same extract and applied to the short column (basic) of a Beckman Model 120B amino acid analyzer equipped with a Packard Tri-Carb radioactivity monitor. Histidine was quantitated by the dot counting method (Beckman Instruction Manual) while the radioactivity of the histidine was obtained by planimetric integration of the Tri-Carb recorder tracing corresponding to the histidine peak. For some of the experiments the total amino acid content of the cold trichloroacetic acid extract was determined by the ninhydrin reaction (Moore and Stein, 1948).

For the chemical assay of RNA, aliquots of 5 ml were taken from the spheroplast suspension at various times and mixed with 5 ml of ice cold 25% trichloroacetic acid. The precipitate was collected and washed once with ice cold 10% trichloroacetic acid. RNA was extracted with 5% trichloroacetic acid at 90° for 20 min, the residue was removed by centrifugation, and the supernatant analyzed for RNA by the orcinol reaction (Mejbaum, 1939).

Nascent protein was obtained in the form of peptidyl-tRNA from the 3–5S region of sucrose gradients containing the polysomal components. Corresponding fractions from three centrifuge tubes were combined, dialyzed against water, lyophilized, and taken up in 0.5 ml of 0.3 N KOH. The sample was incubated at 37° for 16 hr to hydrolyze the RNA and the resultant nucleotides were removed by dialysis against water. Nascent protein in the dialyzed solution was quantitated by the Lowry reaction (Lowry *et al.*, 1951) using 5 × recrystallized pancreatic ribonuclease as a standard.

Results

Polysome Profiles. The identity of the polysomes was verified by incubating lysates from amino acid pulse-labeled cells with very low concentrations of pancreatic ribonuclease

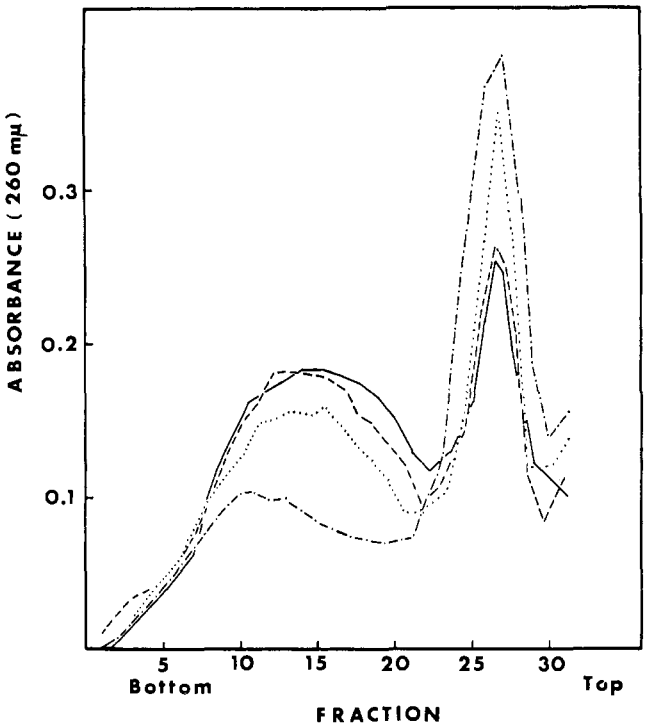

FIGURE 1: Polysome profiles obtained from spheroplasts incubated in SM-5 medium for: 10 min (-·-·-·), 25 min (. . . . .), 45 min (----), and 85 min (———). The low speed supernatant of cell lysates was layered on a 10–30% sucrose gradient and centrifuged for 60 min at 35,000 rpm in an SW-39 rotor.

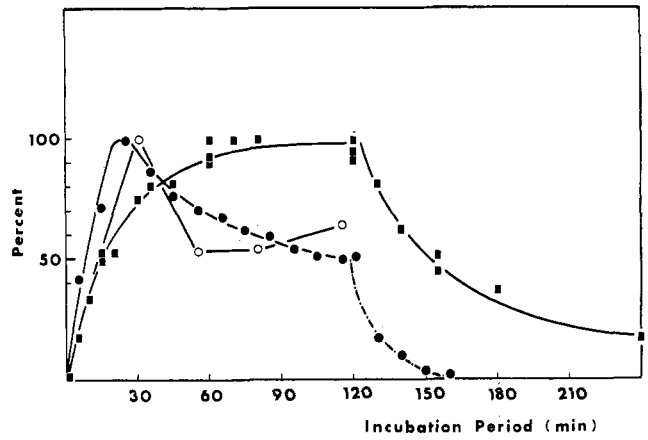

FIGURE 2: Comparison of polysome levels to the relative rates of protein synthesis over 4 hr of spheroplast incubation in SM-5 medium. Each set of data is expressed as the per cent of the maximum value in that set. Polysomes were quantitated from sucrose gradient profiles such as shown in Figure 1 by computing the fraction of total ribosomes contained in the polysome region of the gradient (■——■). The rate of protein synthesis was determined by two different methods: (1) the spheroplasts were labeled continuously with [^{14}C]histidine from 0 to 120 min (●——●) and from 100 to 215 min (●-·-·-●) of incubation; the increment in trichloroacetic acid insoluble counts over a given 10-min interval was divided by the midpoint specific activity of the cellular histidine pool; (2) the spheroplasts were pulse-labeled with [^{14}C]histidine for 7 min and the 4–6-min increment in incorporated counts was divided by the specific radioactivity of nascent protein at 5 min (○——○).

in the cold prior to sedimentation analysis. This treatment resulted in the transfer of practically all material, absorbing at 260 mμ and containing the radioactive label, from the polysome region of the gradient to the monosome region. The small residual amount of ultraviolet absorption remaining in the polysome region after ribonuclease treatment furnished the baseline values for the computation of polysome levels as presented in Figure 2.

At the end of the protoplasting period, spheroplasts do not contain measurable amounts of polysomes. After resuspension of the cells in SM-5 medium, the level begins to rise within a few minutes and reaches a maximum at 50–60 min (Figures 1 and 2). At this time approximately 70% of all ribosomes in the cell exist in the form of polysomes. This maximum level is maintained throughout the 60- to 120-min incubation period and then followed by a rapid decline beyond 125 min.

Rate of Protein Synthesis. The relative rate of protein synthesis at different points in time can be obtained from the amount of radioactivity incorporated into protein during brief pulses with labeled amino acids. Rates thus obtained are only comparable if the labeling kinetics of the amino acid pool are identical at these different time points. We have found, however, that the amino acid pool in spheroplasts expands during the incubation in SM-5 medium (Figure 3) and furthermore that the cellular uptake of amino acids continues after the complete inhibition of protein synthesis by cycloheximide. Both these observations point toward the existence of an "open" amino acid pool. Under such conditions it should still be possible to compute the rate of protein synthesis by correcting the incorporated counts with respect to the time-dependent change in the specific radioactivity of the amino acid pool. This method gave inconsistent results when applied to our organism. The labeling of the amino acid pool during the initial phase of a 5-min pulse proceeded so rapidly as to make a time-wise correlation between its specific activity and the incorporated counts extremely haphazard.

The difficulties experienced with pulse labeling were minimized by an extension of the labeling period, and best results were obtained with continuous labeling. In these experiments, the instantaneous rate of protein synthesis at any given point was computed from the increment in the counts incorporated over a given 10-min interval and divided by the specific activity of the amino acid pool averaged over that interval.

For the time period of 0 to 120 min, the [^{14}C]histidine was added to the spheroplasts immediately upon their suspension in SM-5. At various labeling times, aliquots were removed for the determination of the radioactivity incorporated into trichloroacetic acid insoluble material (Figure 4). Aliquots were also removed for the determination of the specific activity of the histidine pool (Figures 3 and 4) by means of the amino acid analyzer. The radioactivity tracings obtained from the amino acid analyzer revealed that during 2 hr of incubation with [^{14}C]histidine at least 99% of the cold trichloroacetic acid extractable label is present as histidine and therefore a direct radioassay of the trichloroacetic acid extract could substitute for monitoring the label in the amino acid analyzer. The corrected rates of protein synthesis over the first 2 hr of incubation as obtained from 10-min intervals are presented in Figure 2. Rates of

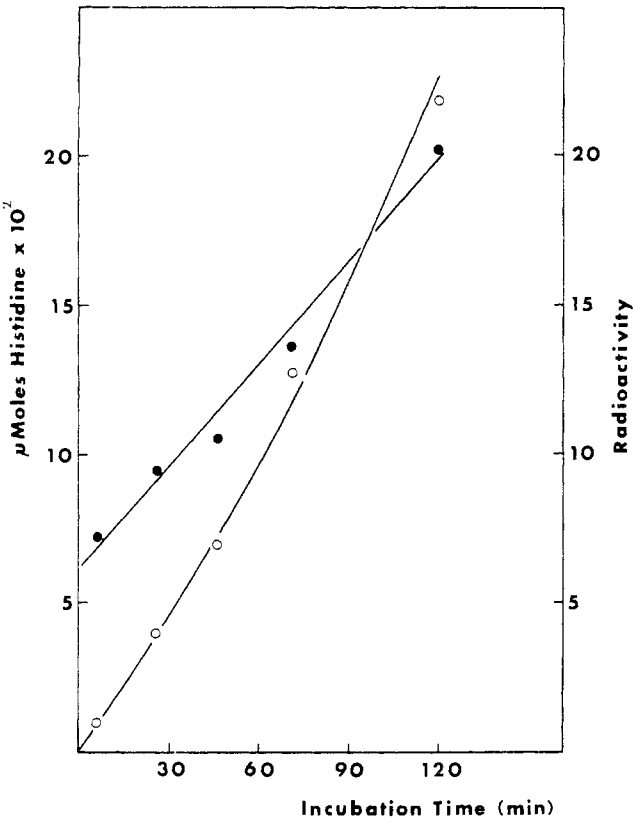

FIGURE 3: Continuous labeling kinetics of the histidine pool and the change in the pool size during the initial 2 hr of spheroplast incubation in SM-5 medium. The trichloroacetic acid soluble radioactivity in histidine is expressed in arbitrary planimeter units (○—○); micromoles of histidine in the trichloroacetic acid extract per aliquot of 10^9 cells (●——●).

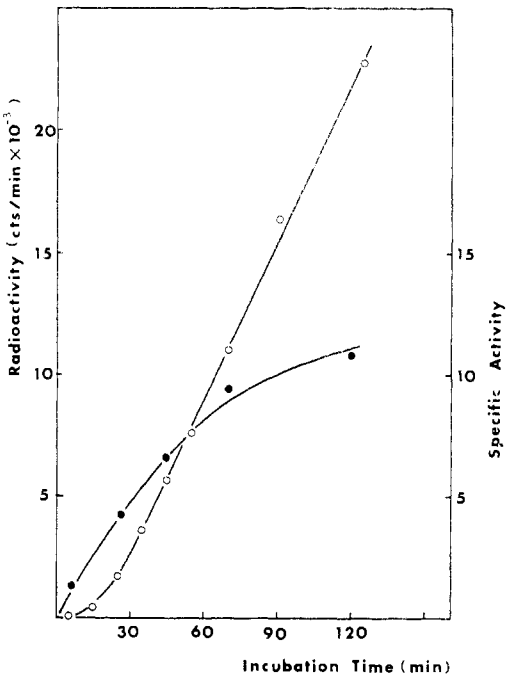

FIGURE 4: Continuous labeling kinetics of protein and changes in the specific activity of the histidine pool during the first 2 hr of spheroplast incubation. Radioactivity in protein (○——○). Specific activity of the cellular free histidine as obtained with the amino acid analyzer, expressed in arbitrary units (●——●).

protein synthesis were also obtained for the second half of the spheroplast incubation by labeling with [^{14}C]histidine during the 100- to 215-min interval, thereby overlapping the initial 0- to 120-min interval. Samples were removed at different labeling times and analyzed for histidine incorporation into trichloroacetic acid insoluble material as well as for the specific radioactivity of the cellular amino acid pool (Figure 5). The latter values were obtained by dividing the radioactivity in soluble histidine by the amount of trichloroacetic acid soluble ninhydrin positive material (Figure 6). The corrected rates of protein synthesis for the second incubation interval are included in Figure 2. A continuous curve was constructed by aligning the overlapping sections of both periods.

The kinetics of histidine labeling revealed that the equilibration of the cellular pool with external histidine occurred rather slowly (Figure 4). In the absence of endogeneous histidine synthesis, as in our auxotroph, such an extended labeling period implied the existence of a relatively large cellular histidine pool. This interpretation is supported by preliminary histidine pulse-chase experiments which indicate that the incorporation of labeled histidine into protein continues for over an hour following the removal of the external label.

It was desirable to verify the rates of protein synthesis in spheroplasts *via* a different experimental approach. Since we were concerned over possible compartmentalization of the free amino acids in the cell, we determined the specific radioactivity of nascent protein, a precursor which is metabolically closer to the finished protein than the cellular free amino acids. Our estimation of the specific activity of nascent protein is based on the assumption that each polysomal ribosome carries one nascent protein molecule ranging in length from a single amino acid at the time of initiation to a length of several hundred amino acids near the point of termination. Thus, for a given size distribution of mRNA molecules which are translated under steady-state conditions and, ignoring the possible existence of inactive polysomes (see Discussion), we can conceptually define an average number of amino acids per nascent peptide attached to one polysomal ribosome. Therefore, the total number of amino acids contained in all the nascent peptides in a cell would be proportional to the number of polysomal ribosomes. Hence, in labeled cells the relative specific activity of the average nascent protein is equivalent to the total radioactivity in nascent protein per ribosome. Values thus obtained can be used to correct the radioactivity which has accumulated in cellular total protein over a given time interval. In order to assure that nascent peptides of different length are as uniformly labeled as the rising specific activity of the amino acid pool permits, a labeling period must be chosen which exceeds the translation time of the longest polypeptide chain.

Nascent protein is released in the form of peptidyl-tRNA by EDTA-induced dissociation of polysomes (Warner *et al.*, 1963; Wettstein and Noll, 1965; Hultin, 1966; Philipps, 1966) and its radioactivity can be readily determined after fractionation of the polysomal components by sucrose density gradient centrifugation (Figure 7). This assay would

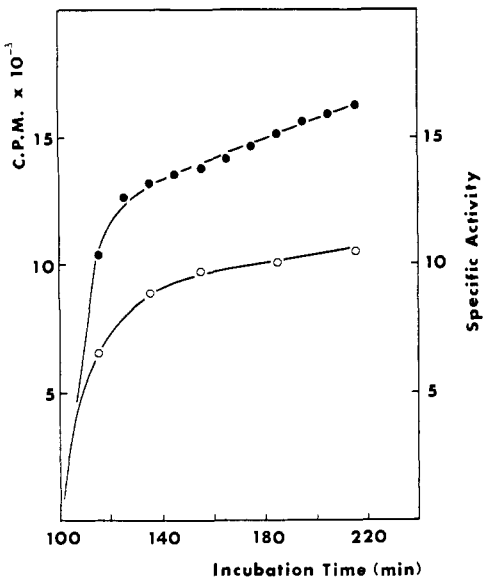

FIGURE 5: Continuous labeling kinetics of protein and changes in the specific activity of the total amino acid pool during the time period of 100–215 min of spheroplast incubation in SM-5 medium. Radioactivity in protein (○——○). Specific activity of the ninhydrin reactive material as expressed in radioactivity in histidine divided by the optical density at 570 mμ (●——●).

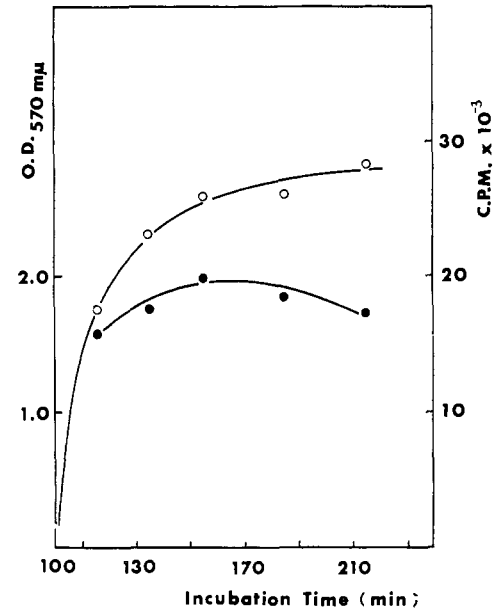

FIGURE 6: Continuous labeling kinetics of the trichloroacetic acid soluble pool and the change in pool size during the time period of 100–215 min of spheroplast incubation in SM-5 medium. Trichloroacetic acid soluble counts per min (○——○). Ninhydrin reactive material expressed in terms of optical density units at 570 mμ (●——●).

only be valid under two conditions, *viz.*, (a) that all label in the peptidyl-tRNA region of the gradient (3–5 S) is exclusively contained in nascent protein and (b) that all or at least a constant portion of nascent protein is released by the EDTA treatment.

In order to demonstrate that the radioactivity in the 3–5S region was entirely contained in peptidyl-tRNA, material from the 3–5S section of the gradient was collected and submitted to three separate treatments: the pH was adjusted to 10.0 with 0.1 M ammonium carbonate buffer and the solution incubated for 60 min at 37°, the solution was made 0.3 N with respect to potassium hydroxide and incubated for 5 hr at 37°, and pancreatic ribonuclease was added to a concentration of 10 μg/ml and the solution incubated for 5 hr at 37°. Analysis of the incubated preparations by sucrose gradient centrifugation revealed that in all three cases the protein label had shifted to the same lower S value. Since all these treatments either resulted in the degradation of tRNA, or cleaved the bond between tRNA and nascent protein (Gilbert, 1963; Bresler *et al.*, 1966), it can be concluded that all label was indeed tRNA bound. With regard to condition b, a proportionate release of peptidyl-tRNA could be assumed if identical yields per polysome of nascent protein were obtained with spheroplasts collected at different times of incubation. Incubation times of 30 and 70 min were chosen because they represent, according to the continuous labeling experiments, two distinct levels of polysome efficiency. The yield per polysome of released nascent protein, as assayed by the Lowry reaction, was indeed found to be the same for both polysome preparations (Table I). The computed average number of amino acid residues per polysomal ribosome is perhaps slightly lower than expected; however, since smaller peptides were undoubtedly lost during the required dialysis steps, the data do not necessarily indicate an incomplete release of peptidyl-tRNA. The radioactivity of the nascent peptides could thus be determined from the zone containing the peptidyl-tRNA (3–5S), while the ultraviolet absorption of the 40S (or 60S) subunit could serve as the reference value for the number of ribosomes.

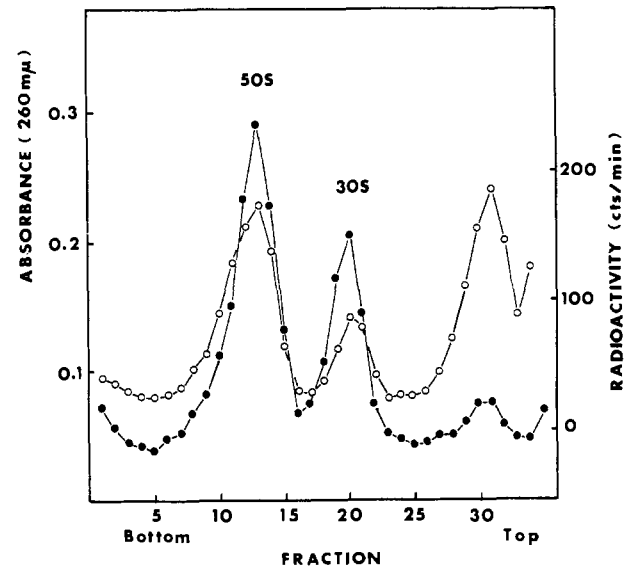

FIGURE 7: Polysomes from cells which had been labeled for 5 min with [^{14}C]histidine were purified by density gradient centrifugation and treated with 0.01 M EDTA and their components separated in a 5–25% sucrose gradient by centrifugation for 4 hr at 45,000 rpm in a SW-50 rotor. Absorbance at 260 mμ (●——●); radioactivity (○——○).

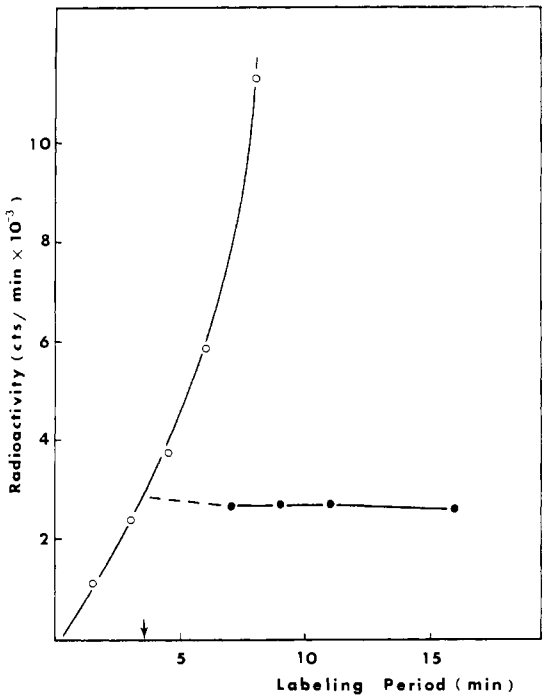

FIGURE 8: Effect of cycloheximide on the incorporation of [^{14}C]-histidine into spheroplast protein. The time of addition of cycloheximide is indicated by the arrow. Rate of incorporation in the presence of 100 μg/ml of cycloheximide (●——●); control rate (○——○).

For the computation of the rate of protein synthesis corrected for the specific activity of nascent protein, the incorporation of [^{14}C]histidine into hot trichloroacetic acid insoluble material was followed over a labeling period of 7 min. The increment in protein labeling over the 4- to 6-min interval was determined and corrected for the specific activity of nascent protein calculated from 5-min samples. With this technique the relative rates of protein synthesis were obtained for four different points during the initial 120 min of incubation (Table II). The results of these experiments, included in Figure 2, were found to agree well with those derived from the continuous labeling experiment.

TABLE I: Balance Sheet for Polysomal Components.[a]

	30 Min	70 Min
Polysomal RNA (OD 260 mμ)	2.48×10^3 μg	5.5×10^3 μg
3–5S RNA (OD 260 mμ)	33.8 μg	66.0 μg
Nascent protein (Lowry)	8.76 μg	17.8 μg
Nascent protein per polysomal RNA	3.53×10^{-3}	3.24×10^{-3}
3–5S RNA per polysomal RNA	1.36×10^{-2}	1.20×10^{-2}
Nascent protein per 3–5S RNA	0.26	0.27
Average number of amino acids per polysomal ribosome	51 residues	47 residues

[a] At 30 and 70 min of incubation in SM-5 an aliquot of 3×10^{10} cells was removed and assayed for the indicated components.

TABLE II: Correction of Incorporation Data for the Relative Specific Activity of Nascent Protein.[a]

Time Point (min)	Incorporated (cpm)	cpm 3–5 S / Ultraviolet 30 S	Relative Rate of Protein Synthesis
30	2450	0.30	8160
55	5300	1.24	4270
80	5500	1.25	4410
115	7400	1.39	5320

[a] After different incubation times in SM-5 medium spheroplast suspensions were labeled for 7 min with [^{14}C]histidine. The relative rate of protein synthesis at 5 min of labeling was determined by dividing the counts per minute incorporated over the 4- to 6-min interval by the relative specific activity of nascent protein at 5 min of labeling.

Although the rate of protein synthesis was determined by two independent methods and in both cases corrected for the specific activity of a protein precursor, the possibility still existed that some of the radioactive amino acid was incorporated in acid-insoluble form *via* a nonpolysomal system. We have ruled out this possibility by demonstrating that cycloheximide, a known inhibitor of polysomal protein synthesis (for a review, *cf.* Sisler and Siegel, 1967), completely inhibits amino acid incorporation in our spheroplast system (Figure 8). It was also noted in this experiment that no measurable loss of incorporated label occurred over a 15-min period following the addition of cycloheximide. Since cycloheximide seems to affect only the synthesis of protein and, in general, not its rate of degradation (Feldman and Yagil, 1969), it was concluded that protein turnover was not occurring to any significant degree and could thus be ignored. Therefore, it was not necessary to correct the calculated rate of protein synthesis with respect to protein turnover.

Of considerable interest is the fact that the observed changes in the rate of protein synthesis, verified by two independent methods, are not relatable to the polysome levels (Figure 2). The rate of synthesis reaches a maximum after 30 min of incubation, followed by a rapid decline to a lower level which then remains relatively constant out to 2 hr. In contrast, the polysome level rises steadily over the first 60 min of incubation and then remains constant out to 2 hr.

RNA Levels. In the experiments discussed above we have determined the relative cellular level of polysomes as the proportion of the total ribosomes which exist in the form of polysomes. This approach is valid only so long as the cellular level of total ribosomes does not change significantly.

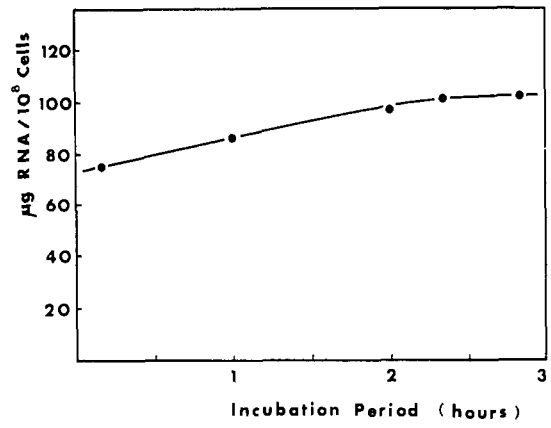

FIGURE 9: Accumulation of total RNA in spheroplasts during their incubation in SM-5 medium as determined by the orcinol reaction.

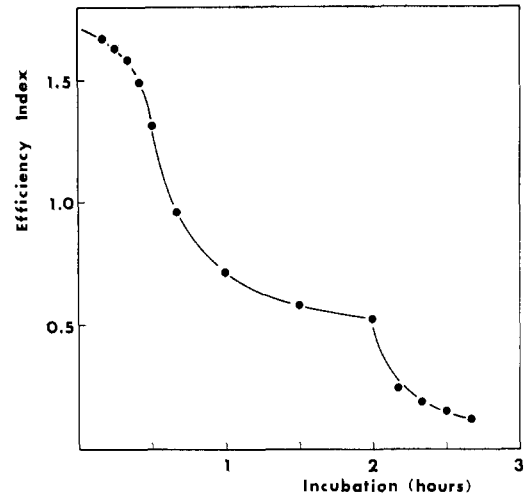

FIGURE 10: Polysome efficiencies over the entire incubation period. The values were obtained by dividing the relative rate of protein synthesis by the corresponding polysome level.

Since the rate of protein synthesis should be compared to the actual level of polysomes, we have measured total RNA at several points during the incubation of the spheroplasts and extrapolated the RNA values to changes in the number of total ribosomes per cell. The results of the orcinol determination are shown in Figure 9. They indicate that the RNA content, and therefore probably also the ribosome content of the cells, increases slightly during the incubation of the spheroplasts. However, this change would only amplify our effect, *i.e.*, the absolute amount of polysomes would actually increase rather than level off beyond the point where the rate of protein synthesis begins to decline.

Discussion

In the present study we have examined the *in vivo* translational efficiency of polysomes as their cellular level passes through a cycle of an initial build up and a subsequent decline during the incubation of yeast spheroplasts (Figure 10).

Polysome efficiency should ideally be defined as the number of peptide chains released per number of polysomes per unit time. Experimentally, however, we determined polysome efficiency as the microgram amount of protein synthesized per microgram amount of polysomal RNA per unit time. By assuming that the size spectrum of messengers does not change during the incubation, it is possible to substitute protein amount for the number of peptide chains. Similarly, there is a constant relationship between the amount of polysomal RNA and the number of polysomes provided their size distribution is invariant. As evident from our polysome profiles (Figure 1), the size distribution does not significantly change during the period of 25 to 120 min and therefore our calculation of polysome efficiency for this interval is equivalent to the above defined "ideal" efficiency. The 10-min polysome profile is slightly shifted toward a larger aggregate size and thus contains a lesser number of polysomes per microgram of polysomal RNA as compared to the other profiles. If a correction were applied it would have the effect of increasing slightly the calculated efficiency over that shown in Figure 10.

Under the assumption that all functional messenger exists in the form of polysomes, our data strongly imply that in our system the rate of protein synthesis varies independently of the level of functional messenger. During the 25- to 65-min phase of the incubation cycle the translational efficiency of polysomes was found to strikingly decrease even before the maximum level of polysomes was obtained. According to the currently accepted tape mechanism of translation (Gierer, 1963; Gilbert, 1963; Warner *et al.*, 1963; Watson, 1963), this decline in the polysome efficiency suggests a decreased frequency of peptide chain initiation for all polysomes, or alternatively, the buildup of a separate population of inactive polysomes. The latter possibility can be ruled out on the basis of an experiment in which polysome runoff was induced by starving the spheroplasts for a carbon source. At any time during the phase of low polysome efficiency, *e.g.*, at 65 min of incubation, starvation lead to the conversion of all polysomes to monosomes in less than 10 min indicating the absence of nonfunctional, "jammed" polysomes. The accumulation of a significant portion of ribosomal aggregates not carrying nascent protein is contradicted by the constancy of the ratio of nascent protein:polysomal ribosome for both 25 min and 60 min of incubation, as presented in Table I.

The observed threefold decrease in the polysome efficiency must therefore be the result of an equivalent decrease in the rate of peptide chain initiation. In the absence of a significant shift in the size distribution of the polysomes a concomitant reduction in the translational speed seems indicated.

The relative rapid loss of polysome efficiency during the early phase of incubation could have been triggered in two basically different ways. One could envision a nonspecific effect caused by the shift from a nonnutrient protoplasting medium to a fully supplemented incubation medium. During the metabolic arrest vital components of the multistep system of protein synthesis could become disarrayed. Following the subsequent return to a steady-state condition an initial "overshoot" in translational efficiency might occur. This interpretation is reminiscent of the oscillations in the activity of certain enzymes along a metabolic pathway

following a substrate induced perturbation of the system (Chance *et al.*, 1964).

Alternatively, it is conceivable that a specific cellular regulatory mechanism sets in after a brief delay and adjusts the rate of protein synthesis to a lower level. The controlling agent might be an unstable repressor which decays over the 90-min protoplasting period, depriving the cell of its controlling effect until it can be resynthesized during recovery. Supporting evidence for the existence of translational repressors has been provided by the studies of other investigators on the regulation of enzyme synthesis (Garren *et al.*, 1964; Kenney and Albritton, 1965; Eliasson, 1967; Leitner, 1967; Tomkins *et al.*, 1969).

It may be argued that the decrease in the polysomal efficiency is merely a pathological response of a steadily deteriorating cell population and is not the result of metabolic regulation. However, such an interpretation is difficult to reconcile with the studies of Hutchison and Hartwell (1967) and with certain of our findings. Aside from the fact that the polysome level remains unchanged during the period of 40 to 120 min of incubation, it can also be seen (Figure 9) that the rate of total RNA synthesis remains constant during this interval. Further, the actual deterioration of the cells is a very characteristic process and its onset is readily recognized at 125 min as witnessed by: (a) a general swelling and vacuolization of the spheroplasts, (b) a curtailing of total RNA synthesis, (c) a rapid loss of the remaining protein synthesizing capacity, and (d) an abrupt loss of polysomes.

In general, our results point out the possibility that severe deviations from metabolic steady state could trigger either specific or nonspecific translational controls. Under such conditions, a constant correlation between messenger levels and the rate of protein synthesis (expressed by k_{sp} of eq 1) would not be obtained, *i.e.*, the originally discussed model would not apply. The determination of messenger levels and half-lives *via* the rate of protein synthesis generally involves the inhibition of RNA synthesis by actinomycin D, analogs, or precursor starvation. Such treatments could conceivably lead to a metabolic stress of sufficient magnitude to introduce errors in the messenger assay through unappraisable variations in k_{sp}. We think that investigations of mRNA levels or turnover *via* protein synthesis in any given system should be preceded by a test for possible variations in the translational efficiency under the condition of the assay.

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