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THE IDENTIFICATION OF A RIBOSOMAL-BOUND β -GLUCOSIDASE*

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Considerable evidence has accumulated in recent years to support the concept that ribosomes are the primary sites of protein synthesis. This view was originally supported by experiments *in vivo* and *in vitro* with C¹⁴ amino acids demonstrating that proteins pass through ribosomes during the course of their synthesis (see review by Novelli¹). More recently, not only has a nascent precursor of soluble proteins been recognized on ribosomes,²⁻⁴ but also specific protein⁵⁻⁷ synthesis has been obtained with *in vitro* experiments in which the specificity for protein synthesis resides in the ribosomes.

Similar evidence has recently been reported for yeast. Protein synthesis is associated with particulate RNA.⁸ Analysis of the ribosome fraction from exponentially growing yeast has revealed one major component having a sedimentation coefficient of 80S.^{9, 10} In experiments with intact cells, C¹⁴ amino acids and S³⁵ are incorporated first into ribosomal protein^{11, 12} and can be chased from these to the soluble proteins.¹² Webster¹³ has demonstrated *in vitro* that amino acids are incorporated into ribosomes in a manner analogous to that of mammalian and plant systems. Finally, the classes of S³⁵-labeled proteins released from ribosomes are identical to the classes of soluble proteins present in the cytoplasm.¹⁴

The essential feature to emerge from the above findings is that ribosomes are involved in determining the amino acid sequence for each particular enzyme. One would therefore expect that at any given moment a fraction of these particles would still be associated with newly synthesized enzyme. A measure of the number of these ribosomal-bound enzymes per cell might therefore provide a minimal estimate of the number of specific enzyme-forming sites (ribosomes) for a given enzyme. In the present communication, we have undertaken an examination of ribosomal particles from a yeast constitutive for β -glucosidase. The preparation of ribosomal-bound enzyme, a description of its properties, and an estimate of its cellular distribution are given.

Materials and Methods.—A diploid yeast (*Saccharomyces dobzhanskii* 1974 $\times$ *Saccharomyces fragilis* 110), constitutive for β -glucosidase synthesis, was grown aerobically at 30°C in synthetic

medium.¹⁵ Growth was measured by following the optical density at 600 m μ in a Beckman Spectrophotometer or by direct count using a Levy hemocytometer. The yeast were harvested during exponential growth, washed twice with cold 6×10^{-4} M Mg cacodylate buffer, pH 7.2, and finally suspended in the same buffer (about 50 mg dry cells/ml). The cell suspension was passed through a French press¹⁶ in the cold two to five times until examination under the phase contrast microscope showed that over 95 per cent of the cells were broken. The suspension was centrifuged at 4°C in the Servall centrifuge at $11,000 \times g$ for 20 min. The precipitate was resuspended in buffer, recentrifuged, and the supernatant fractions combined. The precipitate remaining is referred to as the debris fraction. The supernatant fraction was centrifuged in the preparative Spinco at $105,000 \times g$ for 2 hr, yielding a pellet of ribosomes. The ribosomes were suspended in cacodylic buffer and recentrifuged at $11,000 \times g$ for 20 min to remove denatured material. Purified 80S ribosomes were isolated by centrifugation in an exponential sucrose density gradient employing a swinging bucket head rotor.³

β -Glucosidase was measured by following the hydrolysis of p-nitrophenyl- β -D-glucoside (PNPG) at 400 m μ ¹⁵ in either *M*/15 phosphate buffer, pH 6.8 ($E_{1\text{cm}}^M$ 9.6×10^3), or 0.005 M Mg-cacodylic buffer, pH 6.8. When the soluble enzyme was assayed in cacodylic and phosphate buffers, the ratio of activities was 1.40. Ribosomes, however, were stable in the cacodylic buffer but were broken down in the phosphate buffer. When *M*/15 phosphate buffer, pH 6.8, was used to measure ribosomal-bound enzyme, controls containing ribosome without substrate were included to correct for optical density measurements due to changes in light scattering from breakdown of the ribosomes. In a number of experiments in which enzyme was measured over 6-hr periods or longer, streptomycin (66 μ g/ml) and penicillin (66 μ g/ml) were included to prevent bacterial contamination.

Protein was measured by the method of Lowry *et al.*¹⁷ using crystalline serum albumin as the standard. Ribonucleic acid (RNA) was determined by the orcinol method.¹⁸

Cacodylic acid was obtained from the Fisher Scientific Company. PNPG and carbohydrates were obtained as previously described.¹⁹ Deionized glass-distilled water was used for all experiments.

Results.—(a) *Content of ribosomes per cell:* Chemical analyses were carried out on cells harvested in the logarithmic phase of growth in synthetic medium. At optical densities of 0.40 to 0.65, aliquots of 500–800 ml were removed for chemical

TABLE 1
DISTRIBUTION OF RNA IN YEAST*

	Direct count, cells/ml	Dry weight, mg/ml	Protein, mg/ml	RNA	
				mg/ml	per cent
Cell suspension	5.6×10^7	0.650	0.305	0.085	100
Debris†	0.28×10^7	...	...	0.0195	23.0
Soluble	...	...	...	0.0072	8.5
Ribosomes‡	...	...	...	0.0592	68.5

* Calculated on the basis of a cell suspension of OD = 1.000.

† The debris was removed by centrifugation at $11,000 \times g$ for 20 min and washed twice with cold buffer.

‡ Ribosomes were collected by centrifugation at $105,000 \times g$ for 1 hr.

analysis and 300 ml for dry-weight determinations. The cells were harvested by centrifugation, washed with cold buffer, and disrupted as described in *Materials and Methods*, and the various fractions were analyzed for protein and RNA. Table 1 summarizes the results of several experiments.

The dry-weight content of protein (47%) and RNA (13%) of the diploid is characteristic of that found in numerous strains of yeast.²⁰ It can be seen that the bulk of the RNA (77%) is liberated upon rupture of the cell suspension. In this extract, 89 per cent of the RNA is associated with the ribosomal fraction and 11 per cent is soluble. Similar RNA distributions have been reported in yeast by Osawa.²¹ About 22–24 per cent of the total RNA remains associated with the debris fraction, in various experiments. The number of intact cells in this fraction

ranged from 2 to 5 per cent of the original suspension; a value which is too low to account for the residual RNA. Since the debris fraction contains the cytoplasmic membranes, an attempt was made to determine if ribosomal particles were attached to these. Extended sonic oscillation or treatment of the debris fraction with lipase and proteinases liberates over 95 per cent of the debris RNA, approximately 30 per cent of which can be recovered as ribosomes. This same treatment leads to extensive degradation of ribosomes; thus, the actual content of ribosomal RNA in the debris is considerably higher. Therefore, between 78 and 91.5 per cent of the total RNA is ribosomal. Assuming that the debris RNA is representative of the total cellular RNA, the content of ribosomal RNA is about 1.5×10^{-12} gm/cell.

Ribosomes for chemical analysis were washed by repeated centrifugation (5 times) in the Spinco ultracentrifuge at $105,000 \times g$ for 2 hr. A preparation, which showed a single 80S peak in the analytical centrifuge, had an E_{260}/E_{280} ratio of 1.89, a dry weight of 0.0908 mg per unit E_{260} , and an RNA content of 40 per cent of the dry weight. The chemical composition showed very little change on further recentrifugation. Chao and Schachman⁹ and Yin¹⁰ have observed similar results and have further calculated a molecular weight of yeast 80S ribosomes as 4.1×10^6 to 4.5×10^6 . From the data reported here, the number of ribosome particles per cell of the diploid was calculated to be about 5.5×10^5 .

(b) *Ribosomal-bound β -glucosidase:* Yeast cells containing constitutive β -glucosidase were broken in the French press and the $105,000 g$ pellet was layered on a sucrose density gradient³ and spun at $45,000 \times g$ for 180 min, and then the distribution of enzyme activity and 260 $m\mu$ absorbing material was measured. The results are shown in Figure 1. Most of the enzyme is associated with the soluble protein which is not sedimented under the conditions employed, whereas only a fraction of the activity is observed with ribosomes of the 80S class. The majority of the β -glucosidase activity of the initial ribosomal pellet (99.2%) is thus attributed to contamination by the soluble protein fraction.

The ribosomes from Figure 1 are not completely freed of contaminating soluble protein. The material from fractions 8–10 were combined and the ribosomes collected by centrifugation at $105,000 \times g$ for 180 min. The pellet was resuspended, clarified by low-speed centrifugation, and relayered on a sucrose density gradient.

FIG. 1.—Sedimentation analysis of cell extract. The cell extract was overlaid on a continuous sucrose density gradient (5–20 per cent). Centrifugation 120 min at $45,000 \times g$. Curve A shows the β -glucosidase activity and curve B the absorbance at 260 $m\mu$.

The content of β -glucosidase following repeated density gradient centrifugation is shown in Figure 2. For comparison, the curve expected if each successive centrifugation had the same efficiency of resolving soluble enzyme from ribosomes is shown for the conditions employed in this experiment. After the second density gradient centrifugation, the enzyme associated with the ribosome fraction deviates from the expected exponential dilution. Following centrifugation, a minimum level of 0.00525 enzyme units/ E_{260} was observed. This value, which has been observed on a number of experiments, is not lowered by further washings.

FIG. 2.—Changes of ribosomal β -glucosidase activity after repeated sucrose density gradient centrifugation. Centrifugation conditions are same as in Figure 1. Activities are expressed as enzyme units per E_{260} . Curve A represents the activity of ribosomes from β -glucosidase constitutive cells and curve B the activity of ribosome from β -glucosidase negative ribosomes mixed with supernatant fraction of constitutive cells to give the same activity level as constitutive extracts. The dotted line shows the theoretical dilution calculated from the conditions employed.

FIG. 3.—Effect of parafluorophenylalanine on the ribosomal-bound β -glucosidase. The cells were incubated with 0.05M p-FPA for 5 min. and washed, and the ribosome fraction was collected by centrifugation (B). Density gradient sedimentation conditions are the same as in Figure 1. For comparative purposes, the kinetics of washing of control ribosomes are also included (A).

As a critical test to eliminate the possibility of nonspecific adsorption as an explanation for the ribosomal-bound enzyme, the ribosomes of a β -glucosidase negative cell (*Saccharomyces cerevisiae*) were mixed with the soluble fraction of constitutive cells, separated by density-gradient centrifugation and analyzed. As can be seen in Figure 2, the loss of enzyme from the negative ribosome follows the theoretical exponential dilution. To eliminate the possibility that adsorption is a specific property of ribosomes from constitutive cells, a similar experiment was carried out in which S^{35} -labeled soluble proteins from β -glucosidase constitutive

TABLE 2
AFFINITY CONSTANTS FOR β -GLUCOSIDASE FROM VARIOUS SOURCES

Compound	Soluble* $\times 10^5$	Ribosomal $\times 10^5$	Released $\times 10^5$
PNPG (substrate)	8	8.5	9.3
Glucose	880	1,130	—
Phenyl-S-glucoside	330	330	430
4-Br-phenyl-S-glucoside	33	28	—
4-Bi-phenyl-S-glucoside	5.4	3.8	5.8
3-Cl-4-Me-Phenyl-S-glucoside	12	13	11

* Data from Hu *et al.*¹⁵

cells were mixed with unlabeled ribosomes from the same cells. When the mixture was subjected to the same washing procedure, all detectable traces of radioactivity were removed from the ribosomal fraction.

A direct test was undertaken to determine if the ribosomal-bound enzyme was a precursor of the soluble enzyme. Studies in which cells were briefly exposed to $S^{35}O_4$ or C^{14} amino acids indicate that a small fraction of the proteins of ribosomes has a transient existence of only a few seconds in the growing cell.¹² If the bound β -glucosidase were a portion of this nascent protein fraction, it should have a correspondingly brief existence on the ribosome. A method for measuring this was provided by the finding that the incorporation of p-fluorophenylalanine (p-FPA) into yeast proteins in the place of phenylalanine leads to linear growth and the synthesis of enzymatically inactive protein.²² The incorporation of p-FPA into β -glucosidase leads to an inactive form of the enzyme,²³ and therefore one would expect that it would rapidly lead to the replacement of active ribosomal β -glucosidase by an inactive form of the enzyme. The following experiment was designed to test this possibility. P-FPA was added at a final concentration of 10^{-2} M to a growing culture of cells. After five min, three liter aliquots were removed, chilled with -20°C ice, washed, and broken in the French press, and the ribosomes were collected and subjected to successive density-gradient centrifugation as previously described. The β -glucosidase activity of ribosomes from the p-FPA-incubated cells behaves quite differently from that observed with the control material (Fig. 3). Although the initial activities were very similar, there was no indication of a constant specific activity in the p-FPA-incubated material. After four such centrifugations, the activity was less than 10 per cent of that observed in the control cells. When the final ribosomal fraction was analyzed in an analytical ultracentrifuge, only an 80S component was observed.

Although a dramatic decrease in the ribosomal-bound β -glucosidase was observed in the presence of p-FPA, there was no detectable change in the total level of soluble enzyme. Addition of 10^{-2} M phenylalanine rapidly gave rise to an active form of the bound enzyme; after 50 seconds, the ribosome fraction regained over 50 per cent of the normal level of active β -glucosidase. These experiments indicate that the β -glucosidase which is bound to ribosomes is a precursor of the soluble

TABLE 3
PHYSICAL-CHEMICAL PROPERTIES OF VARIOUS β -GLUCOSIDASES

Properties	Soluble	Ribosomal	Released
pH optimum	6.4-6.6	6.4-6.6	—
ΔH (cal/mol)	16,200	15,200	—
S_{20}	14	80	14.3

enzyme and further eliminate an *in vivo* nonspecific adsorption, since in the p-FPA experiments ribosomes essentially negative for β -glucosidase activity were isolated from cells having normal levels of soluble enzyme.

(c) *Characterization of ribosomal-released enzyme:* We have previously demonstrated that chelating agents release soluble protein from ribosomes and that the pattern of ribosomal-bound protein is identical to that found normally in the cytoplasm.¹⁴ In attempting to determine if the soluble and ribosomal-bound β -glucosidase are identical, we have examined the specificity and physical-chemical properties of these as well as the enzyme released by 1 *M* borate. The specificity data, recorded as affinity constants for the substrate (PNPG) and other β -glucosides, are summarized in Table 2. Although these values range over 100 fold, from 4×10^{-3} *M* for phenyl-S- β -D-glucoside to 4×10^{-5} *M* for 4 bi-phenyl- β -D-glucoside, there is no significant difference between soluble, ribosomal, or released enzyme. A similar conclusion is evident from examination of the physical-chemical properties of these enzyme activities (Table 3). The pH optimum and ΔH are identical for the soluble and ribosomal enzyme. The sedimentation constants of the soluble and ribosomal enzyme were determined by moving boundary sedimentation; a partition cell was used to measure the sedimentation constant of the released enzyme. The ribosomal-bound enzyme sediments at the same velocity (80S) as the E₂₆₀ material. The enzyme released from ribosomes has the same sedimentation value as the soluble enzyme. From these data, it seems reasonable that the ribosomal-bound enzyme is identical to the soluble enzyme.

(d) *Latent β -glucosidase:* The latency of a portion of the ribosomal-bound β -glucosidase was initially observed when enzyme activity was compared under conditions maintaining ribosome integrity (cacodylic buffer) and under conditions favoring ribosome degradation (phosphate buffer). The activities for the ribosomal-bound enzyme were 0.00525 units/E₂₆₀ and 0.0133 units/E₂₆₀ for cacodylic and phosphate buffers respectively. Since the ratio of the cacodylic to PO₄= activities at pH 6.8 for the soluble enzyme was 1.40, the corrected activity of the enzyme active in intact ribosomes is 0.00375 units (phosphate buffer)/E₂₆₀. Thus, only 28 per cent of the ribosomal-bound enzyme is active in intact particles. The remaining 72 per cent is latent and demonstrable only when the particles are degraded. Increases of 3- to 6-fold in β -galactosidase activity have been reported when ribosomes of *Escherichia coli* are treated with specific anti- β -galactosidase serum.²⁴ Noninduced, inducible cells contained about one molecule of ribosomal-bound β -galactosidase per cell.

(e) *Cellular distribution of β -glucosidase:* The number of total enzyme molecules per cell can be readily calculated from the differential rate of β -glucosidase synthesis and the properties of the purified enzyme. The molecular weight of the enzyme is 330,000 and its turnover number for PNPG in phosphate buffer, pH 6.8, is 13.2×10^4 enzyme units/min/mg protein.¹⁵ Thus, from the Δ enzyme/ Δ OD of about 240,²⁵ and from the data in Table 1, it follows that the total number of molecules of enzyme per cell is about 62,000.

Assuming (i) identical turnover numbers for all β -glucosidases, (ii) no more than 1 molecule of β -glucosidase per ribosome, and (iii) no selection of specific ribosomal types during isolation, the cellular distribution of enzyme can be estimated. From the above data and those of Table 1, there is one molecule of

ribosomal-bound enzyme per 6.9×10^5 ribosomes or 0.8 molecules per cell.

Discussion.—Numerous claims have been made for protein and enzyme association with the ribosome and microsome fraction. However, in a number of cases, either contamination from soluble protein or protein derived from other cellular components was found to be adsorbed on these particles either prior to or during their isolation. In the present example, such contamination must be seriously considered, since only a small fraction of the total β -glucosidase is bound to the 80S particles. For the following reasons, we believe that the binding observed in the present experiments represents the *in situ* situation: (a) 80S yeast ribosomes contain a residual β -glucosidase activity which cannot be removed by density-gradient sedimentation without disrupting the integrity of the particles; (b) in control experiments with S^{35} proteins or β -glucosidase-negative ribosomes, density-gradient sedimentation removes all activity from the ribosome fraction; (c) *in vivo* brief exposure to p-FPA gives rise to ribosomes essentially negative for β -glucosidase activity in cells containing normal levels of soluble enzyme. Similar studies have led to the identification of enzymes and specific proteins on ribosomes or microsomal particles. These include hemoglobin,² albumin,²⁶ amylase,^{4, 27, 28} aldolase,⁷ β -galactosidase,²⁴ RNase,^{29–31} α -chymotrypsinogen,⁴ peroxidase,³³ and antibodies.³⁴ Further, the proteins released by disruption of the particles from guinea pig pancreas,⁴ bovine pancreas,³⁵ and yeast¹⁴ closely resemble the soluble proteins from the supernatant fraction.

It seems reasonable to assume that the proteins released following disruption of ribosomal particles are primarily newly synthesized enzymes. Demonstrations from *in vivo* pulse-labeling experiments that the highest specific activity was associated with the proteins^{3, 4, 35} and α -chymotrypsinogen⁴ released from these particles support this view. We have previously reported that in yeast labeled amino acids are incorporated first into the 80S ribosome fraction and that upon addition of unlabeled amino acids, this label is transferred to the soluble protein fraction.¹² In the present experiments with β -glucosidase, the results with p-FPA, leading to the formation of inactive enzyme, lead to the conclusion that the ribosomal-bound enzyme represents newly synthesized material.

In the present experiments, approximately one molecule of ribosomal-bound β -glucosidase was found per cell. Since enzymic activity would appear only late in synthesis of nascent protein, one would expect the total number of templates specific for one enzyme to exceed the number of these templates retaining completed enzyme. An estimate of the total number of β -glucosidase templates per cell can be obtained from the following assumptions: (i) specific ribosomes are involved in the synthesis of the majority of the cellular proteins; (ii) all of the ribosomes are active in protein synthesis; (iii) for nonrepressed enzymes, the rate of peptide bond formation is constant for all proteins. Based on the molecular weight of 100,000 as average for yeast cell proteins, and on the data in Table 1, there are approximately 3.3×10^7 molecules of protein per cell. Thus β -glucosidase represents 0.18 per cent of the protein population. From this, it follows that about 100 ribosomes per cell participate in the synthesis of β -glucosidase. The number of β -glucosidase templates per cell therefore could range from a minimum of 1 to possibly 100.

The association of latent enzymes with ribosomal particles appears to be a

widespread phenomenon. RNase has been reported to be latent in particles from a variety of organisms,²⁹⁻³² including yeast.³⁶ Pancreas microsomes contain a latent amylase.²⁸ In *E. coli*²⁴ and in the results reported here for yeast, the majority of the β -galactosidase and β -glucosidase associated with ribosomal particles are latent. The activation of these enzymes following ribosomal degradation is presumably due to an increased availability of enzyme to substrate. The existence of these latent enzymes suggests that the newly synthesized enzymes have a finite period of existence on the ribosomal particle and are removed from their template by some rate-limiting processes. The presence of active and specifically associated forms of β -glucosidase on ribosomes further implies that there may be discrete stages in enzyme release from these particles. Whatever the mechanism of the release may be, the present results suggest that claims for enzyme synthesis with ribosomal particles should be treated with caution, since the conditions employed for amino acid incorporation frequently lead to ribosome degradation and activation of latent enzymes.

Summary.—The distribution of β -glucosidase has been analyzed in a constitutive strain of yeast (*Saccharomyces dobzhanskii* $\times$ *Saccharomyces fragilis*). There are approximately 62,000 molecules of enzyme per cell, of which one molecule of enzyme is firmly bound to the 80S ribosome particles. Approximately $3/4$ of the bound enzyme is latent and can be recognized upon disruption of the particle by metal binding agents. The enzyme released from the particles is identical to the soluble enzyme. Inhibition experiments with an amino acid analogue, p-fluorophenyl-alanine, suggest that the ribosomal-bound β -glucosidase is a precursor of the soluble enzyme.

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AMINO ACID TRANSFER FROM AMINOACYL-RIBONUCLEIC ACIDS TO PROTEIN ON RIBOSOMES OF *ESCHERICHIA COLI**

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We are continuing attempts to understand the mechanism by which peptide bonds are formed in protein synthesis. Progress depends largely on the characterization of the enzymic processes involved in polypeptide synthesis from the amino acid esters of soluble ribonucleic acid (sRNA), the biosynthetically active amino acids. In a previous study,^{1, 2} we used the aminoacyl-sRNA microsome system of rat liver. The recently developed *E. coli* ribosome system^{3, 4, 17} seemed to hold more promise. The present report deals with an analysis of the components of the *E. coli* aminoacyl-sRNA ribosome reaction.

Methods.—Preparation of ribosomes: *E. coli* B was grown in a Biogen (American Sterilizer Co., Erie, Pa.), using a medium composed of 1 per cent dextrose, 1 per cent yeast extract, 0.25 *M* potassium phosphate pH 6.5; it was continuously harvested in a refrigerated Sharples centrifuge at the end of logarithmic growth. The cells were washed once and stored at -20° in 100 gm batches of paste. Ribosomes were prepared as required from 100 gm of bacteria by grinding with 250 gm of alumina (Alcoa A-303) and extracting with 300 ml of 0.01 *M* Tris HCl, pH 7.4, and 0.01 *M* magnesium acetate. After centrifuging first at 10,000 × *g* for 20 minutes and then the super