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RIBOSOME-BOUND β-GALACTOSIDASE

BY D. B. COWIE, S. SPIEGELMAN,* R. B. ROBERTS, AND J. D. DUERKSEN†

DEPARTMENT OF TERRESTRIAL MAGNETISM, CARNEGIE INSTITUTION OF WASHINGTON, D. C.,
AND DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILLINOIS, URBANA

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Considerable evidence has been accumulated to show that ribonucleoprotein particles (ribosomes) provide sites for protein synthesis in animal cells.[1] Studies of the incorporation of radioactive tracers by *Escherichia coli* also indicated that the ribosomes of bacteria are active in protein synthesis (McQuillen, Roberts, and Britten[2]). These authors showed that in growing bacteria, a quantity of material roughly equal to the protein synthesized in three seconds was transiently associated with the ribosomes before being released to the soluble protein fraction of the cell. Since the rate of protein synthesis in *E. coli* is about 0.02 per cent per second, 0.06 per cent of any *particular* protein might be expected to be found transiently associated with the ribosomes. A series of experiments was started to determine whether β-galactosidase showed the same transient association with ribosomes as was indicated for proteins in general. The first experiments showed that a small fraction of the enzyme was bound to the ribosomes, and it is the purpose of the present paper to describe some of the properties of this ribosome-associated enzyme. Furthermore, the results suggest a general procedure for isolating specific ribosomes.

Materials and Methods.—E. coli strains: Three strains of *E. coli* differing in their β-galactosidase-synthesizing properties were used, namely ML 30 (inducible), ML 308 (constitutive), and W2214 (absolute negative).

Growth conditions: Cells were grown at 37°C in a vigorously aerated synthetic medium (C) of the following composition: 2 *g* NH_4Cl, 6 *g* Na_2HPO_4, 3 *g* KH_2PO_4, 3 *g* NaCl, 0.01 *g* Mg as $MgCl_2$, 0.026 *g* S as Na_2SO_4, 900 ml H_2O, and 10 ml. of 10 per cent maltose.

Enzyme induction and assay: Thiomethyl-β-D-galactoside (TMG) or thioisopropyl-β-D-galactoside (TIPG) at 5×10^{-4} *M* were used as inducers of β-galactosidase synthesis. Assays of β-galactosidase on ribosomal preparations were performed with the following mixture buffered at pH 7.4; 0.0027 *M* ortho-nitrophenyl-β-D-galactoside (ONPG), 0.05 *M* NaCl, 0.01 *M* trishydroxymethylaminomethane

(Tris), 0.004 *M* succinic acid, and 0.01 *M* magnesium acetate. The hydrolysis of the ONPG was followed in a Beckman spectrophotometer at 420 mμ. The sodium chloride, tris-succinate mixture was used in place of the more commonly employed phosphate buffer because of the known[3] instability of ribosomes in the presence of phosphate. The rate of ONPG hydrolysis is the same in the two buffer systems.

Preparation of wall-free cell juice: Exponentially growing cultures of *E. coli* were harvested and washed once in a Tris buffer adjusted to pH 7.6 containing 0.01 *M* Tris, 0.004 *M* succinic acid, and 0.01 *M* magnesium acetate (TSM). Following the wash, the cells were resuspended in 10 ml of the same buffer. The cells

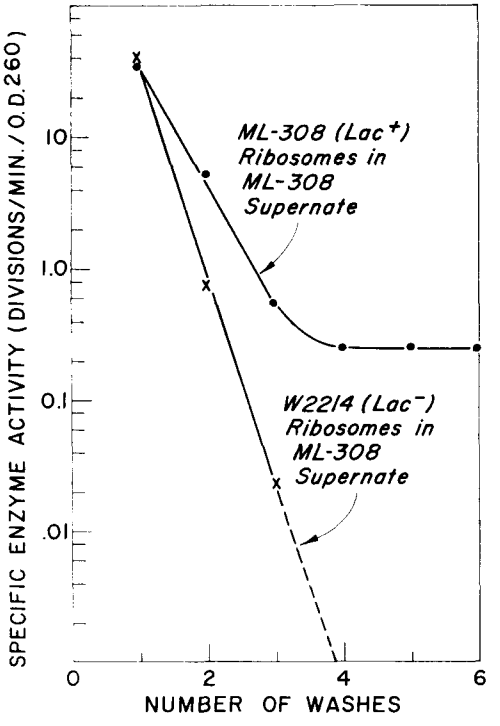

FIG. 1.—The effect of successive washings by centrifugation on the specific enzyme activity of ribosomes from genetic positives (●) and ribosomes from genetic negatives (X), both initially suspended in an extract containing large amounts of active β-galactosidase.

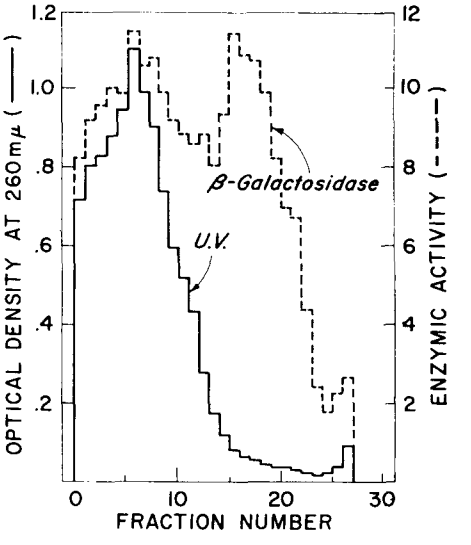

FIG. 2.—Sedimentation pattern of Lac+ ribosomes washed three times prior to being layered on the sucrose gradient (3 to 20 per cent). The run was made at 37K for 80 min.

of this suspension were ruptured by extrusion through a small orifice under pressure (approximately 15,000 lb/sq. inch) in a modified French pressure cell. The extruded material was centrifuged for five minutes at 40,000 rpm in the angle head rotor of the Spinco Model L centrifuge to remove whole cells, cell walls, and other large fragments.

Antisera: β-galactosidase antiserum was prepared by injecting rabbits with 10 mg of purified (90 per cent) *E. coli* β-galactosidase. Chicken anti-rabbit serum was kindly furnished us by Dr. Alan Boyden, Rutgers University, New Brunswick, N. J.

Results.—Enzymatic activity of ribosomes: Centrifugation of a wall-free cell

juice for 45 minutes at 40,000 rpm in the angle head rotor of the Spinco Model L centrifuge gives a pellet (40K 45P) which contains more than 90 per cent of the 70S and 85S ribosomes of the cell. Such a centrifugation leads to a useful separation of the major ribosome components from the bulk of the soluble proteins and smaller particles and yields material suitable for further purification.

One technique for purifying large quantities of 85S and 70S ribosomes involves repeated washing in TSM. Figure 1 compares the amounts of enzymic activity found in the pellet fractions (40K 45P) in successive washings of ribosomes derived from ML 308, the constitutive mutant (circles). It will be noted that by the fourth washing a constant specific enzymic activity (enzyme units per O.D. at 260 mμ) is achieved.

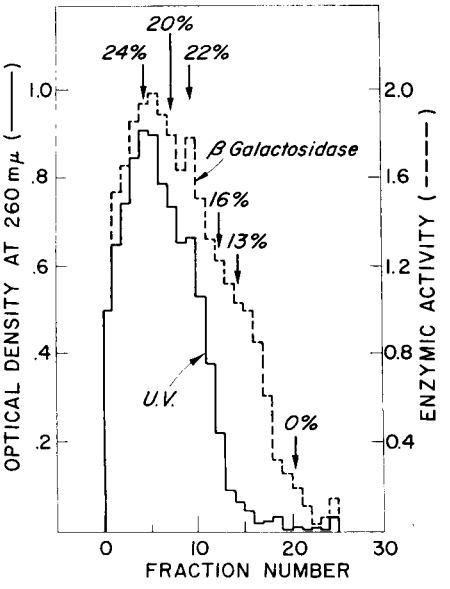

FIG. 3.—Sedimentation pattern of the ribosomes in the first 13 fractions of Figure 2. The numbers denote per cent increases in enzyme activity when the corresponding fraction was incubated with anti-β-galactosidase serum.

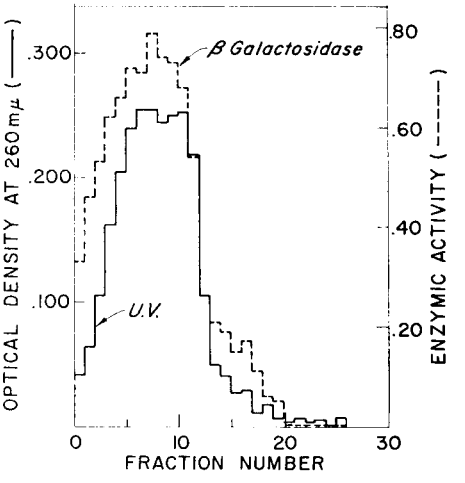

FIG. 4.—Sedimentation pattern of the ribosomes in the first 13 fractions of Figure 3.

Such data do not necessarily establish a specific association between a fraction of the enzyme molecules and the ribosomes. For example, some enzyme molecules could be nonspecifically but firmly adsorbed to the surface of the ribosomes. Alternatively, the enzymic activity of the pellet may not be associated with the ribosomes but may reside in aggregates possessing an average sedimentation constant roughly equivalent to the larger ribosomes.

The first of these possibilities was excluded by the following experiment. An extract rich in enzyme but free of ribosomes was prepared from the constitutive strain, ML 308 (Lac^+), by centrifuging the cell juice for five hours at 40,000 rpm. Purified ribosomes prepared from the absolute lactose-negative strain W2214 (Lac^-) were suspended in this extract. The resulting mixture was subjected to the

successive centrifugations in TSM. Figure 1 shows that the enzyme associated with the genetically negative ribosomal pellet behaves quite differently from that observed with genetically competent material. Initially, the specific enzymic activities of the two ribosomal pellets are about equal. In the lower curve, there is no suggestion of an approach to a constant specific activity on repeated washings. By the fourth step, the Lac^- ribosomes reach a specific activity (not indicated on the graph) which is less than 0.001 units of enzyme per optical density unit. Such enzymic activities are too low for accurate measurement and correspond to levels considerably less than 1/100 of the constant specific activities attained with the Lac^+ ribosomes.

Centrifugation of a layer of ribosome suspension through a sucrose gradient in the swinging bucket rotor of the Spinco centrifuge[4] provides a much better indication that the enzyme is actually associated with the ribosomes.

Figure 2 shows the distribution of the enzymic activity and optical density of a ribosome preparation (subjected first to two washes as described above) after being layered on the sucrose gradient and centrifuged for 45 minutes at 37,000 rpm. The enzyme is distributed roughly equally between the ribosomal and supernatant fractions. Figures 3 and 4 illustrate how successive sedimentations through sucrose gradients lead to the elimination of the soluble enzyme component from the ribosome fraction. The first 13 fractions of the run described in Figure 2 were pooled and the ribosomes collected by centrifugation (40K 45P). They were then resuspended and layered on a new sucrose gradient and centrifuged. The resulting profile of O.D. at 260 mμ and of enzyme activity is given in Figure 3. Comparatively little enzyme is found unassociated with the ribosome peak although there is clearly a contamination of free enzyme. A repetition of this procedure on the first 13 fractions of Figure 3 is shown in Figure 4. Here, there is an excellent correlation of enzyme activity with ribosome peaks in the density gradient.

Nature of the ribosome-associated enzyme: The sedimentation patterns described above encouraged the belief that some enzyme molecules are in physical association with the larger ribosome particles. It was of interest to look for some feature other than the sedimentation characteristics which would serve to distinguish these molecules from those which are in the soluble fraction. A series of experiments were, therefore, performed examining the effect of a variety of agents on the residual ribosomal enzyme activity. Methods of disrupting ribosomes (RNAase, versene, or citrate) which have been shown to be capable of releasing latent RNAase activity of the ribosomes of *E. coli*[5, 6] did not cause any increase in the β-galactosidase activity of highly purified ribosomal preparations. Several attempts to activate such preparations by the addition of the galactoside inducers, TMG and TIPG, were also unsuccessful.

The effect of adding specific antiserum was examined with the hope that it might serve as a specific means for removing the associated enzyme from the ribosomes. Table 1 shows that exposure of purified ribosomes to anti-β-galactosidase results in a striking increase in enzymic activity. In a series of similar experiments using comparably purified ribosomes, increases of between four- and sixfold were often observed. This activation is unique for the specific anti-β-galactosidase serum; normal rabbit serum and nonhomologous antisera (e.g. anti-alkaline phosphatase) have no effect. Further, the increase in activity is confined to ribosome-bound

TABLE 1

EFFECT OF RABBIT SERUM UPON RIBOSOME ASSOCIATED AND SOLUBLE β-GALACTOSIDASE

Fraction	Rabbit serum added	Enzyme activity
Ribosomes (uninduced ML 30)	0	0.04
Ribosomes (" ")	Anti-β-galactosidase	0.15
Ribosomes (" ")	Normal	0.05
Soluble (" ")	0	0.092
Soluble (" ")	Anti-β-galactosidase	0.090

Ribosome fractions were purified by successive centrifugations to constant specific activity and enzyme was assayed with ONPG according to the procedures described under *Methods*. Soluble enzyme was obtained by removal of ribosomes by means of a sucrose gradient swinging bucket centrifugation. Assays were continued until linear rates were well established.

enzyme and is not observed with the soluble enzyme as shown in Figure 3. Aliquots taken from fractions corresponding to different portions of this O.D. profile were tested for their ability to be activated by the specific β-galactosidase antiserum. The increases over controls are recorded in Figure 3 over the appropriate parts of the profile.‡ Fractions corresponding to the ribosome region exhibit the antiserum-activating effect. As one proceeds to fractions closer to the free soluble region, however, the degree of activation falls and finally becomes zero. Thus, the soluble enzyme and the enzyme associated with the ribosomes differ not only in their sedimentation rates but also in their response to antibody.

Two other possible explanations for such activation are (1) that the addition of ribosomes activates in some manner β-galactosidase present in the antiserum which is not detectable in their absence or (2) that the presence of ribosomes nonspecifically inhibits the activity of β-galactosidase and this inhibition can be reversed with specific antiserum. To test the validity of the first suggestion, purified ribosomes prepared from the Lac$^-$ strain (W2214) were incubated with the anti-β-galactosidase serum. These mixtures were assayed for enzymic activity and none was found. It is concluded from such experiments that the observed increase in activity is not due to a latent enzyme in the antiserum. To examine the second possibility, the following experiment was performed. A soluble enzyme fraction was prepared from fully induced cells by exhaustive centrifugation of the cell-free extract to remove the ribosomal components. Purified ribosomes from a Lac$^-$ strain of *E. coli* (W2214) were introduced into this soluble enzyme extract. The ribosomes were then purified in the usual fashion by successive centrifugations. The purification was stopped when the specific enzymic activity corresponded to that at which pronounced activation by antiserum is observed with Lac$^+$ ribosomes (see Table 1). The resulting mixture was then exposed to antiserum and the enzyme activity measured. No activation of the enzyme was ever observed. In a typical experiment, 0.092 div./min were observed in the absence of antiserum and 0.090 div./min in its presence. Since the Lac$^-$ ribosomes did not activate or depress the soluble enzyme activity, one is inclined to believe that the phenomenon is unique for ribosome-associated enzyme.

Mechanism of antibody action: Antibody was added to the ribosome preparations with the hope that the antibody might dislodge the enzyme molecule from the ribosome and thereby expose the active regions. A second possibility was that the antibody might help to shape the associated polypeptide without removing it from the ribosome. The sedimentation characteristics of the enzyme-antibody complex were therefore examined.

If the antiserum removes the enzyme from the ribosomes, one should no longer observe a peak of enzymic activity in the ribosome region. This possibility was tested under various conditions differing widely with respect to the amount of soluble β-galactosidase present. In one case, a soluble protein fraction from the constitutive mutant, containing a small quantity of ribosomes, was incubated with antiserum at room temperature for several hours. The amount of antiserum added was greatly in excess of that required to precipitate all the enzyme present. The mixture was then layered on a sucrose gradient and centrifuged at 37K for 90 minutes. A companion tube was run with enough purified ribosomes to show the position of the ribosome peak by its O.D. at 260 mμ. Figure 5 shows the distribution of enzymic activity observed (dashed line) and of the O.D. at 280 mμ (light line) indicative of the protein distribution. Superimposed is the distribution of O.D. at 260 (heavy line) obtained in the companion tube. More than 99 per cent of the enzyme activity was precipitated as a pellet, and virtually none was found in the region corresponding to the soluble protein. There is, however, still a peak of enzyme activity in the region corresponding to the ribosomes.

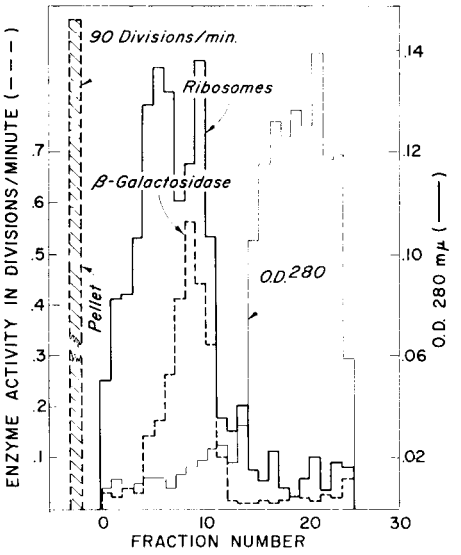

FIG. 5.—The effect of anti-β-galactosidase serum on the distribution of enzyme activity in a sucrose gradient sedimentation pattern of a small amount of ribosomes in the presence of excess enzyme. A companion tube was run to provide the position of the ribosomes as given by heavy line histogram.

The response of the ribosome-enzyme complex to antiserum was also examined using ribosome preparations at various stages of purification. In all cases, the same pattern was observed. Any soluble enzyme present was removed as a pellet, but there remained a residual peak of enzyme activity in the sedimentation pattern regions where the 70S and 85S ribosomes are located.

It is evident that antibody easily forms large precipitable aggregates with soluble enzyme but not with the ribosomal enzyme. Further, the activation effect of anti-serum does not seem to be produced by a removal of the enzyme molecules from their association with ribosomes. Were this the case, the enzyme either would precipitate rapidly, as does the soluble enzyme-antibody complex, or would show a decreased sedimentation constant if it were detached but not able to form an aggregate.

The association of the antibody with the ribosomes was demonstrated by the use of another antibody against the rabbit γ-globulin. A purified ribosome preparation was incubated for one hour in the presence of excess rabbit anti-β-galactosidase serum and then centrifuged for one minute at 25K. As expected, no detectable enzyme was removed from the supernatant, enzyme activities of the supernatants being 4.2 before and 4.7 per 0.1 ml after the centrifugation. In a control incubation and centrifugation with soluble enzyme, virtually all enzymic activity was removed

from the supernatant. Subsequently, excess chicken anti-rabbit serum was added to the mixture of ribosomes and anti-β-galactosidase rabbit serum. After an hour's incubation, this mixture was subjected to a centrifugation (25K for 1 minute). Examination of the supernatant revealed that 94 per cent of the ribosome-bound enzyme had been precipitated. The enzyme removed from the supernatant was found in the pellet fraction. Examination of the ribosome content of the supernatant revealed that less than one per cent had been removed. Hence, less than one per cent of the ribosomes possess the β-galactosidase enzyme.

The amount of ribosome-associated enzyme in different states of induction: It was of interest to examine the effect of induction on the amount of β-galactosidase found associated with the ribosome fraction. Table 2 summarizes the results of a

TABLE 2

β-GALACTOSIDASE ACTIVITY ASSOCIATED WITH RIBOSOMAL PARTICLES OF *E. coli*

Strain	Divisions per minute per O.D. unit at 260 mμ		
	ML 30 (basal)	ML 30 (induced)	ML 308 (constitutive)
	0.017	0.34	0.21
	0.018	0.60	0.17
	0.020	0.46	0.25
	0.016	0.46	0.23
Average	0.018	0.47	0.22

Ribosome fractions were purified by successive centrifugations to constant specific activity and enzyme was assayed with ONPG according to the procedures described under *Methods*.

series of experiments examining the enzymic activity of ribosomes prepared from cultures of noninduced, fully induced, and constitutive cells. The ribosomes were prepared and purified to constant specific enzymic activity by the methods described. It is evident that increased capacity to synthesize enzyme is accompanied by a considerable increase in the amount of ribosome-bound enzyme. A 25-fold increase in the enzymic activity of the ribosome fraction is observed in going from the noninduced to the fully induced state. During this same period, the enzyme content per cell increases by a factor of about 200. It is of interest to note that although the constitutive strain synthesizes about five times as much enzyme per cell as a fully induced cell, this difference is not reflected in an increased amount of ribosome-bound enzyme. On the contrary, this difference was consistently less by a factor of two in the constitutive mutant as compared with the inducible variety.

Discussion.—The experiments described provide strong evidence that the ribosomes carry a small fraction of the β-galactosidase of the cell, possessing three characteristics which distinguish it from the free enzyme molecules: (*a*) it sediments much more rapidly, possessing a sedimentation coefficient of about 70S; (*b*) the ribosome-bound activity is increased by exposure to specific antibody; (*c*) it is not precipitated by an antiserum which removes all of the soluble enzyme present in the reaction mixture. From the results described above, it is not possible to decide whether the enzyme is transiently or permanently associated with the ribosome in the living cell. Experiments to be reported later suggest that both classes are present.

It is of some interest to consider briefly the numerical relations of the partitioning of enzyme molecules between the ribosomal and soluble fractions. For such calculations, we assume that the molecular weight of the RNA in the 70S ribosomes is

1.8×10^6 and that 1 mg of ribosomal RNA/ml will have an optical density of 24. Thus, a solution of O.D. equal to 1 will contain 1.4×10^{13} 70S particles. The turnover number of purified β-galactosidase, measured with orthonitrophenyl-β-D-galactoside, is 4,000 moles/sec per mole of enzyme.[7] Under the conditions of assay, 1 $m\mu M$ of orthonitrophenol yields an O.D. of 0.000143 at 420 mμ. Using these numbers, one can calculate the number of ribosomes per enzyme molecule from the specific activities of the purified ribosomal preparations. This computation leads to a value of 5×10^3 ribosomes per enzyme molecule for the inducible strain in the noninduced state. Cells growing in C-medium are estimated to contain approximately $5–10 \times 10^3$ large ribosomes per cell. It is concluded that there is approximately one enzyme molecule per cell bound to the ribosomes in the noninduced strain. This increases by an order of magnitude upon full induction.

The kinetics of this increase are of great interest and are being investigated. The available data suggest that the increase is a fast process completed in about three minutes. The relative rate of enzyme synthesis in constitutive mutants is usually between 5 and 10 times that seen in fully induced inducible strains. It is evident (Table 2) that this higher rate cannot be ascribed to a higher content of ribosomes which can specifically retain β-galactosidase activity.

It must be emphasized that it is difficult to make unambiguous estimations of the absolute activities of the ribosome-bound enzyme molecules. It seems likely, however, that the orders of magnitude computed from these assays can be taken seriously. It is of some interest that the numbers obtained are what might have been expected from a reasonably simple interpretation of ribosome function. If the ribosomes carrying a given protein in association are the only ones concerned with its synthesis, one would conclude that one to ten ribosomes may be active at any given moment in the synthesis of a particular protein. Thus, the several thousand ribosome of an *E. coli* cell could synthesize roughly a thousand different enzymes which should be adequate to carry out the various cell functions. The data described, however, do not eliminate the possibility that a given ribosome may be concerned with the synthesis of a variety of proteins at different times of its existence.

The experiments reported here raise the question of whether other enzymes can be found associated with the ribosomes and whether these also possess similar distinguishing features. Preliminary experiments along these lines in *E. coli* indicate that the same situation obtains with a variety of enzymes. One in particular, alkaline phosphatase, for which an antiserum was available, also exhibited the antibody-activating effect. Halvorson[8] and his co-workers have uncovered a ribosome-bound fraction of β-glucosidase in yeast. It is again of interest to note that these investigators compute from their data that there is approximately one enzyme molecule bound to the ribosome fraction per cell.

It is evident that the use of specific antiserum may greatly aid the preparation of a ribosome suspension containing a particular protein in bound form and free of its soluble counterpart. Furthermore, the possibility exists of developing a general procedure for isolating ribosomes associated with specific proteins. This is suggested by the experiments in which the ribosome-bound enzyme was precipitated by first complexing with rabbit anti-β-galactosidase and then exposing the complex to an antibody directed against the rabbit γ-globulin.

Summary.—Experiments are described which provide evidence that a certain fraction of the β-galactosidase molecules of the cell are carried on the ribosomes. This fraction corresponds (in order of magnitude) to one molecule per cell in non-induced, inducible cells and rises to between 10 and 20 per cell for the fully induced and constitutive states. In addition to possessing an apparent higher sedimentation coefficient, the ribosome-bound enzyme molecules are distinguishable from their soluble counterparts in their response to specific anti-β-galactosidase serum. Antiserum precipitates the soluble enzyme without affecting the observed activity. Exposure of the ribosome-bound enzyme to antiserum results in a three- to sixfold rise in activity which is not accompanied by the formation of a precipitable aggregate. It was found that the complex can, however, be precipitated by the addition of an antiserum (chick anti-rabbit) directed against the antibody. The latter reaction suggests a means for the isolation of specific ribosomes.

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* Department of Microbiology, University of Illinois, Urbana, Illinois.

† This work was done while J. D. Duerksen was a Research Fellow at the Carnegie Institution of Washington. Present address: Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas.

‡ Aged preparations lose their ability to respond to the specific antibody. One preparation which showed a fourfold increase in the presence of antibody showed no increase after two weeks storage at −20°C.

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