group were obtained and an increase in "small" antibody was found, the response consisted predominately of the rapidly sedimentating "large" antibody (Fig. 2). The deficiency in converting to "small"-antibody synthesis in the tertiary response of the allogeneic chimeras might possibly be related to the small amount of this antibody produced in the primary response. To test this, groups of normal mice were injected with an antigen dose designed to yield a weak primary response with an amount of "small"-antibody production comparable to that obtained with the allogeneic mice. The normal mice were then given a third course of *S. typhi* antigen, and the titrations for antibody and sucrose gradient studies were performed. This response (data not shown) to the third antigen injection also consisted predominately of "small" antibody, similar to that shown for the normal control group in Figs. 1*A* and 2. Thus, the low amount of "small" antibody in the allogeneic chimeras after the primary injection was not the factor that prevented maximum conversion to "small"-antibody synthesis after the third antigenic stimulus.

Finally, the absence of 2-ME–resistant antibody in the primary response of chimeras (Fig. 1*B*) does not agree completely with the sucrose gradient results (Fig. 2), which show the presence of "small" antibody. Therefore, some caution should be applied in the current usage of "small" or "large" antibody terminology to denote 7*S* or 19*S* antibody, respectively, when the analysis is based on 2-ME–inactivation studies alone. In this regard, Rocky and Kunkel (*8*) have reported the presence in human serum of antibodies having intermediate sedimentation coefficients of 9*S* to 15*S* which are also sensitive to 2-ME treatment. The antibodies discussed in this report may thus represent a broad range of molecular size, as the sucrose density-gradient studies alone do not permit distinction between 7*S* and 9*S* molecules, and similarly, in the more rapidly sedimentating zone, distinction between 15*S* and 19*S* antibody molecules would not be possible.

Thus, allogeneic chimeras possess an abnormal mechanism for the production of humoral antibody. Also, additional studies have revealed that this defect is present in varying degrees among such mice; great latitude exists from truly abnormal to almost normal ability to convert to small-antibody formation. Whether this variability is related to the status of the immunologic tolerance between host and graft tissues in these animals is not known. Such chimeras may be used as models for the study of the sequential conversion of synthesis of large antibody to that of the smaller type; the physiology of this process in normal animals is not yet clearly understood (*9*).

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1. Recommended by G. Snell [*Transplant.* **2**, 655 (1964)], the new terminology for tissue transplantation has been used in this report. Homologous, previously used to denote genetic differences within a species, has been replaced by allogeneic; heterologous, previously used to denote genetic differences between species, is replaced by xenogeneic; and isologous, once used to denote the same genetic origin, is replaced by isogeneic.
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Polyribosomes from Escherichia coli: Enzymatic Method for Isolation

Abstract. *Polyribosomes can be rapidly extracted from* Escherichia coli *by sequential passage of the cells through solutions of a chelating agent and lysozyme in a centrifugal field. Biosynthesis of protein in whole cells and in a cell-free system occurs almost exclusively in the polyribosomes.*

Although techniques for the isolation of polyribosomes from animal tissues have been developed by Wettstein *et al.* (*1*) for rat liver, Penman *et al.* (*2*) for HeLa cells, and Gierer (*3*) and Marks *et al.* (*4*) for rabbit reticulocytes, it has proved more difficult to obtain them from *Escherichia coli* cells.

Staehelin *et al.* (*5*) demonstrated the existence of polyribosomal aggregates in *E. coli*, and Schaechter (*6*) has extracted polyribosomes from a number of bacterial species, including *E. coli*, by gentle lysis of the whole organisms in a French pressure cell. Kiho and Rich (*7*) have also been able to obtain polyribosome-rich extracts of *E. coli* by treatment of the cells with penicillin and subsequent lysis of the protoplasts by detergent.

We now have isolated polyribosomes from *E. coli* by a technique (*8*) for producing spheroplasts of this organism. The spheroplasts produced by the original method will not yield significant amounts of polyribosomes, apparently because during the formation of spheroplasts, EDTA (disodium ethylenediaminetetraacetate) can effect chelation of intracellular divalent cations and thus break down the polyribosomes. If the spheroplasts are incubated in a growth medium, polyribosomes, presumably produced from newly synthesized messenger RNA (*9*), can be isolated.

If the cells could be briefly exposed to EDTA and lysozyme in succession, rather than together, the enzyme might be able to act on the cell wall without the undesired accompanying depletion of intracellular cations (*10*). Such treatment was achieved by sedimenting the bacterial cells through sucrose solutions of different densities with EDTA in an upper and lysozyme in a lower layer. The resulting cells are not true spheroplasts, but their cell walls are weakened sufficiently to be readily opened by detergents. Removal of unbroken cells and cellular debris then yields an extract rich in polyribosomes.

The technique is simple, rapid, and reproducible; the protein synthesizing activity of crude extracts of *E. coli* is concentrated in these polyribosomes both in vivo and in vitro.

Escherichia coli was grown in Medium A of Davis and Mingioli (*11*) (the disodium citrate being omitted) with 0.2 percent glucose added; the cultures (100 ml) were incubated at 37°C on a rotary shaker. During exponential growth the cells were harvested by centrifugation and suspended in magnesium-tris buffer (15 m*M* magnesium acetate, 5 m*M* tris buffer, *p*H 7.6 at 23°C). One milliliter of the cell extract [at 20 to 40 mg (wet weight)/ml] was layered over four layers of sucrose solutions in a 10-ml cellulose tube. These layers were (from the bottom up): (i) 2 ml of 25 percent sucrose in the magnesium-tris buf-

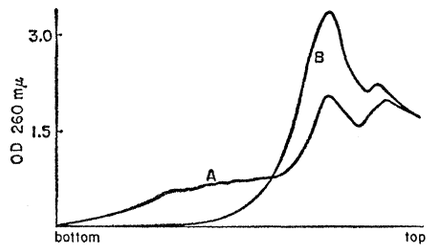

Fig. 1. The effect of ribonuclease on *Escherichia coli* polyribosomes. An extract was prepared from exponentially growing cells and incubated for 10 minutes at 0°C, (*A*) in the absence of ribonuclease, and (*B*) in the presence of ribonuclease (2 μg/ml). Both extracts were then examined by the sucrose density-gradient technique.

fer; (ii) 2.5 ml of 15 percent sucrose in 0.03*M* tris buffer, *p*H 8.2 at 23°C, and 1 mg of lysozyme per milliliter (twice recrystallized, Nutritional Biochemicals) freshly dissolved; (iii) 2.5 ml of 10 percent sucrose in 5 m*M* EDTA; and (iv) 1.5 ml of 5 percent sucrose in 5 m*M* tris buffer, *p*H 7.9 at 23°C. The tubes were placed in an HS-swinging bucket head in a Sorvall RC-2 centrifuge, the head was brought to 20,000*g* with maximum acceleration for 2 minutes, and the centrifuge was decelerated with the brake on. All operations up to this point were carried out at room temperature; subsequent steps were at 0°C. The sucrose solutions were decanted, and the cell pellets were suspended in a small volume of the magnesium-tris buffer; sodium deoxycholate (10 percent) was added to a final concentration of 0.25 percent and left standing for 5 minutes. Unbroken cells and cellular debris were removed by centrifugation at 20,000*g* for 10 minutes. The supernatant was collected.

For analysis of the suspension on sucrose density gradients, 0.2 to 0.4 ml was layered directly on a linear (15 to 30 percent) sucrose gradient in the buffer of Nirenberg and Matthaei (*12*) (0.06*M* KCl, 0.01*M* magnesium acetate, 0.006*M* 2-mercaptoethanol, 0.01*M* tris buffer, *p*H 7.6 at 23°C), in the Spinco SW 39 head. The tube was centrifuged for 1 to 1¼ hours in the Spinco Model L preparative ultracentrifuge at 39,000 rev/min. The bottom of the tube was then punctured, and the contents were pumped through a Gilford spectrophotometer with a finger pump. Optical density was monitored continuously with a graphic recorder. Amino acid incorporation in vitro was assayed by the methods of Nirenberg and Matthaei (*12*). Radioactivity incorporated into protein was measured by collection of the material precipitable by hot acid onto Millipore filters and counted in a Nuclear-Chicago end-window gas flow counter. RNA was determined spectrophotometrically after partial alkaline hydrolysis, an extinction coefficient of 32 cm^2/mg at 260 mμ being used (*13*). DNA was determined by the diphenylamine reaction (*14*).

The treated cells may be lysed by 0.25 percent sodium deoxycholate, 0.1 percent sodium dodecyl sulfate, or 0.5 percent Brij 58 (Polyoxyethylene-20 cetyl ether; Atlas Chemical) without any significant differences in recovery of polyribosomes or distribution of ribosomes on the gradient. Varying the deoxycholate concentration from 0.05 percent to 0.5 percent had no significant effect.

In a series of 15 experiments with cells grown on minimal medium and harvested during the middle of the log phase of growth, the polyribosomes constituted 47 ± 5 percent of the total extracted ribosomes, as determined by measuring the relative areas under the polysomal and monosomal regions in gradient sedimentation. In four experiments with cells grown in nutrient broth a similar percentage was obtained (46 ± 3 percent). The efficacy of the technique was dependent on temperature: if the procedure was carried out at 0°C, there was no significant lysis of the treated cells by detergent. The deoxycholate lysate of exponentially growing cells contained 40 to 50 percent of the total cellular RNA and no detectable DNA.

Figure 1*A* shows a typical sucrose density-gradient profile of the extract obtained by this method from exponentially growing cells. Very mild treatment by ribonuclease (2 μg/ml for 10 minutes at 0°C) led to complete conversion of the polyribosomal material (Fig. 1*B*) to monosomes. Deoxyribonuclease (20 μg/ml for 10 minutes at 0°C) did not change the sedimentation profile, although this treatment was sufficient to reduce appreciably the viscosity of an S-30 fraction (supernatant of a crude cell extract centrifuged at 30,000*g*) prepared as described by Nirenberg and Matthaei (*12*).

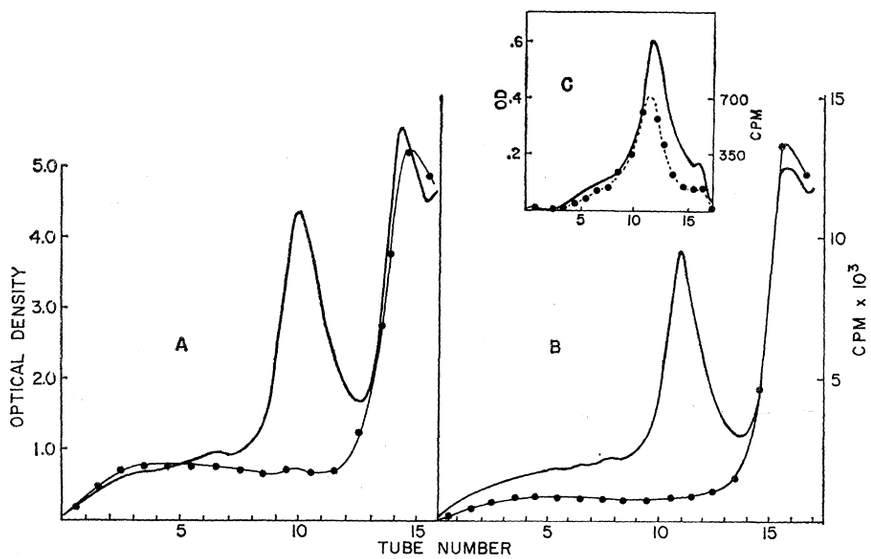

Fig. 2. Amino acid incorporation in whole cells. (*A*) Cells of *E. coli* growing exponentially at 37°C were exposed to 2 μc of C^{14}-leucine (1.3 μc/μg) for 15 seconds and rapidly chilled; an extract was then made as described. A sample of the extract was centrifuged for 1¼ hours at 39,000 rev/min on a linear sucrose density gradient (15 to 30 percent). Samples (4 drops each) were collected and precipitated with 5 percent trichloroacetic acid and carrier protein; the material precipitable by hot acid was collected on Millipore filters and counted. (*B*) Experimental procedure as in *A*, except that at the end of 15-second exposure to C^{14}-leucine, a 200-fold excess of unlabeled leucine (0.04 m*M*) was added to the cells for an additional 3 to 5 seconds. (*C*) (Inset) Tubes 10 and 11 from the sucrose gradient of Fig. 2*A* were collected, combined, and centrifuged at 35,000 rev/min for 2½ hours in 2-ml cellulose tubes in the Spinco Model L preparative centrifuge. At the end of the centrifuge run the pellet was resuspended in magnesium-tris buffer, layered on a 15 to 30 percent sucrose gradient, and centrifuged for 1¼ hours at 39,000 rev/min. Samples (four drops each) were collected, precipitated, and counted, as above.

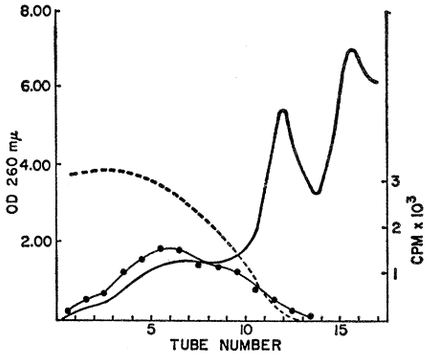

Fig. 3. Incorporation of amino acids by *E. coli* polyribosomes in vitro. An extract from *E. coli* cells was applied to a 15 to 30 percent linear sucrose gradient and run for 1 hour at 38,000 rev/min. Samples (four drops each, approximately 0.30 ml) were incubated with 105,000*g* supernatant (0.2 ml/ml), adenosine triphosphate (1 m*M*), C^{14}-leucine (1.3 μc/μg, 8 μM), and 0.05 m*M* of the other 19 amino acids. Incubation was terminated at 45 minutes by the addition of 5 percent trichloroacetic acid. Material precipitable by hot acid was collected on Millipore filters and counted. The dashed line represents the specific activity of each fraction as counts per minute per absorbancy unit at 260 mμ.

Kiho and Rich (*7*) found that when *E. coli* spheroplasts were exposed to labeled amino acids for 30 seconds and were subsequently lysed, most of the label appeared in either the polyribosomes or the soluble protein, but appreciable amounts were also found in the monosome region. With our technique, exposure of exponentially growing cells to a labeled amino acid for very short periods of time (10 to 15 seconds) resulted in the appearance of virtually all the label in the polyribosomes and the soluble fraction (Fig. 2*A*). The specific radioactivity (count/min per unit of optical density at 260 mμ) of the polyribosomes was five to six times greater than that of the monosomes and accounted for more than 80 percent of the label incorporated into the ribosomes.

In order to determine how much of the label in the 70*S* region was due to overlap from the soluble protein and polyribosome areas, the monosome area was collected, sedimented, and layered on a second sucrose density gradient. Only about 50 percent of the radioactivity apparently associated with the monosomes actually resedimented with the monosomes (Fig. 2, inset), the rest being found in the contaminating polyribosome and soluble fractions. Thus relatively few of the monosomes carry nascent chains.

It seems unlikely that monosomes not carrying nascent protein arise by breakdown of the polyribosomes during the isolation procedure, for Kiho and Rich (*7*) demonstrated that mild ribonuclease treatment resulting in polyribosome breakdown does not remove nascent protein from the monosomes. Loss of nascent protein chains during polyribosome breakdown by other mechanisms, however, cannot be ruled out.

When cells which had been exposed to labeled amino acid, as already described, were subsequently given a large excess of unlabeled amino acid for 3 to 5 seconds, about 50 percent of radioactivity shifted from the polyribosome region to the soluble protein region without detectable appearance in the monosome region (Fig. 2*B*). Since approximately one half was removed in 3 to 5 seconds, the average time required to synthesize a protein molecule in vivo is approximately 6 to 10 seconds. This estimate agrees with those of McQuillen *et al.* (*15*) and Goldstein *et al.* (*16*).

Gilbert (*17*) demonstrated that most of the protein-synthesizing activity of an extract of *E. coli* prepared by alumina grinding resided in the polysomal region. Because such extracts contain relatively few polyribosomes, it is difficult to calculate the specific incorporating capacity of the ribosomes in polysomes. Because our technique allows the isolation of appreciable amounts of polyribosomes, the ability of these ribosomal aggregates to synthesize protein in vitro can be accurately determined. When samples from a gradient were collected and incubated in an amino acid–incorporating system in vitro (*12*), virtually all the protein-synthesizing activity was found in the polyribosome region (Fig. 3), and the incorporation ability per ribosome (count/min per optical density unit) was the same for both large and smaller polysomes. Wettstein *et al.* (*1*), using rat-liver ribosomal aggregates, have obtained similar data. From the data of Schlessinger (*18*), we calculate that membrane-attached polyribosomes from *Bacillus megaterium* incorporate amino acids in vitro to roughly the same extent as do the *Escherichia coli* polyribosomes prepared by our method.

It is difficult to estimate to what extent the cells attacked by lysozyme release their components. A priori it might be expected that there are all gradations in the extent of damage to cells, from those releasing only small molecules to those releasing polyribosomes. Thus there may be a preferential release of monosomes resulting in a higher ratio of monosome to polysome than exists in the cell. However, the monosomes seen, as mentioned above, probably do not arise by polysome breakdown during preparation. These monosomes are competent to function with polyuridylic acid in extracts, and in vivo they can accept natural messenger (*19*). Thus it would appear that the bacterial cell has a steady-state reserve of functional ribosomes. This conclusion was also reached by Goldstein *et al.* (*16*).

By our method we can isolate polyribosome-rich extracts from intact growing cells of *E. coli* without prolonged treatment and apparently without extensive breakdown to monosomes. It should therefore be possible to perform experiments on the biosynthesis of polyribosomes, as reported for rat liver (*20*), and to investigate more closely relatively transient events in the control of protein synthesis. The technique is also applicable to nongrowing cells.

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