SENSITIZATION OF BACTERIAL RIBOSOMES FOR IN VITRO AMINO ACID INCORPORATION*

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The addition of the supernatant of a high speed centrifugation of bacterial extracts to suspensions containing bacterial ribosomes, transfer RNA, synthetic or native polynucleotides, and an energy generating supply stimulates the incorporation of amino acids into acid-insoluble polypeptides (1, 2, 3, 4). The isolation and characterization of the stimulating factors present in the high speed supernatant would be facilitated by reducing the background incorporation of unstimulated ribosomes without impairing their ability to respond. We present a method for preparing sensitized ribosomes by sedimenting them in a high ionic strength solution whose density is greater than that of the average protein.

Methods: Cells of wild type Escherichia coli strain K-12, grown in a glycerol medium were employed (5). Ribosomal extracts were prepared in TSM buffer (0.01 M magnesium acetate, 0.004 M succinic acid, 0.01 M tris(hydroxymethyl)aminomethane, 0.006 M 2-mercaptoethanol; pH 7.4) by the method of Tissières, et. al. (1).

A linear gradient of CsCl was prepared in plastic centrifuge tubes by mixing CsCl solutions of 1.3 g/cm^3 and 1.6 g/cm^3 made in

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TSM buffer containing 0.03 M magnesium acetate. Concentrated solutions of CsCl and magnesium acetate were added simultaneously to chilled ribosomal preparations to make a solution of density 1.3 g/cm^3 and 0.04 M in Mg^{++}. Volumes of 0.1 to 0.4 ml of these ribosomes were layered on top of the gradient. The preparation was centrifuged at 35,000 rpm for 2 to 6 hours in a swinging bucket rotor SW-39 in a Spinco L centrifuge. Samples were recovered by puncturing the bottom of the tube and collecting drop fractions, or for prolonged centrifugations, by slicing the tube and removing the pellet.

Ribosomal content was determined by the optical density at 260 mμ. Fractions containing ribosomes were pooled, diluted in TSM buffer, and centrifuged at 39,000 rpm for 120 min. The pellet, now completely colorless and transparent, was resuspended, recentrifuged to insure removal of CsCl, and used for incorporation studies. Ribosomes washed an equal number of times in TSM, but not exposed to the salt density gradient, were also employed.

Transfer RNA was prepared from E. coli by the method of Ofengand, et. al. (6). Polyuridylic acid was a gift from Dr. W. E. Razzell, or was purchased from Miles Laboratories. The supernatant of the first 39,000 rpm centrifugation of the original cell-free extract was centrifuged at 45,000 rpm for 90 min. to remove traces of ribosomes, then dialysed 18 hours against TSM buffer.

The complete incorporation mixture in 1 ml final volume contained up to 5 mg ribosomes, up to 0.2 ml dialysed supernatant, 1.1 mg transfer RNA, 0.45 $O.D._{260m\mu}$ units of polyuridylic acid, 100 μmoles tris, 10 μmoles magnesium acetate, 60 μmoles KCl, 6 μmoles 2-mercaptoethanol, 5 μmoles phosphoenolpyruvate, 1 μmole ATP, 0.03 μmoles each of GTP, CTP, and UTP, 20 μg

pyruvate kinase, and 9 mμmoles uniformly labeled C^{14}-phenylalanine (21 μc/μmole); pH 7.8.

Mixtures were incubated at 37° C. The reaction was stopped with one ml of 15% TCA. The precipitate after centrifugation was dispersed in 1 M NaOH, reprecipitated in 5% TCA, and heated for 15 min. at 90° C. Samples were chilled, filtered through millipore membrane filters, washed, dried, and counted in a Nuclear Chicago flow counter with an efficiency of 20-30 percent. A background of 16 cpm and a zero time value of 3 to 7 cpm have been subtracted from the data presented.

TABLE 1

Incorporation of C^{14}-phenylalanine into acid-insoluble product by ribosomes prepared by washing in buffer or by centrifugation in a CsCl gradient.

Ribosomes (mg) Gradient-treated	Buffer-washed	Supernatant (ml)	Acid-insoluble Product (counts/min)
		Preparation 1	
5	-	-	<1
5	-	0.15	1759
-	5	-	65
-	5	0.15	283
5*	-	0.15	55
		Preparation 2	
0.22	-	-	5
0.22	-	0.15	1028
-	0.22	-	139
-	0.22	0.15	316

*Polyuridylic acid was omitted from this sample, and 0.5 μmoles of all l-amino acids other than phenylalanine was added.

Results: The results in Table 1 indicate that the incorporation of amino acids into protein by gradient-treated ribosomes is essentially nil in the absence of the supernatant fraction, and is markedly stimulated by its presence. Presumably, the reduction of background incorporation by unsupplemented gradient-treated ribosomes is effected by the removal of traces of supernatant components which otherwise adhere to washed ribosomes. In support of this hypothesis, we note that in studies of ribosomal-bound enzymes (5, 7), this and similar methods of preparing ribosomes have been used to remove contaminant proteins.

Some preparations show an increase in their absolute ability to carry out incorporation (Table 1). We find an optimum ratio for supernatant to ribosomes, above which an increase in the relative amount of supernatant results in decreased incorporation (Table 2). This suggests that salt-gradient treatment removes an

TABLE 2

Effect of supernatant fraction on incorporation of C^{14}-phenylalanine into acid-insoluble product by gradient-treated ribosomes.

Gradient-treated ribosomes (mg)	Supernatant (ml)	Acid-insoluble product (counts/min)
2	-	1
1	0.05	1260
1	0.1	1170
1	0.2	310
-	0.2	41
2	0.2	2665
2*	0.2	66

*Polyuridylic acid was omitted from this sample, and 0.5 μmoles of all l-amino acids other than phenylalanine was added.

inhibitor or nuclease, or releases inactive messenger-RNA from ribosomes, thus revealing new attachment sites for added polyuridylic acid. Restoring the supernatant fraction not only provides the additional factors necessary for incorporation, but may also reintroduce the antagonizing agent.

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