[3] Cell-Free Protein Synthesis Directed by Messenger RNA

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Assay Method

Principle. Cell-free extracts of *Escherichia coli* incorporate C^{14}-amino acids into protein. Under certain conditions this amino acid incorporation is dependent on the addition of synthetic[1-3] or natural[1,4] messenger RNA, thus providing a sensitive assay for such RNA fractions. In the absence of added messenger RNA, amino acid incorporation presumably is directed by preformed and newly synthesized endogenous messenger RNA. Preincubation of extracts with DNase prevents new messenger RNA synthesis and depletes endogenous messenger RNA. C^{14}-Amino acid incorporation by these extracts is therefore dependent on added messenger RNA and over a certain range is proportional to the amount added.

E. coli extracts ("preincubated" S-30 fractions) are incubated with an ATP-generating system, a C^{14}-amino acid, nineteen C^{12}-amino acids, and messenger RNA. After incubation, proteins are precipitated and treated to remove C^{14}-amino acyl transfer RNA and free C^{14}-amino acid. The C^{14} in the washed protein precipitates then is determined.

Reagents

1. Mix I contains: 10.0 ml. of 2 *M* Tris buffer, pH 7.8; 2.0 ml. of 1.4 *M* magnesium acetate; 5.0 ml. of 2 *M* KCl; 3.0 ml. of 6.66 ×

[1] M. W. Nirenberg and J. H. Matthaei, *Proc. Natl. Acad. Sci. US.* **47**, 1588 (1961).
[2] J. H. Matthaei, O. W. Jones, R. G. Martin, and M. W. Nirenberg, *Proc. Natl. Acad. Sci. US.* **48**, 666 (1962).
[3] J. F. Speyer, P. Lengyel, C. Basilio, and S. Ochoa, *Proc. Natl. Acad. Sci. US.* **48**, 63 (1962).
[4] A. Tsugita, H. Fraenkel-Conrat, M. W. Nirenberg, and J. H. Matthaei, *Proc. Natl. Acad. Sci. US.* **48**, 846 (1962).

10^{-2} *M* ATP, Na salt, and 2.0×10^{-3} *M* GTP, Na salt. Store in 5-ml. aliquots at −20°.

2. Phosphoenolpyruvate kinase, crystalline, 10 mg./ml., obtained as a suspension in $(NH_4)_2SO_4$ solution (California Biochemical Corp.).
3. 7.5×10^{-2} *M* phosphoenolpyruvate, K or Na salt, crystalline (California Biochemical Corp.) Store in small aliquots at −20°. The tricyclohexylammonium salt of phosphoenolpyruvate inhibits C^{14}-amino acid incorporation and should be converted to the potassium salt as follows: 5 g. of phosphoenolpyruvate, tricyclohexylammonium salt, are dissolved in minimal quantities of H_2O at 3°, and the solution is added to a washed Dowex 50 (H^+) column of approximately 2.5×15 cm. Phosphoenolpyruvic acid is eluted with H_2O, and the pH of the eluate is determined rapidly with pH paper. When the pH of the eluate drops to 2, collection of the solution is begun, after the pH rises to 4, collection is discontinued. The phosphoenolpyruvic acid solution is converted to the potassium salt by the addition of *M* KOH with vigorous stirring. The pH of the final solution should be 5.5 to 6.0.
4. 2-mercaptoethanol.
5. C^{14}-L-Amino acid. 10^{-2} *M*, 4 mc./millimole.
6. Mixture of nineteen C^{12}-L-amino acids, minus the appropriate C^{14}-amino acid, 2×10^{-3} *M* (each amino acid).
7. Poly U (Miles Chemical Co., Clifton, New Jersey), 30 O.D. units/ml. at 260 mμ, or other messenger RNA preparation. Poly U solutions should be stored at −20° in small aliquots. It is important to note that poly U undergoes a conformational change at 6°, so that solutions which have been frozen and thawed may have lower absorbancies at 260 mμ.
8. "Preincubated" S-30 or S-100 and W-RIB enzyme fractions, described below.

Procedure. (*a*) For ten reaction mixtures, prepare mix II each day as follows: 250 μl. of mix I; 1 μl. of 2-mercaptoethanol; 250 μl. of phosphoenolpyruvate, 4 μl. of phosphoenolpyruvate kinase suspension; 250 μl. of C^{12}-amino acid mixture, and 50 μl. of C^{14}-amino acid solution.

(*b*) Add components of each fraction mixture in the following sequence: 80 μl. of mix II; appropriate amount of H_2O to make final volume 0.25 ml.; 5 to 20 μl. of poly U solution (15 to 20 μg. of poly U or 100 μg. of natural messenger RNA usually saturates a reaction mixture); and *E. coli* extracts (0.5 to 2 mg. of protein). Reaction mixtures are incubated at 37° for 15 minutes. C^{14}-Amino acid incorporation should be

dependent on the addition of messenger RNA, and, when limiting amounts of messenger RNA are added (see Remarks), incorporation should be proportional to the amount of messenger RNA added.

(*c*) Protein is precipitated at the end of incubation by the addition of 3.0 ml. of 10% trichloroacetic acid at 3°, and precipitates are washed by the method of Siekevitz.[5]

For exploratory work and preparation of extracts, the following fast procedure[6] for washing protein precipitates may be used for all C^{14}-amino acids incorporations *except* C^{14}-*tryptophan.* Reaction mixtures deproteinized by the addition of 10% trichloroacetic acid are placed in a water bath at 90° to 95° for 20 minutes to hydrolyze amino acyl transfer RNA. Tubes then are chilled in ice for 30 minutes. Protein precipitates are dispersed by stirring or by vigorous agitation, and each suspension is filtered under suction through a Millipore filter (HA Millipore filter, 25 mm. in diameter, 0.45-μ pore size, held in an appropriate Millipore filter funnel, Millipore Co., Bedford, Massachusetts). Each precipitate then is washed rapidly with five 5-ml. aliquots of cold 5% trichloroacetic acid. If the radioactivity of the washed protein precipitate is to be counted in a thin-window gas-flow B-scaler, the Millipore filter is glued with rubber cement to a disposable planchette, dried for 5 to 10 minutes under an infrared lamp, and counted. Curled Millipore filter edges should be reglued, for they will tear windows of gas-flow counters.

Growth of *E. coli*

Escherichia coli W-3100 (other strains may be used) are grown at temperatures between 25° and 37° (30° is advantageous) in a medium containing 8 g. of nutrient broth (Difco Co., Detroit, Michigan) and 5 g. of glucose per liter. A 60% solution of glucose is autoclaved and is added to autoclaved nutrient broth solution after both have cooled. A typical preparation consists of five 19-l. carboys, each containing 15 l. of nutrient broth–glucose solution and equipped for aeration. About 1 g. of Antifoam A (Dow-Corning Corp.) is suspended with stirring in approximately 50 ml. of H_2O, and the suspension is centrifuged at $3000 \times g$ for 10 minutes to remove undissolved lumps. The supernatant solution of antifoam is autoclaved, and approximately 1 to 2 ml. are added to each carboy before inoculation with bacteria. Additional antifoam may be needed to prevent foaming during growth of bacteria; however, minimal amounts should be used. One liter of nutrient broth–glucose solution in a 6-l. Ehrlenmeyer flask is inoculated with *E. coli* grown on the surface

[5] P. Siekevitz, *J. Biol. Chem.* **195, 549** (1952).
[6] M. W. Nirenberg, unpublished data.

of an agar slant, and the flask is aerated by shaking at 25° for 8 to 12 hours. While the culture is still in the logarithmic growth phase, 200-ml. aliquots are added to each carboy. The carboys are vigorously aerated for 1.5 to 6 hours, depending on the temperature of the medium and the degree of aeration. At 30°, 2.5 to 3.0 hours of growth will suffice. *It is important to follow cell growth carefully so that aeration may be stopped at the proper time.* Aliquots of cultures should be removed at intervals so that turbidities may be determined spectrophotometrically. Aeration should be stopped when a reading of 25 to 35 Klett spectrophotometer units at 520 mμ is obtained, compared to a sterile nutrient broth–glucose solution set at zero. The carboys then are chilled in ice water, and the cells are harvested by means of a refrigerated Sharpless centrifuge. A yield of 0.5 to 0.75 g. of packed cells, wet weight, per liter of medium is optimum. *When more cells are obtained, the extracts often are less active.* The packed cells are washed by rapid suspension in 3 vol. (w/v) of 0.01 *M* Tris, pH 7.8, 0.014 *M* magnesium acetate, and 0.06 *M* potassium chloride at 3°, and are centrifuged at 15,000 × *g* in preweighed tubes for 15 minutes at 3°. The supernatant solutions are decanted, and the pellets are drained. Centrifuge tubes again are weighed to obtain wet weights of packed cells. Washed, packed cells may be frozen quickly and stored for several months at —20° if desired.

Preparation of Extracts

Step 1. All operations, unless specified, are carried out in a cold room or at 3°. Cells may be disrupted either by grinding with alumina in a mortar, or with a French press (American Instrument Co.). If the alumina grinding method is to be used, about 30 g. of fresh or thawed packed cells are transferred to a large prechilled, unglazed porcelain mortar (20 cm. in diameter), and 30 g. of alumina A-301 (Aluminum Corporation of America) are added. The cells are ground vigorously with a pestle into a thick paste; cracking, popping noises should be produced during grinding. As the cells break, the paste becomes more fluid, and an additional 30 g. of alumina are slowly added to keep the paste thick and to maintain the popping noise. After about 15 minutes of grinding, 60 ml. of "standard buffer" containing 0.01 *M* Tris, pH 7.8, 0.014 *M* magnesium acetate, 0.06 *M* potassium chloride, and 0.006 *M* 2-mercaptoethanol freshly prepared are added to the mortar, and the paste is suspended evenly by gently stirring. Alumina, intact cells, and debris are removed by centrifugation at 20,000 × *g* for 20 minutes. The supernatant fluid is decanted, and the pellet is discarded. Cells to be broken in a French press should be evenly suspended in 1.5 to 2.0 vol. of "standard buffer" (w/v) and disrupted with 18,000 p.s.i. in a pre-

chilled cylinder. Additional 2-mercaptoethanol (6 micromoles/ml. of extract) is added after the cells have been broken.

Step 2. Extracts disrupted in either manner are treated as follows: Two micrograms of pancreatic DNase (twice crystallized, Worthington, Biochemical Co.) are added with gentle mixing to each milliliter of *E. coli* extract. (*Caution:* Some commercial DNase preparations are contaminated with pancreatic RNase, which strongly inhibits C^{14}-amino acid incorporation into protein.) Before the DNase is added, the extract obtained by alumina grinding should be noticeably viscous and should contain some gelatinous clots. Although the extract is maintained at 3°, 5 minutes after addition of the DNase the viscosity and clots disappear. The extract is centrifuged at $30{,}000 \times g$ for 30 minutes at 3°. The supernatant solution is removed by aspiration to within 1 cm. of the pellet and again is centrifuged at $30{,}000 \times g$ for 30 minutes. The upper four-fifths of the supernatant solution is removed by aspiration, and henceforth will be referred to as the *S-30 fraction.*

At this stage the activities of 25 to 150-μl. aliquots of the S-30 fraction should be assayed rapidly to determine how much C^{14}-phenylalanine can be incorporated into protein in the presence and in the absence of poly U. One milligram of S-30 protein added to a 0.25-ml. reaction mixture containing 15 to 20 μg. of poly U should incorporate 1 to 20 millimicromoles of C^{14}-phenylalanine into protein in 15 minutes at 37°. If, *in the absence of poly U,* 1 mg. of S-30 protein directs more than 0.1 millimicromole of C^{14}-phenylalanine into protein, the "preincubation" procedure described in step 3 should be followed. (Step 3 usually is necessary.) If less than 0.1 millimicromole of C^{14}-phenylalanine is incorporated in the absence of poly U, the S-30 fraction may be dialyzed for 8 hours as described in step 3.

For routine messenger RNA assays and many amino acid incorporation studies, ribosomes need not be separated from the S-30 fraction. If the S-30 preparation at this stage can be used for assays, the dialyzed extract should be divided into 1- to 3-ml. aliquots (one tube of extract per experiment), frozen quickly, and stored until needed.

Step 3. "Preincubation." S-30 extracts carried through this step are almost completely dependent on added messenger RNA.

The components of a reaction mixture appropriate for "preincubation" of 100 ml. of S-30 are: 10 ml. of *M* Tris, pH 7.8; 2.0 ml. of 0.14 *M* magnesium acetate; 4.0 ml. of 0.02 *M* ATP (neutralized); 12 ml. of 7.5×10^{-2} *M* phosphoenolpyruvate, K or Na salt; 1 mg. of pyruvate kinase, crystalline; 0.04 ml. of 2-mercaptoethanol; and 1 micromole each of twenty L-amino acids. After addition of 100 ml. of S-30 fraction, the reaction mixture is incubated at 37° for 80 minutes. The reaction mix-

ture then is cooled to 3° and dialyzed against 120 vol. of "standard buffer" at 3° for 8 hours. The dialyzing medium should be changed once. In most cases the "preincubated" S-30 fraction can be used after dialysis without further preparation to assay messenger RNA. The extract is divided into 1- to 3-ml. aliquots and frozen quickly for storage.

One milligram of dialyzed "preincubated" S-30 protein added to a 0.25-ml. reaction mixture containing 15 to 20 μg. of poly U (see Remarks) should incorporate 1 to 40 millimicromoles of C^{14}-phenylalanine into protein during 15 minutes of incubation at 37°.

Step 4. If washed ribosomes and supernatant solution are required, the dialyzed S-30 extract is centrifuged at 105,000 × *g* for 120 minutes in a Spinco Model L preparative ultracentrifuge at 3°. The upper four-fifths of the supernatant solution is aspirated (S-100 fraction). The lower fifth of the supernatant solution is decanted and discarded. Ribosomal pellets are suspended in the initial volume of "standard buffer" by *gentle* homogenization (four to five passes) in a Potter-Elvejehm homogenizer. The ribosomal suspension is centrifuged again at 105,000 × *g* for 2 hours, and the supernatant solution is decanted and discarded. The ribosomal pellets are suspended, as described previously, in one-fourth the original volume of "standard buffer," and the suspension is centrifuged at 10,000 × *g* for 5 minutes to remove aggregates. The washed ribosomal fraction will be designated W-RIB. W-RIB and S-100 fractions are divided into small aliquots, frozen quickly, and stored until needed.

Remarks

Stability of E. coli Extracts. The extracts appear to retain more activity if they are frozen rapidly in either dry ice–acetone mixture or in liquid N_2 than if they are frozen slowly at —20°. Little loss in activity of S-30 fractions can be detected after storage in liquid N_2 refrigerators (Linde Corp.) for 6 months and longer. S-30 fractions stored at —20° lose less than 5% activity per week. S-100 and W-RIB preparations also retain more activity when stored under liquid N_2 than when stored at —20°.

Some amino acid-activating enzymes may be more labile than others; thus, S-30 and S-100 fractions are not frozen and thawed more than once. However, W-RIB fractions may be frozen and thawed several times without undue loss of activity.

Template Activities of Poly U Preparations. Optimal amounts of poly U or natural messenger RNA required for reaction mixtures may depend on the nuclease content of *E. coli* extracts.[2] Another variable factor related to the template activity of poly U is the average molecular

weight of each poly U preparation. Poly U molecules composed of less than one hundred uridylic acid residues are almost completely inactive.[2] Therefore, poor C^{14}-phenylalanine incorporations need not be ascribed always to inactive *E. coli* extracts.

Most poly U preparations contain traces of nucleases; to prevent degradation, solutions may be stored in small aliquots at −20° and kept at 3° when thawed.