CELL-FREE PROTEIN SYNTHESIS DEPENDENT ON TRANSFER RNA

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Protein synthesizing systems are conveniently prepared from bacteria (Kameyama et al., 1960) and mammalian cells (Schweet et al., 1958; von Ehrenstein and Lipmann, 1961) and are widely used for studies of amino acid incorporation, in vitro. The necessary component parts of a complete system are ribosomes, message RNA (m-RNA), transfer RNA (t-RNA), amino acids and several enzyme systems. Removal of any component from an otherwise functional system permits a study of the particular component by restoring it in a known and controlled way. For example, the system is made dependent on added m-RNA by a preincubation, and this has been a valuable tool for studying the coding properties of synthetic polyribonucleotides (Nirenberg and Matthaei, 1961). Other investigators (Yamane and Sueoka, 1963; and Bennett, 1963) have studied incorporation of C^{14}-amino acids from labelled amino acyl t-RNA added to a cell-free system. These systems contained endogenous t-RNA to which the labelled amino acid from the added amino acyl t-RNA may be transferred by amino acyl t-RNA synthetase (Yamane and Sueoka, 1964). For example, the transfer of C^{14}-leucine between two species of leucyl t-RNA obscures the specificity between a triplet on m-RNA and its recognition by an adaptor molecule. The enzyme preparations of Nathans and Lipmann (1961) and Yamane and Sueoka (1963) involve removing ribosomes from cell lysates by centrifuga-

tion and fractionating the supernatant on DEAE cellulose. The recovered enzyme fraction is free of t-RNA, and if it is recombined with washed ribosomes a cell-free system lacking t-RNA can be obtained. We have required a more convenient procedure for removing t-RNA and report here a simple method of gel-filtration for preparing an amino acid incorporating system from Escherichia coli, free from endogenous t-RNA and dependent on an added source of both m-RNA and t-RNA.

A 30,000 x g supernatant was prepared from Escherichia coli A19, a ribonucleaseless mutant obtained from Dr. S. Spiegelman, according to the method of Nirenberg (1963). About 40 grams of frozen cell paste from early log phase cells plus 4 ml. of standard buffer (tris-HCl 0.01M, pH 7.8, 0.01M magnesium acetate, 0.06M KCl and 0.006M mercaptoethanol) were ruptured at 10,000 psi in a French press (American Instrument Co.) precooled at -20°C. The homogenate was diluted with 20 ml. of the buffer and treated with 200 μg of pancreatic DNAase (twice crystallized, Worthington Biochemical Co.) for 10 min. at 4°C. The suspension was centrifuged at 22,000 x g for 20 minutes. The supernatant fluid was centrifuged again at 30,000 x g for 30 minutes. This supernatant was carefully removed leaving 0.5 ml. above the pellet, giving about 18 ml. with ultraviolet absorption of 125 optical density units/ml. at 280 mμ and 233 at 260 mμ.

To destroy endogenous m-RNA the preparation was then incubated at 37°C. for 80 min. in the following medium: 2.0 ml. of phosphoenolpyruvate, K salt (0.06M); 0.02 ml. of pyruvate kinase (10 mg/ml.); 1.5 ml. of ATP (neutralized, 0.04M); 1 ml. of CTP (0.01M); 0.1 ml. of GTP (0.1M); 0.5 ml. of twenty L-amino acids (0.01M each) and 6 ml. of 0.1M tris buffer (pH 7.8 with 0.01M magnesium acetate, 0.05M KCl, and 0.006M mercaptoethanol). After incubation, a portion of the supernatant was dialyzed overnight at 4°C. against the standard buffer to obtain a preparation containing endogenous t-RNA. The components in the remaining incubated super-

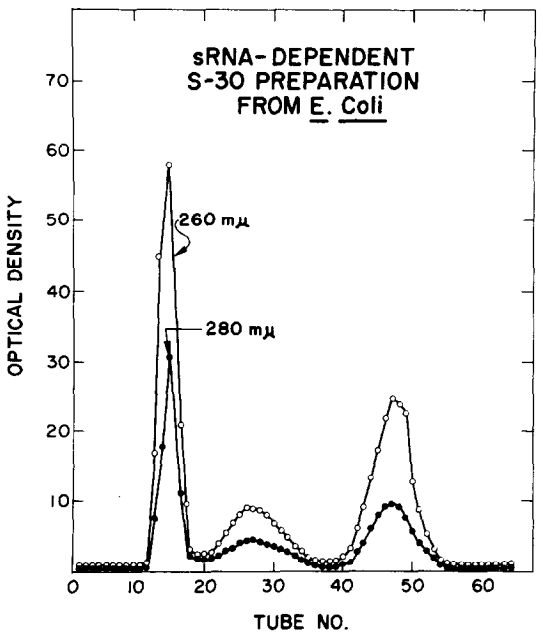

Fig. 1. Gel filtration of E. coli extracts with Sephadex G100 (see text for details).

natant (27 ml.) were separated on a Sephadex G100 column (2.5 x 95 cm.; void volume, 140 ml.) at 4°C. with the standard buffer at a flow rate of approximately 1 ml/min. The results of gel-filtration are shown in Fig. 1. The excluded peak with a A_{280}/A_{260} ratio of 0.54 contained ribosomes and enzymes active for amino acid incorporation into polypeptides. Density gradient centrifugation of the preparation in a 5 to 20% sucrose gradient revealed the absence of ultraviolet absorption in the 4 S region. The second peak with A_{280}/A_{260} ratio of 0.48 was t-RNA, and the third peak contained smaller components of the mixture. The ribosome-enzyme peak was pooled and stored in liquid nitrogen to maintain activity.

Poly U-directed phenylalanine incorporation with the preparations and t-RNA prepared from E. coli A-19 by the method of Holley (1961) is shown in Fig. 2. The preparation prior to gel filtration contained endogenous

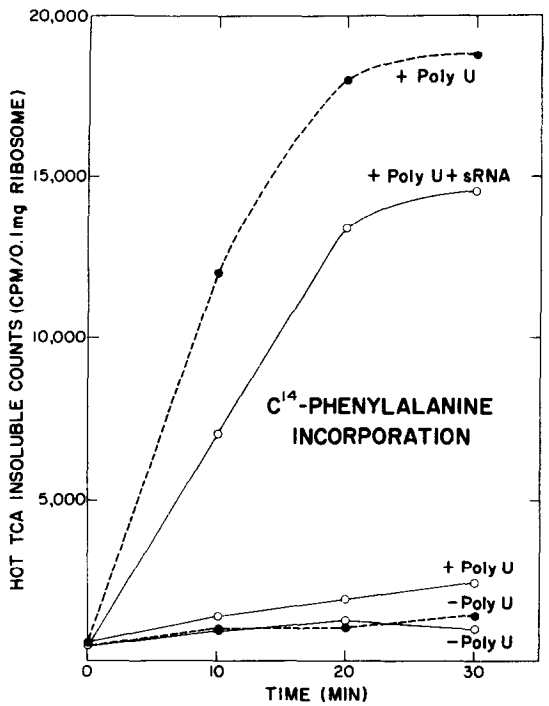

Fig. 2. Poly U stimulated C^{14}-phenylalanine incorporation with the cell-free preparation before (dotted lines) and after (solid lines) Sephadex G100. The incorporation studies were carried out according to the method of Nirenberg (1963). A typical reaction mixture in a total volume of 0.55 ml contained the following components: the cell-free preparation, 0.5 mg protein; poly U, 2 A_{260} units; t-RNA (E. coli A19), if used, 10 A_{260} units; C^{14}-phenylalanine (350 mc/mmole), 0.5 μc plus 19 other C^{12}-amino acids; GTP and an ATP-generating system.

t-RNA and showed dependence on only m-RNA (the dotted lines), whereas the preparation obtained from gel-filtration was dependent on both t-RNA and m-RNA (solid lines). Similar results were obtained when poly C was used as the message to direct proline incorporation with the preparation from gel filtration (Fig. 3). Active proline incorporation was observed only if both poly C and t-RNA were added. Omission of either one resulted in no proline incorporation into hot trichloroacetic acid (TCA) insoluble counts. The saturation curve of this cell-free system with t-RNA in poly C translation is shown in Fig. 4. While cold TCA insoluble counts

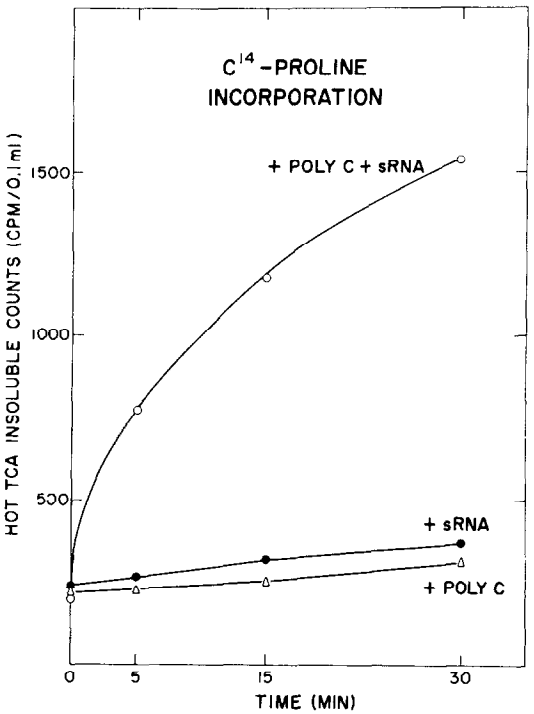

Fig. 3. Poly C-stimulated C^{14}-proline incorporation. Reaction conditions were identical to those described in Fig. 2 except that poly C was heated at 90°C. for 5 min. and rapidly chilled prior to use.

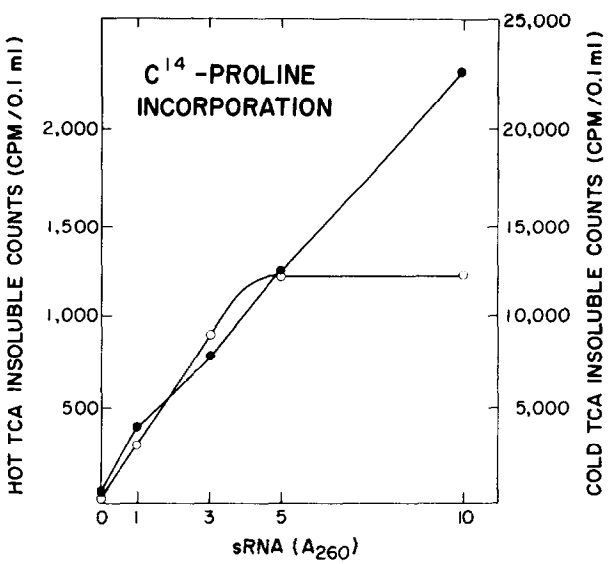

Fig. 4. Saturation of the cell-free system with t-RNA. Reaction conditions were similar to those in Fig. 2. Cold TCA insoluble counts were obtained by deproteinizing 0.1 ml of the reaction mixture with 1 ml of cold 10% TCA. Hot TCA insoluble counts were obtained by heating at 90 to 95°C. for 20 minutes.

(representing both proline-charged t-RNA and synthesized polypeptide) increased linearly with t-RNA concentration, the hot TCA insoluble counts (polyproline) reached a maximum with about five A_{260} units of t-RNA in the incubation mixture. The described system has maintained activity for periods of several months when stored in liquid nitrogen. We have successfully used the preparation to determine the efficiency of different t-RNA preparations in translating polyribonucleotides of known sequence (Hung, 1966).

SUMMARY

Endogenous transfer RNA was removed by gel filtration from 30,000 x g extracts of Escherichia coli. The preparations were active for cell-free protein synthesis after the addition of both t-RNA and message RNA. The system provided a method to study various t-RNA preparations in the translation of specific messages.

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