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An efficient in vitro total reconstitution of the Escherichia coli 50S ribosomal subunit

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ABSTRACT

A new, relatively simple technique for the total in vitro reconstitution of E. coli 50S ribosomes has been developed. It is a two-step procedure like that previously reported by Nierhaus and Dohme [Proc. Natl. Acad. Sci. 71, 4713 (1974)], but it differs in a number of important aspects. Ribosomal RNA is prepared by direct phenol extraction of 70S particles to minimize nuclease fragmentation. A mixture of 50S proteins is prepared by acetic acid extraction and immediate removal of the acetic acid by thin film dialysis. The resulting protein mixture is soluble and stable. Separate RNA and protein fractions are mixed, incubated first at 44°C in 7.5 mM Mg^{2+}, and then at 50°C in 20 mM Mg^{2+}. The resulting 50S particles comigrate with native 50S particles in analytical gradients. They range from 50 to 100% active in five different functional assays. This is a fairly stringent test of the effectiveness of reconstitution since 50S particles derived from highly active vacant couples were used as a control.

INTRODUCTION

Many studies of the synthesis, assembly, structure, and function of the Escherichia coli 50S ribosomal subunit require a reliable method of total reconstitution. However, the history of attempts to develop or use reconstitution procedures has been rather frustrating. The reconstitution system developed for the Bacillus stearothermophilus 50S particle by Nomura and Erdmann[1] has been applied successfully to the 50S subunit of other Bacillus species, but unfortunately it does not work with E. coli[2]. A one-step reconstitution for the E. coli 50S was reported by Maruta et al[3], but could not be reproduced by other laboratories.[4,5] Although Nierhaus and Dohme described a two-step reconstitution procedure for the E. coli 50S particle in 1974[6,7], to date no application

of this method has been published by any other laboratory. This is truly surprising in view of the obvious applications of a reconstitution system. We have made numerous attempts to implement the Nierhaus and Dohme precedure. None were successful, nor were minor variations on the published procedures. However by introducing less destructive techniques for the preparation of rRNA and 50S proteins, we have been able to develop a new method that successfully reconstitutes E. coli 50S subunits. This method has been tried and implemented successfully in other laboratories[8].

MATERIALS AND METHODS

Isolation of Ribosomes

E. coli MRE600 cells were grown in modified L-broth to the early log phase (A_{660} = 0.5), slow cooled to 15°C, and harvested on ice. The fresh cells were ground with alumina and extracted in Buffer A (10 mM Tris-HCl pH 7.6; 10 mM $MgAc_2$; 60 mM NH_4Cl; 6 mM β-mercaptoethanol). The alumina and cell debris were removed by centrifugation. Crude 70S ribosomes used in the preparation of RNA were obtained by pelleting the S30 extract (Beckman 50.2Ti rotor, 45,000 rpm, 2 hours) and resuspending the pelleted material in Buffer A. Vacant 70S couples were further purified by washing the crude 70S particles in Buffer A, then in Buffer A containing 1.5 M NH_4Cl, and finally suspending in TMAI (Buffer A containing 30 mM NH_4Cl)[9]. Ribosomal subunits were obtained by zonal centrifugation[10] in 1.0 mM Mg^{++}. The ribosome suspension in TMAI was applied directly to the low magnesium sucrose gradient, since dissociation to subunits is fast.[11] Fractions containing the 50S and 30S subunits were pooled, the magnesium concentration was raised to 10 mM, and the subunits were concentrated by centrifugation. All particles were resuspended in TMAI at 45 mg/ml and stored at -40°C.

Preparation of RNA

Total ribosomal RNA was extracted from fresh crude 70S ribosomes with redistilled phenol following Traub et al[12]. A suspension of crude 70S particles was diluted to 400 A_{260}

units per ml with TMAII (TMAI containing 1.0 mM $MgAc_2$). An equal volume SCE buffer (150 mM NaCl; 15 mM NaCitrate; 10 mM Na_2EDTA pH 7.0), 0.1 volume 10% SDS, and 0.2 volume 2% bentonite were added, and the mixture agitated on a vortex mixer. One volume SCE-saturated phenol was added and the mixture agitated thrice for one minute. The two phases were separated by low speed centrifugation and bentonite was added to the aqueous phase. The phenol extraction was repeated three times. RNA was precipitated from the aqueous phase by addition of two volumes cold ethanol (-20°C). Any phenol present was removed by repeated alcohol precipitation. Total ribosomal RNA was stored as an ethanol precipitate at -20°C. Before use, the precipitate was pelleted, dessicated, and resuspended in Buffer R (10 mM Tris-HCl pH 7.6; 4 mM $MgAc_2$; 0.2 mM Na_2EDTA). It was stored frozen at -40°C.

Ribosomal RNAs were purified by sucrose gradient centrifugation. All sucrose solutions were treated with 2% bentonite. Total RNA was resuspended in SCE and separated on a 5-20% sucrose gradient in SCE. Fractions containing the component RNAs were pooled, the sucrose dialyzed out, and the RNA precipitated with several volumes of cold ethanol. Thereafter it was treated in the same manner as total RNA.

Pure 5S RNA was obtained from Boehringer Mannheim, or prepared by the method of Erdmann et al[13].

For use in reconstitution, RNA was resuspended in Buffer R at approximately 400 A_{260} units/ml. The integrity of all RNA used in this study was ascertained by gradient gel electrophoresis[14]. The 23S fraction of total RNA was determined by comparing the area under the 23S peak to the total area in a sucrose gradient. (Data not shown). We found that 58% of the total rRNA was 23S RNA.

Preparation of 50S Proteins

One-tenth volume of 1.0 M magnesium acetate and 2.2 volumes glacial acetic acid were added to one volume of a 45 mg/ml 50S subunit suspension. This was stirred for 45 minutes at 4°C[15]. Precipitated RNA was removed by low speed centrifugation. The supernatant was fast-dialyzed[14] against a gradient of acetic acid in Buffer P (5 mM Tris-HCl pH 7.6; 2 mM $MgAc_2$; 1 mM β-mer-

captoethanol) starting at 10% acetic acid by volume (pH 2.3) to a final concentration of 0.01% and a final pH of 6.0. The gradient dialysis took one hour, and the resultant protein solution (TP50) was dialyzed against Buffer P, pH 6.0, for an additional twenty minutes. This procedure generally gave a protein solution 17 mg/ml by the Lowry determination[16], or A_{230}= 5.3. TP50 solution was stored at 4°C or frozen at -40°C and had no observable precipitate. Two dimensional gel electrophoresis[17, 18] showed that all of the 50S proteins were present in this solution (Fig. 1).

Reconstitution

Twenty microliters of a concentrated buffer solution (0.09 M Tris-HCl pH 7.6; 2.4 M NH_4Cl; 0.034 M $MgAc_2$; 1.0 mM Na_2EDTA; 7.7 mM β-mercaptoethanol) were added to 15 µl Buffer R at 4°C containing a maximum of 3.8 A_{260} units total RNA. 85 µl of TP50 solution were added and the mixture was agitated. The final concentrations of reaction components were 20 mM Tris-HCl pH 7.6; 7.5 mM $MgAc_2$; 0.4 M NH_4Cl; 2.0 mM β-mercaptoethanol; 0.2 mM Na_2EDTA. The reaction vessel was closed and incubated at 44°C for 30 minutes. 1.5 µl 1 M $MgAc_2$ were added and the mixture incubated at 50°C for 2 hours. The reaction was stopped by placing the vessel on ice.

Duplicate samples of 50 µl were withdrawn for use in the poly(phe) synthesis assay. For other functional assays and analytical sucrose gradients, larger scale reactions were used. After incubation the samples were cooled and centrifuged (Beckman Ti60 rotor, 19,000 rpm, 12 hours) and the 50S pellet washed and resuspended in TMAI. We could scale up the procedure to a total volume of 5 milliliters without a loss in efficiency.

Assays

The poly(U)-dependent poly(phe) synthesis performed was a modification of the Nirenberg assay[19]. Each 250 µl reaction contained 50 µl of the reconstitution mixture, one A_{260} unit 30S ribosomes, 250 µg *E. coli* tRNA, 20 µl S100 enzymes, 5 µg pyruvate kinase, and 250 µg poly(U). The final concentrations were 50 mM Tris-HCl pH 7.6; 20 mM $MgAc_2$; 250 mM NH_4Cl; 1 mM ATP;

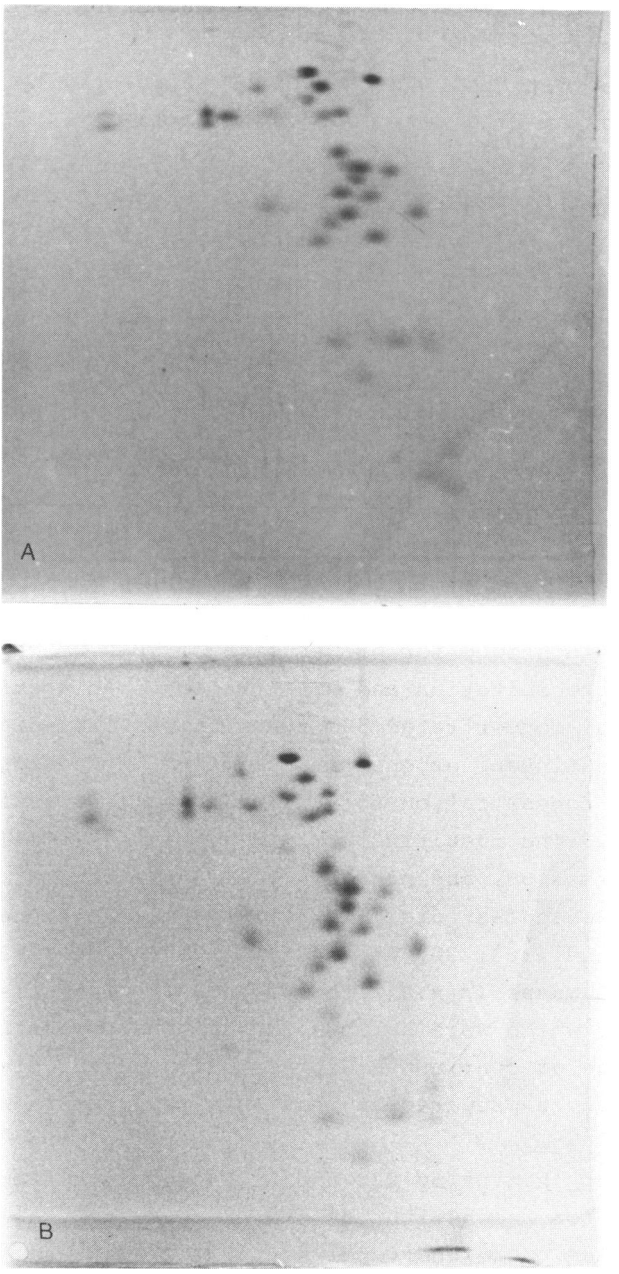

Fig. 1. Analysis of 50S ribosomal proteins by two-dimensional gel electrophoresis.
a) TP50 prepared as described in Materials and Methods
b) TP50 from purified reconstituted 50S ribosomes.

0.03 mM GTP; 7.5 mM PEP; 50 µM [^{12}C]amino acids; 5 µM [^{14}C]phenylalanine (100 mCi/mmol, 0.125 µCi per reaction), and 6 mM β-mercaptoethanol. The amount of radioactivity incorporated in 30 minutes at 37°C was measured.

Other assays used to characterize reconstituted 50S particles include elongation factor G-dependent GTPase activity in TMAI[20], non-enzymatic phe-tRNA^{phe} binding[21], RNAse protection of phe-tRNA by 50S ribosomes[22], and stimulation of dihydrostreptomycin binding to 30S by 50S subunits[23].

RESULTS

Protein and RNA Preparation

In the Nierhaus and Dohme reconstitution procedure[6,7] a 50S ribosomal protein mixture is suspended in a urea buffer after acetone precipitation. Our major difficulty with this procedure was insolubility of the proteins upon removal of the urea by dialysis. To circumvent this problem, we developed a technique for preparation of TP50 which avoids deliberate precipitation and denaturation. An acetic acid extract[6,7] of concentrated 50S subunits was fast-dialyzed against a continuous exponential gradient decreasing in acetic acid concentration to a final pH of 6.0, as in Materials and Methods. The resulting protein mixture is completely soluble at this pH, and performs well in subsequent reconstitution. Step-wise fast dialysis against decreasing percentages of acetic acid (10%, 3%, 1%, 0.3%, etc.), or dialysis by conventional means (3 x 4 liters Buffer P, pH 6) also gave TP50 samples which were soluble and capable of reconstitution. The advantage of continuous exponential gradient dialysis is that TP50 can be prepared in less than three hours with minimal handling.

We found that using 23S and 5S RNA derived from 50S subunits[6,7] for reconstitution experiments inevitably led to particles with poor functional activity. This was true regardless of the method used for protein preparation. Gel electrophoresis of this RNA showed significant degradation of the 23S RNA, as was reported by Ceri and Maeba[24]. However, total RNA extracted from crude 70S ribosomes exhibits little, if any, degradation (see Fig. 2). Individual components of

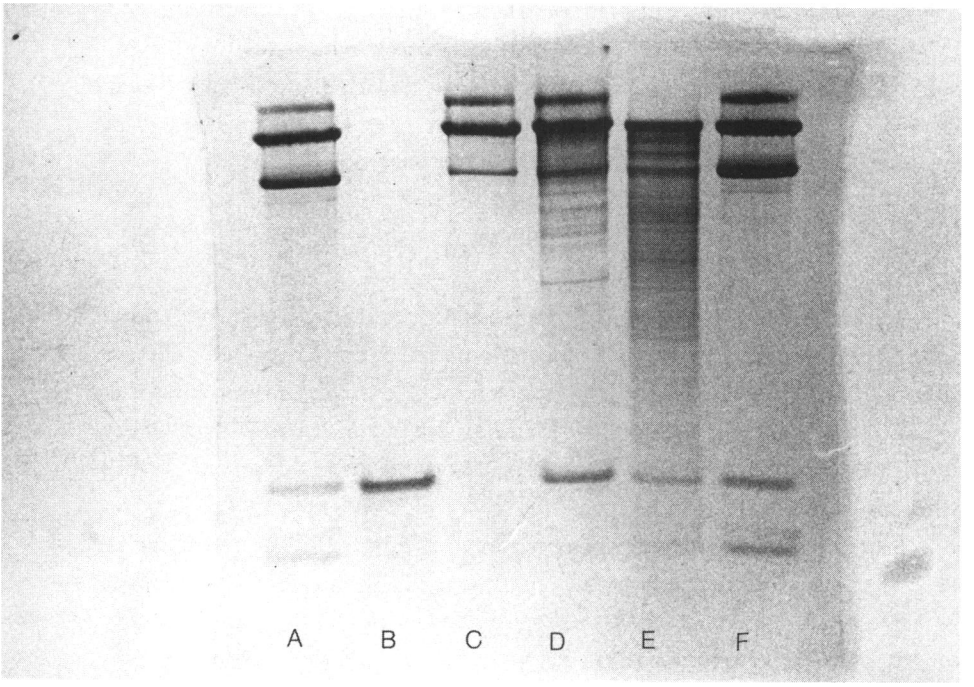

Fig. 2. Analysis of RNA by gradient gel electrophoresis. a,f) total rRNA. b) 5S RNA. c) 23S RNA. d) RNA from control 50S. e) RNA from reconstituted particles.

total RNA can be subsequently purified without significant damage. Either total RNA or 23S and 5S purified from 70S ribosomes work successfully in our reconstitution procedure.

Reconstitution Procedure

50S ribosomes are reconstituted by incubating a mixture of TP50 and ribosomal RNA under the following conditions:

First step:	Tris-HCl pH 7.6	20 mM
	$MgAc_2$	7.5 mM
	NH_4Cl	400 mM
	β-mercaptoethanol	2 mM
	Na_2EDTA	0.2 mM

incubate 30 minutes, 44°C

Second step: Increase $MgAc_2$ to 20 mM

incubate 120 minutes, 50°C

A measure of the absolute extent of reconstitution was routinely obtained by comparing activity in poly(U)-directed

poly(phe) synthesis with that of a control 50S sample. Aliquots taken directly from the reaction vessel were added to a mixture containing the requisite ingredients and excess 30S ribosomes. TP50 and/or total RNA did not interfere with the results of this assay. Activity was expresses as cpm TCA-insoluble [^{14}C]phe incorporated per A_{260} unit 23S RNA present in the sample, assuming A_{260} 23S = 0.58 A_{260} total 70S RNA.

Direct assay of the crude reconstitution mixture tends to underestimate the activity of the particles since any fragmented RNA which fails to reconstitute contaminates the sample. Therefore, for all subsequent assays of functional activity reconstituted particles were purified by pelleting and resuspension prior to assay.

Several assays were performed to ensure the reassembled 50S particles were competent in known 50S functions. Results are shown in Table I. We have included in this table the activities in these assays of salt-washed tight couples (prepared according to reference 9) to demonstrate the relative activity of the 50S control particles and to allow absolute comparison of the efficiencies of reconstitution procedures in various laboratories.

Purified reconstituted particles show 85 to 100% of the activity of control 50S in poly(phe) synthesis, non-enzymatic binding of phe-tRNA, and stimulation by 50S of dihydrostreptomycin binding. The EF-G dependent GTPase activity of the reassembled subunit was 51% that of the 50S control. However, addition of 30S subunits, which generally have no effect on this activity in low concentrations of monovalent cations[25], stimulated GTPase to 77%. Since this behavior is observed in a sensitive assay of a strictly 50S function, it is possble that the reconstituted particles exist in a slightly perturbed form that approximates but is not identical to the true structure. Coupling with the 30S subunits may induce the reassembled particles to assume a more nearly native structure. Half-maximal activity was also observed in the RNAse protection experiment. Phe-tRNA is more accessible to RNAse in the couples formed with reconstituted 50S, which is consistent with a looser structure for the couple than in the controls.

TABLE I

FUNCTIONAL ACTIVITIES OF RECONSTITUTED 50S SUBUNITS

Assay	30S	70S	50S control	50S R[a]
A. poly(phe) synthesis cpm [^{14}C]poly(phe)				
direct assay	419	--	62,140 (100)[e]	38,254 (62)
pure particles	419	81,052	62,140 (100)	53,040 (85)
B. EF-G dependent GTPase cpm [^{32}P]GTP[b] hydrolyzed				
-30S	--	93,888	61,624 (100)	31,533 (51)
+30S	6533	--	57,460 (100)	42,668 (77)
C. phe-tRNA binding cpm [^{14}C]phe-tRNA[c]				
non-enzymatic	3923	5840	5680 (100)	5523 (97)
RNAse protected	190	6384	6219 (100)	3172 (51)
D. dihydrostreptomycin(Dsm) cpm [^{3}H]Dsm[d] bound	3878	12,326	11,156 (100)	11,663(102)

[a] 50S reconstituted with total RNA

[b] 10-20 cpm/pmol γ[^{32}P]GTP

[c] 183 cpm/pmol [^{14}C]phe-tRNA

[d] 608 cpm/pmol [^{3}H]Dsm

[e] Numbers in parentheses indicate activity in percent of the 50S control.

Efficient reconstitution of the 50S ribosome from pure RNA and TP50 seems to require two incubation steps as reported by Nierhaus and Dohme[6,7]. The first incubation is at lower temperature and lower magnesium than the second. We examined a number of experimental variables in an attempt to identify the optimum conditions. In each case below, we measured reconstitution efficiency as a function of a single variable by assaying the activity of the reconstitution mixture in poly(phe) synthesis.

Magnesium. Figure 3 shows the relative activity of the reconstituted particles as a function of magnesium concentration for each of the two steps. At 44°C maximum activity occurred in 7.5 mM Mg^{2+} in contrast to the optimal condition of 4 mM found by Nierhaus and Dohme, which in our hands gave negligible particle formation. This discrepancy may result from different methods of preparing the reaction components. Note in Figure 3 that optimal conditions of reconstitution remained the same whether 23S and 5S or total rRNA was used.

Temperature. A study of the effect on reconstitution of

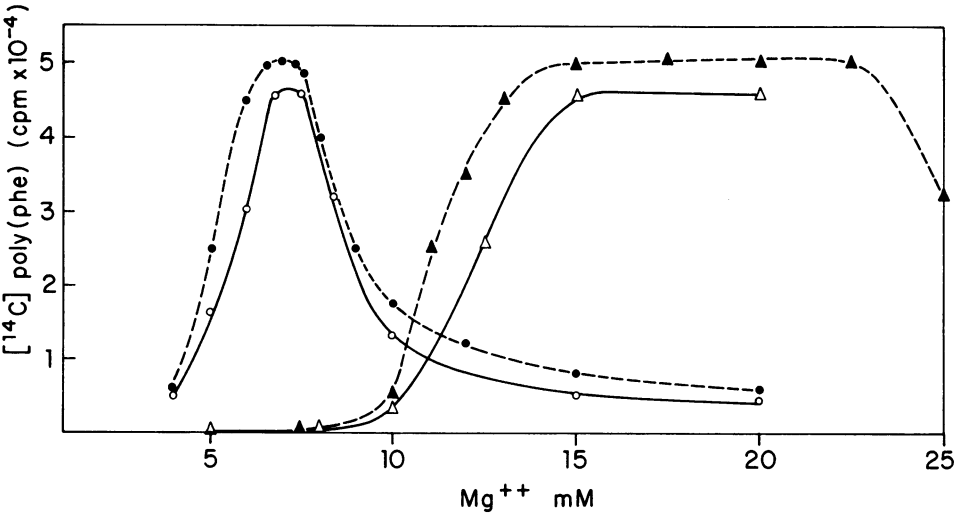

Fig. 3. Magnesium dependence of reconstitution. Reaction mixtures were incubated for 30 minutes at 44°C (first step) and then for 120 minutes at 50°C (second step) under the following conditions: Circles (O-O,●-●) indicate variable magnesium in the first step, 20 mM Mg^{++} for the second step. Triangles (Δ-Δ, ▲-▲) indicate 7.5 mM Mg^{++} in the first step, variable Mg^{++} in the second. Closed symbols (●-●, ▲-▲) indicate that total RNA was used; open symbols (O-O, Δ-Δ) indicate that 23S + 5S RNA was used.

both magnesium and temperature for the first step (Fig. 4) demonstrated that 7.5 mM Mg^{++} and 44°C are indeed the optimal conditions. The second incubation was insensitive to magnesium concentration in the range 14 to 22 mM at the optimal temperature of 50°C.

Time. Kinetic studies at the optimal conditions show that a 30 minute incubation for the first step and 120 minutes for the second are sufficient to yield maximal reconstitution.

Salt. The effect of NH_4Cl concentration on reconstitution is demonstrated in Figure 5. Relatively high ionic strength optimizes this process as is the case in the reassembly of E. coli 30S subunit[26] and the B. stearothermophilus 50S.[1] This study supports the assertion by Nierhaus and Dohme[6,7] that 0.4 M NH_4Cl yields optimal results for E. coli 50S subunits. Variations in the concentration of Na_2EDTA had no effect up to 0.6 mM.

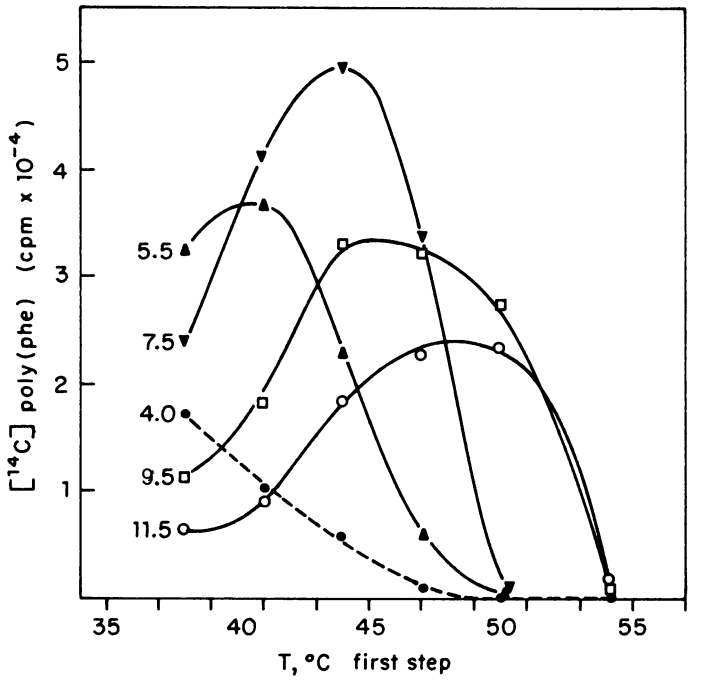

Fig. 4. Temperature and magnesium dependence of reconstitution for the first incubation step. The optimal temperature and Mg^{++} concentration for the first step were determined with the second step at 50°C and 20 mM Mg^{++}. The magnesium concentrations for the first step are: 4.0 mM (O-O), 5.5 mM (▲-▲), 7.5 mM (▼-▼), 9.5 mM (□-□), and 11.5 mM (O-O).

pH. Changing the pH of the reaction mixture did not alter reconstitution efficiency significantly. Lower pH values are slightly more effective.[14]

Protein to RNA Ratio. Varying the concentration of 23S RNA present in the reconstitution mixture yielded maximal activity at a protein to RNA molar ratio of 2.4. We found that a three to six fold excess of 5S RNA over 23S was required to give maximal activity when a mixture of 23S and 5S was used. When total RNA was used, no additional 5S was necessary to give optimal results.

30S Subunit. Addition of 30S particles to the reaction mixture did not stimulate activity, rather, at a 2-fold molar excess of 30S ribosomes over 23S RNA, reconstitution was inhibited 30%. (Data not shown).

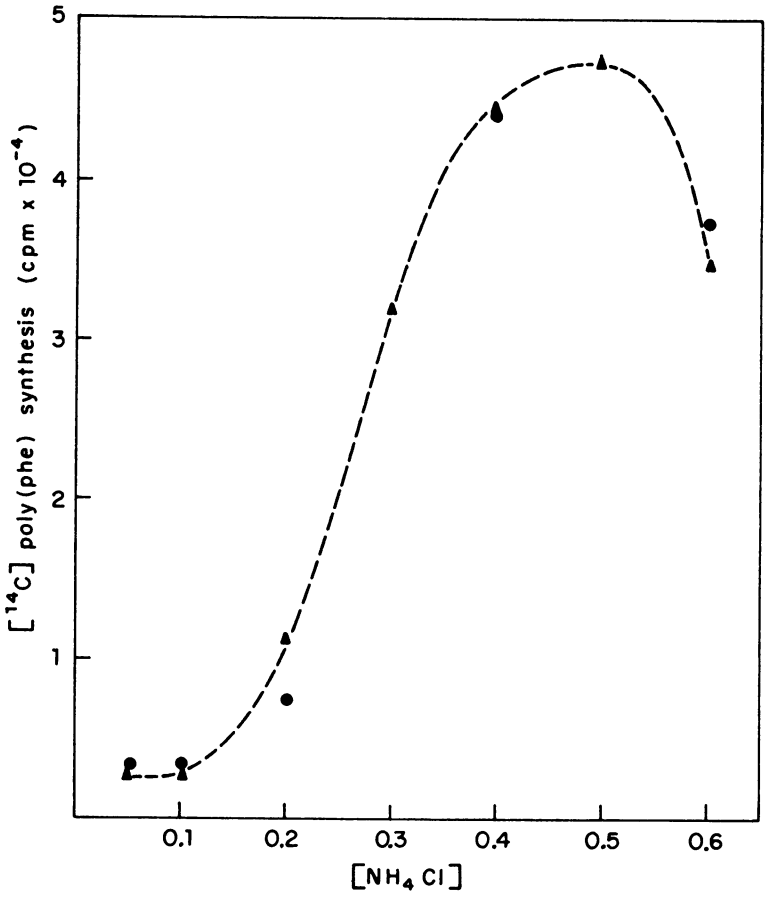

Fig. 5. NH_4Cl dependence of reconstitution. Particles were reassembled with total RNA (▲-▲), or 23S + 5S RNA (●-●).

Particle Characterization

Reconstituted particles, either taken directly from the reaction mixture or isolated by centrifugation, co-migrated in a sharp peak with radioactive 50S controls in analytical sucrose gradients (Fig. 6). In addition, when subunits were reconstituted with ^{32}P labeled 23S or 5S RNA, the radioactivity migrated with the absorbance peak of marker 50S ribosomes (Fig. 6). The sedimentation coefficient of the reassembled particles, determined by analytical centrifugation in a Spinco Model E centrifuge, was 50S.

All proteins present in TP50 can be extracted from isolated reconstituted particles, as shown by 2D gel electro-

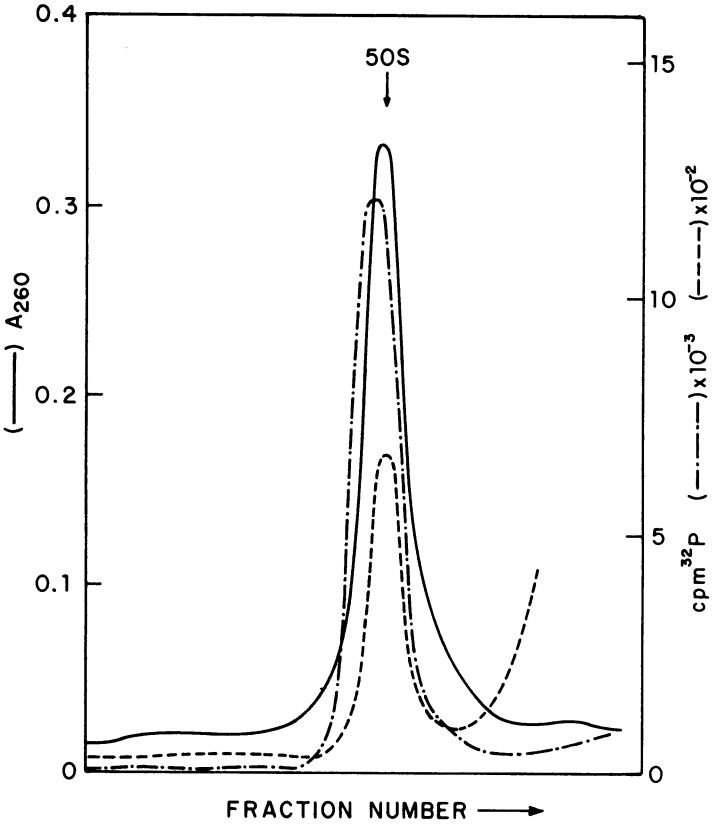

Fig. 6. Analytical sucrose gradients of reassembled particles. Approximately 3 A_{260} units of particles were layered over a 5-20% (w/w) sucrose gradient. Centrifugation was for 2 hours 40,000 rpm in a Beckman SW 50.1 rotor. Particles were reconstituted with total rRNA (——), total rRNA + [^{32}P]23S RNA (-·-·-), or total rRNA + [^{32}P]5S RNA ---). The arrow indicates the position of the control 50S peak in a similar gradient.

phoresis (Fig. 1). Electrophoresis of the RNA from reassembled particles showed some degradation of the 23S RNA. However, the RNA from control 50S ribosomes was similarly degraded (Fig. 2).

DISCUSSION

The work presented here demonstrates a rapid new method of total reconstitution of E. coli 50S ribosomal subunits from rRNA and unfractionated 50S proteins. The technique incorporates a variation on the two step system

devised by Nierhaus and Dohme, in the context of revised methods of preparation of the ribosomal components. Two principles guided these preparations: 1) prevention of damage to the RNA by extraction from crude 70S particles into a nuclease free environment as quickly as possible, and 2) maintaining the 50S proteins in solution at all times and limiting their exposure to denaturing agents.

It is known that the susceptibility of 23S RNA to degradation is increased in the isolated 50S particle.[24] Previous work had shown that B. stearothermophilus can be reassembled more efficiently using 23S from total RNA.[27] Thus we decided to use RNA obtained from crude 70S ribosomes isolated from fresh, early log E. coli.

Ribosomal proteins were obtained by a fast, gentle technique that yields a solution sufficiently concentrated that acetone precipitation is not necessary. The two classical techniques for preparation of ribosomal proteins, lithium chloride-urea extraction and acetic acid extraction/acetone precipitation, were unsatisfactory. We found the LiCl-urea system time consuming and inefficient in protein extraction from the RNA. Also, the extracted protein behaved inadequately in reconstitution. The best activity we could obtain was only 30% that of the control 50S in the poly(phe) synthesis assay. The acetic acid/acetone technique, although efficient in protein extraction, gave TP50 impossible to keep in solution in the absence of urea. Therefore, we extracted with acetic acid and eliminated the acetone precipitation. By starting with a concentrated suspension of 50S subunits and removing acetic acid by dialysis, acetone precipitation was obviated.

Dohme and Nierhaus conclude on the basis of studies of intermediates in reconstitution that two steps are required for the process of assembly to occur[7]. The results presented in this paper substantiate that a two step method is indeed competent in total reconstitution. However, as careful examination of the data given in Figure 4 and in reference 7 reveals, at magnesium concentrations and temperatures for the first step that approach the conditions of the second step, reassembly does take place, albeit to a limited extent.

Further exploration of these conditions may prove a one step total reconstitution procedure feasible.

The reconstitution of the 30S ribosome enabled many structural and functional studies to be done that were previously impossible. Mapping the assembly of the 30S proteins with the 16S RNA is an obvious example (see reference 28 for a review). Reconstitution allows the incorporation and function of individual proteins to be examined [28]. Likewise, the distances between pairs of components cannot be measured without the ability to reassemble the labeled molecules into a viable particle. This is an integral requirement for topographical studies such as neutron scattering interference[29] and fluorescence energy transfer[30] experiments. Reconstitution of chemically modified components permits investigation of the relationship of assembly to function[28]. It is our hope that with this reconstitution procedure, these techniques may now be applied to the study of the E. coli 50S ribosome.

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