Properties of 30S Ribosomal Particles Reconstituted from Precursor 16S Ribonucleic Acid†

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ABSTRACT: The precursor 16S RNA (p16 RNA), which is formed in RC-relaxed strains of *Escherichia coli* during methionine deprivation, was used to reconstitute 30S ribosomes *in vitro*. The p16 RNA, which is nonmethylated and contains excess oligonucleotides at the 3′- and 5′-ends of the molecule, reacts efficiently with 30S ribosomal proteins and forms a homogeneous 30S particle. These particles, however, are deficient in the protein S-3. The particles containing p16 RNA are inactive in poly(U)-directed poly(phenylalanine) synthesis. This inactivity results from the inability of the particles to bind Phe-tRNA and 50S ribosomes. Particles with p16 RNA will bind to poly(U). The inactivity of the p16 RNA containing particles in binding Phe-tRNA is correlated with their deficiency in protein S-3, which has been shown by others to be involved in tRNA binding to the 30S ribosome.

The *in vitro* reconstitution of 30S ribosomes from their molecular components (24) has provided a powerful tool for studying ribosome assembly, structure, and function. From these studies, the role of some of the ribosomal proteins has been deduced (Nomura *et al.*, 1969; Randall-Hazelbauer and Kurland, 1972; Van Duin and Kurland, 1970; Van Duin *et al.*, 1972), as have some of the features of the assembly process itself (Traub and Nomura, 1968b). We have been concerned with how this *in vitro* system approximates the process of ribosome biosynthesis. Although many of the intermediate steps *in vitro* resemble those which can be demonstrated in growing cells, significant differences exist between the reconstitution reaction and the assembly which takes place intracellularly. One such difference is that ribosomes are biosynthesized from a precursor form of 16S RNA. This RNA is submethylated and contains excess oligonucleotides at both the 5′- and 3′-ends of the molecule (Brownlee and Cartwright, 1971; Hayes *et al.*, 1971; Lowry and Dahlberg, 1971; Sogin *et al.*, 1971).

We have reconstituted particles with a form of the precursor 16S RNA (p16 RNA), which was isolated from a methionine starved "relaxed" mutant of *Escherichia coli*. The aims were to determine if the precursor would serve as a substrate in such a reaction, and if the assembly process was substantially different from that employing mature 16S RNA (m16 RNA). We have already reported that there is no apparent difference in the thermal energy required for assembly from either p16 RNA or m16 RNA (Wireman and Sypherd, 1974). In this paper we will show that particles made with p16 RNA are inactive in poly(uridylic acid)-directed protein synthesis, and we will describe several properties of these particles which bear on their inactivity.

Materials and Methods

Buffers and Reagents. The following buffers were used: TM4 (0.01 M Tris-HCl (pH 7.8)–0.3 mM $MgCl_2$), TM2 (same as TM4 but with 0.01 M $MgCl_2$), TMK (0.01 M Tris-HCl (pH 7.8)–0.001 M $MgCl_2$–0.10 M KCl), TM2K (same as TMK but with 0.01 M $MgCl_2$), TSES (0.01 M Tris-HCl (pH 7.8)–0.10 M NaCl–0.001 M EDTA–0.1% sodium dodecyl sulfate), E (0.04 M Tris–0.02 M sodium acetate–0.001 M EDTA; adjusted to pH 7 with glacial acetic acid (Bishop *et al.*, 1967), AEN (0.01 M sodium acetate (pH 5.4)–0.001 M EDTA–0.10 M NaCl), and BIII and BIV were those described by Traub and Nomura (1968b).

Unlabeled and ^{3}H-labeled poly(U) were purchased from Miles laboratories; urea, enzyme grade, was purchased from Schwarz-Mann; [^{3}H]uracil (18 Ci/mmol) was also purchased from Schwarz-Mann. Pancreatic DNase (electrophoretically purified) and pancreatic RNase were purchased from Worthington Biochemical Corp.

Growth Conditions. For the preparation of m16 RNA or ribosomes from exponentially growing cells, *E. coli*, D-10 was grown to mid-log phase in minimal medium (Sypherd and Strauss, 1962) supplemented with 1% glucose and 50 μg of methionine/ml. In order to accumulate precursor RNA under conditions of relaxed control of RNA synthesis, D-10 was grown as above with methionine reduced to 4 μg/ml. Under these conditions there was sufficient methionine present for the cell density to reach about 2×10^8 cells/ml before depletion of methionine. Accumulation of relaxed RNA during the period of methionine starvation was allowed to continue for 90 min before the cells were harvested. In order to differentially label RNA accumulated during exponential growth and during methionine starvation, the minimal medium was modified to contain 5 mM phosphate and 2 μg of uracil/ml. During exponential growth the cells were labeled with 5 μCi of [^{3}H]uracil/ml and during methionine starvation with 50 μCi of $^{32}PO_4$/ml of media. There was essentially no cross-labeling of the two RNA forms (Figure 4).

Purification of rRNA. The standard method of rRNA purification was by phenol extraction of the total cell lysate. The frozen cell pellets were resuspended in TM4 buffer and broken by passage through a French pressure cell at 8000 psi. Electrophoretically purified DNase was added to 5 μg/ml and unbroken cells and cell debris were removed by centrifugation at 10,000 rpm for 10 min. The supernatant was adjusted to con-

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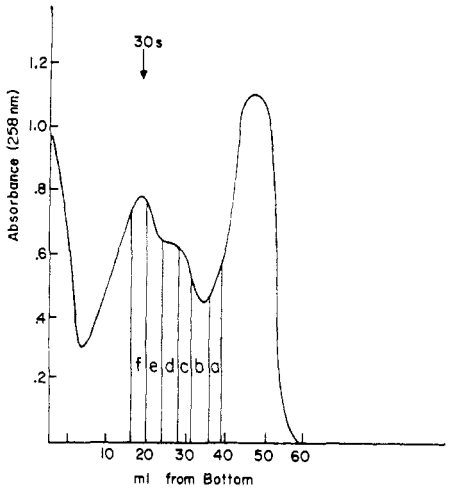

FIGURE 1: Zone sedimentation of the crude extract of *met*-starved *E. coli* D10. The sedimentation was performed in TM4 buffer with a gradient of 5–20% sucrose. The SW27.1 rotor was spun at 25,000 rpm for 16 hr so that 50S ribosomes were at the bottom of the tube. The gradient was fractionated by pumping it through an ISCO fractionator. The fractions were pooled as indicated by a–f and each pool was analyzed as indicated in Figure 2.

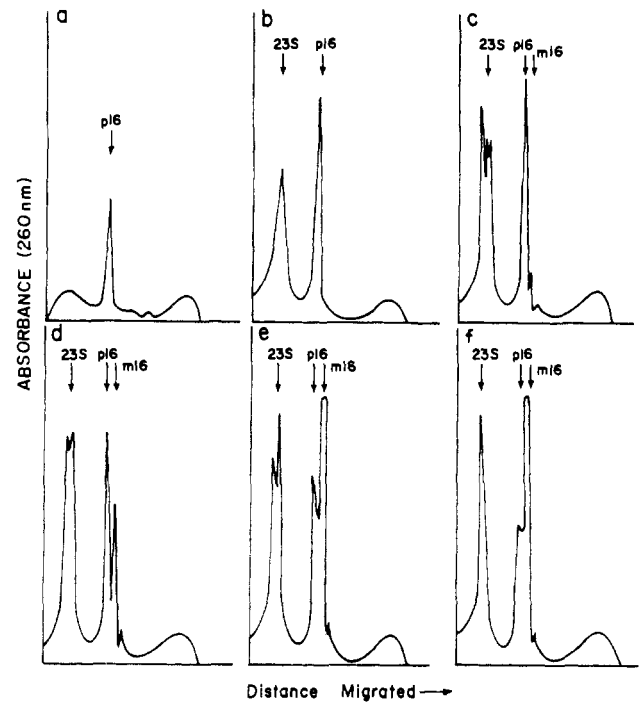

FIGURE 2: Polyacrylamide gel electrophoretic analysis of the pooled fractions a–f from Figure 1. The pooled fractions were shaken with E buffer and 0.1% sodium dodecyl sulfate then run on 2.8% acrylamide gels for 4.5 hr. The gels were then scanned in a quartz cell at 260 nm on a recording spectrophotometer. Panels a–f correspond to the fractions indicated in Figure 1.

tain 1% sodium dodecyl sulfate, 10 mM EDTA, and 100 mM NaCl. The aqueous solution was shaken for 20 min at 4° with an equal volume of water-saturated, distilled phenol. The extraction was repeated once and the aqueous phase was precipitated two times with ethanol. The RNA precipitate was resuspended in AEN buffer and the 16S RNA purified by centrifugation on 5–20% linear sucrose gradients containing AEN buffer. The 16S RNA pooled from the gradients was concentrated by precipitating two times with ethanol, followed by a second sucrose gradient centrifugation. The final 16S RNA was resuspended in 5 mM potassium phosphate buffer (pH 7.8) and stored at −70°. The effectiveness of the phenol extraction procedure was determined by employing ribosomes labeled with [^{3}H]uracil and [^{14}C]leucine. From the amount of radioactivity from the [^{14}C]leucine in the extracted RNA, there was less than 1 molecule of protein/10 molecules of RNA, on the average.

For purification of p16 RNA relatively free of m16 RNA, the cell lysate was first centrifuged on 5–20% linear sucrose gradients containing TM4 buffer. The regions of the gradient containing p16 RNA free of m16 RNA were pooled and further purified free of p23 RNA or tRNA on sucrose gradients as described above (see Figures 1 and 2).

Preparation of Ribosomes. The standard method of ribosome purification by ammonium sulfate washing and precipitation and separation of subunits by zonal centrifugation has been described (Miller and Sypherd, 1973). It was found, however, that greater stability of the 30S and 50S subunits could be achieved by modification of the above procedure to use TMK buffer in place of TM4 and TM2K buffer in place of TM2.

Preparation of 30S Proteins. Proteins were extracted from 30S ribosomes by the LiCl–urea method. Purified 30S subunits, stored in TMK buffer, were mixed with an equal volume of 8 M urea and 4 M LiCl. It was necessary to use highly purified urea (enzyme grade) and treat the LiCl with Norit A as described by Traub *et al.* (1971). The mixture was stored on ice for 36–48 hr. The precipitated RNA was removed by centrifugation at 30,000 rpm for 30 min at 0°. The upper two-thirds of the supernatant fluid was removed and dialyzed against 100 volumes of buffer BIII for 3 hr. The dialysis was repeated two more times and the protein solution was stored in aliquots at −70°. The protein concentration (1–4 mg/ml) was determined by the Folin method using bovine serum albumin as a standard.

Reconstitution. The conditions used for the reconstitution of 30S ribosomes were essentially those described by Traub and Nomura (1968b). The 16S RNA preparations were stored in 5 mM phosphate buffer (pH 7.8). The RNA was mixed with appropriate amounts of buffer BIII or BIV to make the final concentrations in the reconstitution reaction 5 mM PO_4^{2-}, 20 mM $MgCl_2$, 0.03 M KCl, and 6 mM mercaptoethanol after addition of 30S protein solution in BIII. The standard reaction volume was 0.5 ml containing 0.5–1.5 A_{260} units of 16S RNA. The reconstitution mixture was preincubated at 40° for 2 min prior to the initiation of the reaction by addition of 30S proteins. At the conclusion of the reaction, usually 20 min, the reaction mixture was cooled in an ice bath for 5–10 min and then layered directly on 5-ml 10–30% sucrose gradients containing TM4 buffer and centrifuged 2.5–3 hr at 49K rpm in a SW50.1 rotor. The products were recovered by fractionation on an ISCO density gradient fractionator, then pooled, and used immediately or stored at −70°. Activities of ribosomes reconstituted with m16 RNA, when recovered in this way, were somewhat lower than those obtained by pelleting directly from the reconstitution reaction.

Activity Assays. The binding of [^{3}H]poly(U) to ribosomes determined by the filter binding method of Moore (1966). The binding of [^{3}H]Phe-tRNA was measured by the method of Nirenberg and Leder (1964), as modified by Jost *et al.* (1968). [^{3}H]Phe-tRNA from *E. coli* was kindly provided by Dr. Kivie Moldave.

The components for measuring the poly(U)-directed synthesis of poly(phenylalanine) were essentially those of Hosokawa *et al.* (1966) with the following modifications. A soluble enzyme–tRNA fraction (S100) was prepared from early log D10 cells grown in 1% trypton and 0.2% yeast extract. No preincubation was necessary since there was less than 1%

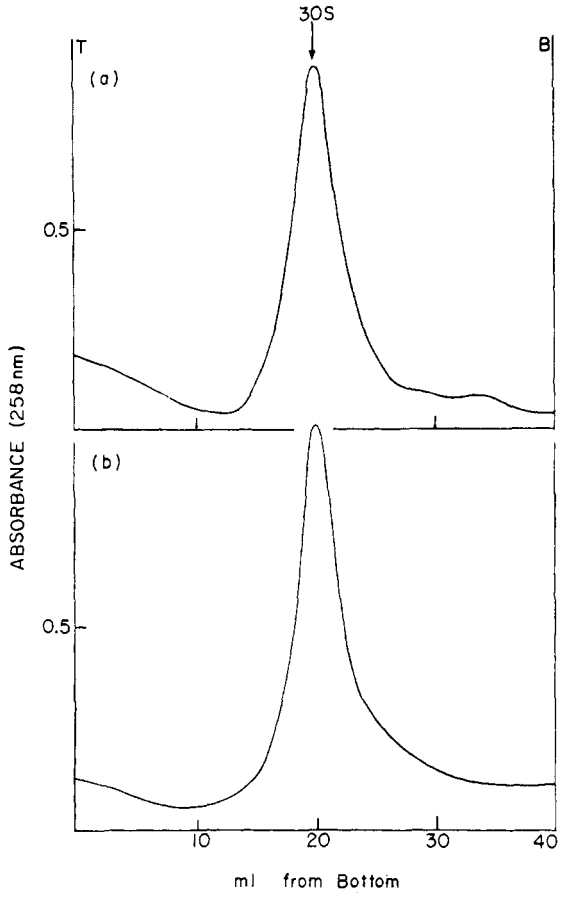

FIGURE 3: Sedimentation profiles of particles reconstituted with p16 (a) and m16 (b) RNA. The RNAs were purified by preparative polyacrylamide gel electrophoresis and reconstituted with 30S ribosomal proteins as described in Methods. In each reconstitution mixture 17 A_{260} units of RNA was incubated with 1020 μg of 30S protein in a total volume of 2 ml. The reconstitution mixture was incubated at 40° for 20 min, then layered on a 10–30% sucrose gradient on TM4 and centrifuged at 27,000 rpm in SW27.1 rotor for 16 hr. The gradients were analyzed by pumping through an ISCO fractionator.

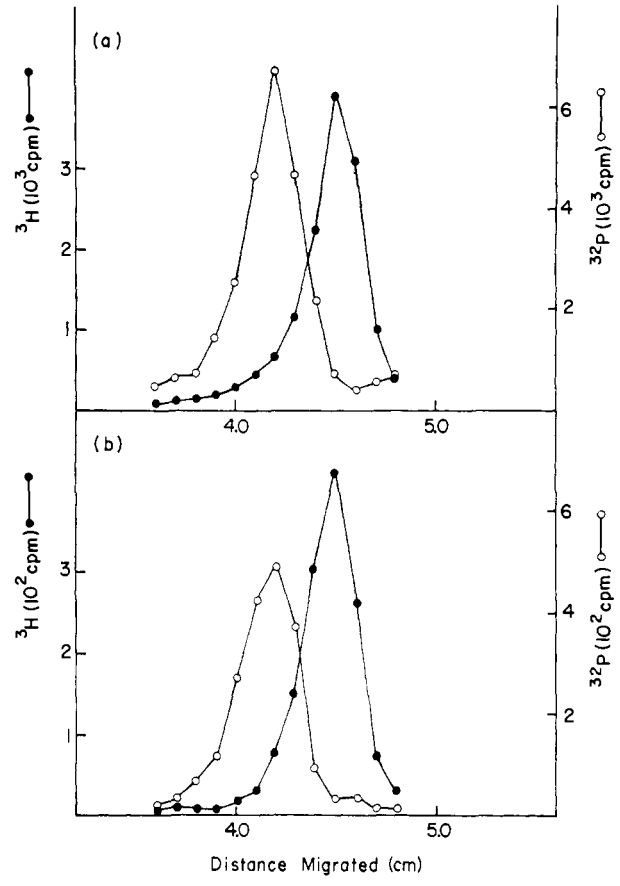

FIGURE 4: Polyacrylamide gel electrophoresis of p16 RNA and m16 RNA before (a) and after (b) reconstitution. RNA was extracted from a culture of *E. coli* D10 which had been labeled with [^{3}H]uracil (●) during exponential growth and $^{32}PO_4^{2-}$ (○) during methionine starvation. The mixture of p16 and m16 RNAs was prepared from a total phenol extract by sedimentation in a sucrose gradient and recovery of the 16S region. The RNA mixture was then reconstituted with 30S ribosomal proteins. RNA was extracted from the reconstituted particles for the analysis shown in part b.

endogenous activity. The amounts of S100 and poly(U) were titrated for each preparation of S100 and used in a range that gave a proportional response to 30S ribosomes. The standard reaction was 0.25 ml and gave a linear response in incorporation to 30S ribosomes up to 0.3 A_{260} unit. The reaction at 37° was initiated with poly(U) and stopped after 30 min by the addition of 2 ml of 10% CCl_3COOH containing 1 mg/ml of phenylalanine. The hot CCl_3COOH insoluble product was determined by boiling for 10 min followed by extensive washing of the precipitate with 5-ml aliquots of 5% CCl_3COOH (total about 40 ml) on glass fiber filters.

Assay for Resistance to Ribonuclease. The determination of pancreatic RNase resistance was done according to the method of Hayes and Hayes (1971). Ribosomes or RNA was incubated for 2 min in a standard volume of 8 ml containing 5 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, and 50 mM NaCl at 25°. An aliquot was withdrawn followed by the addition of 2 μg of pancreatic RNase/A_{260} unit of ribosomes or RNA. The standard reaction was 0.5–1.0 A_{260} unit of RNA and 1–2 μg of RNase. Samples of 0.9 ml were withdrawn at various times and precipitated with 2 ml of 10% CCl_3COOH containing 1 mg of bovine serum albumin/ml. The precipitates were collected on glass fiber filters and washed extensively with 5% CCl_3COOH.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels were prepared by the method of Bishop *et al.* (1967) but were preswollen. The standard gels were 2.8% acrylamide with a diameter of either 0.6 or 0.9 cm and were 10 cm long. When gels were to be counted, they were sliced into 1-mm slices and dried on filter paper squares.

Proteins were run in the two-dimensional gel system of Kaltschmidt and Wittmann (1970) as described by Miller and Sypherd (1973).

Results

Physical Properties of Reconstituted Particles. A "relaxed" methionine auxotroph was deprived of methionine for 90 min, and the precursor of 16S RNA was extracted from particles recovered from zonal centrifugation. Figure 1 shows the A_{260} profile of such an extract when the centrifugation time was sufficiently long to sediment the 50S subunits. Pooled fractions from each area designated in the figure were extracted with phenol and detergent. The RNA was then analyzed on polyacrylamide gels (Figure 2). The pooled fraction showing the highest purity of p16 (*e.g.*, Figure 2a–c) was saved for future experiments, and the other fractions were discarded. The p16 RNA and the m16 RNA recovered from a parallel preparation with nonstarved cells were used to reconstitute 30S ribosomes by the Traub and Nomura method (1968a,b). Figure 3 shows the sucrose gradient profiles of the reconstituted particles. Both p16 and m16 RNA produced relatively homogeneous 30S particles. The reconstitution process did not result in the

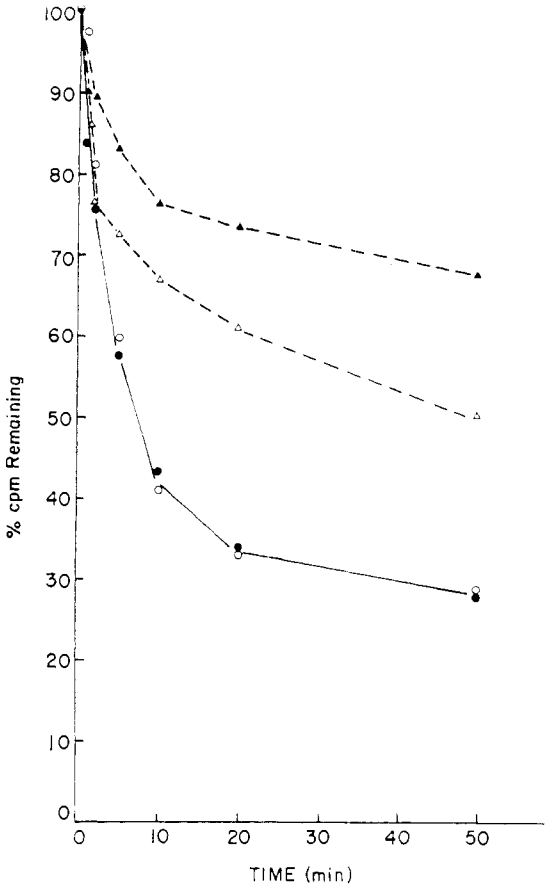

FIGURE 5: The sensitivity of reconstituted particles and RNA to pancreatic RNase. A mixture of p16 RNA and m16 RNA reconstituted with 30S ribosomal proteins. The reconstituted particles and the free RNAs were subjected to RNase digestion as described in Methods. Triplicate samples were taken at intervals, precipitated with cold 5% CCl_3COOH, and the remaining insoluble radioactivity determined: p16 RNA (○); m16 RNA (●); particles with p16 RNA (△); particles with m16 RNA (▲).

conversion of p16 RNA to the mature form as shown by acrylamide gel electrophoresis of RNA extracted from the reconstituted particles (Figure 4).

The two types of reconstituted particles were compared in their sensitivity to ribonuclease. Figure 5 shows that p16 and m16 RNA are degraded to the same extent. Reconstitution of these RNAs into particles results in considerable increase in resistance to RNase degradation, but particles containing p16 RNA are more labile than those containing m16 RNA. The basis of this greater lability of the particles with p16 RNA is not known, but it may result from the formation of a less compact structure during the assembly process.

The protein composition of particles reconstituted with p16 RNA was determined. Reconstitutions were carried out with purified p16 and m16 RNAs and the proteins were extracted with hot trichloroacetic acid. The proteins were then iodinated with ^{131}ICl and ^{125}ICl, respectively. The radioactive proteins were mixed together with excess nonradioactive 30S protein and then separated by two-dimensional acrylamide gel electrophoresis (Miller and Sypherd, 1973). Stained spots were cut from the gels and counted by liquid scintillation spectroscopy. The data in Table I show that particles formed with p16 RNA are deficient in protein S-3 and have somewhat reduced amounts of protein S-6 when compared with particles made with m16 RNA. All the other proteins are present in the p16 RNA particles in about the same amounts as in the control particles.

TABLE I: Proteins on the p30 Particle.

Protein No.	Ratio[a]
S2	0.86
S3	*0.41*
S4	1.25
S5	1.10
S6	0.73
S7	0.97
S8	1.02
S9	1.03
S10	0.91
S11	1.17
S12	0.88
S13	1.10
S14	0.87
S15	0.99
S16	1.16
S17	1.11
S18	1.07
S19	0.95
S20	1.34
S21	0.94

[a] Ratio of ^{131}I cpm (p30 proteins)/^{125}I cpm (m30 proteins) from a two-dimensional gel. The ratios were normalized to deviate around a mean value of 1.0. The actual ratios ranged from 0.18 (S3) to 0.60 (S20).

Activity of Reconstituted Particles. Particles reconstituted with p16 RNA were examined for their ability to carry out certain steps in protein biosynthesis. The data in Table II show that 30S particles made with highly purified p16 (*i.e.*, greater than 94% pure, expt 3) have no appreciable activity in

TABLE II: Poly(U)-Dependent Poly(phenylalanine) Incorporation Activity of Particles Reconstituted with p16 and m16 RNA.

Expt No.	Particle	Act. (10^4 cpm/A_{260})	% Act.
1	Native 30S	4.70	100
	Recon (m16)	1.71	36.4
	Recon (p16)	1.00	21.3
2	Native 30S	2.63	100
	Recon (m16)	1.96	74.5
	Recon (p16)	0.72	27.4
3	Native 30S	3.87	100
	Recon (m16)	3.08	79.8
	Recon (p16)	0.23	6.0

[a] Expt 1: Reconstitution reaction was done with 52 μg of total 30S proteins/A_{260} unit of RNA. The p16 RNA preparation was contaminated with 21% m16 RNA. The incorporation assay contained 0.09–0.20 A_{260} unit of particles and the background was 325 cpm. Expt 2: Reconstitution with 47 μg of 30S protein. The p16 RNA contained 15% m16 RNA. The incorporation assay contained 0.05–0.20 A_{260} unit of particles and the background was 375 cpm. Expt 3: Reconstitution with 40 μg of 30S protein. The p16 RNA contained 9% m16 RNA. The incorporation assay contained from 0.02–0.20 A_{260} unit of particles and the background was 308 cpm.

TABLE III: [^{3}H]Poly(U) Binding Activity of Particles Reconstituted with p16 and m16 RNA.

Expt No.[a]	Particle	Binding (10^4 cpm/A_{260})	% Act.
1	Native 30S	14.5	100
	Recon (m16)	8.7	60.0
	Recon (p16)	8.5	57.6
2	Native 30S	22.8	100
	Recon (m16)	17.9	78.5
	Recon (p16)	18.6	81.6

[a] Expt 1: Same particles used in Table IV, expt 1. The input [^{3}H]poly(U) was 1.4 μg with a specific activity of 4.2×10^4 cpm/μg. Input of particles was 0.11–0.50 A_{260} unit. Background binding (−30S particles) was 523 cpm. Expt 2: Same particles used in Table V, expt 2, and shown in Figure 3. A standard binding assay as above with 0.03–0.12 A_{260} unit of particles. The background was 719 cpm.

poly(uridylic acid)-directed incorporation. In many similar tests (*i.e.*, expt 1 and 2, Table II) apparent activity of the p16-containing particles was never greater than the degree of contamination of the p16 RNA preparation by m16 RNA. We conclude from these experiments that ribosomes formed from p16 RNA are incapable of carrying out all the steps required for peptide bond formation.

The ability of p16-containing particles to bind poly(uridylic acid) was tested by the filter assay of Moore (1966). Table III shows that the particles reconstituted with p16 RNA are as efficient in binding poly(uridylic acid) as those formed with m16 RNA, although both are less active than mature ribosomes.

Although the p16 RNA containing particles will bind to mRNA, they apparently will not form an active complex with 50S ribosomes. Sucrose gradient analysis of a total poly(U)–poly(phenylalanine) incorporating mixture showed that p16 RNA containing particles were not detected at significant levels as active 70S (or larger) particles (Figure 6).

In addition to their apparent inability to bind 50S ribosomes and make active monosomes, the particles made with p16 RNA are defective in binding Phe-tRNA. Table IV shows that ribosomes reconstituted with p16 RNA have between 14 and 21% of the binding capacity of native 30S ribosomes. A polyacrylamide gel analysis of the p16 RNA used in these experiments showed that the contamination with m16 was about 20%. Therefore, the particles apparently have no binding activity which can be ascribed to the p16 RNA containing particles alone. From these data, we conclude that ribosomes which contain p16 RNA are inactive in polypeptide formation because they cannot carry out the formation of a complete complex with aminoacyl-tRNA and 50S ribosomes.

Discussion

The possibility that p16 RNA is a preferred substrate for the in vitro assembly of ribosomes has been studied by us (Wireman and Sypherd, 1974) and by Lowry and Nomura (Lowry, 1972). From such studies there are no data which suggest that the precursor form of RNA lowers the activation energy or interacts more completely with proteins. However, it is clear that some form of precursor is involved in the early stages of ribosome biosynthesis *in vivo* (Adesnik and Levinthal, 1969; Hecht and Woese, 1968; Sypherd and Fansler, 1967; Mon-

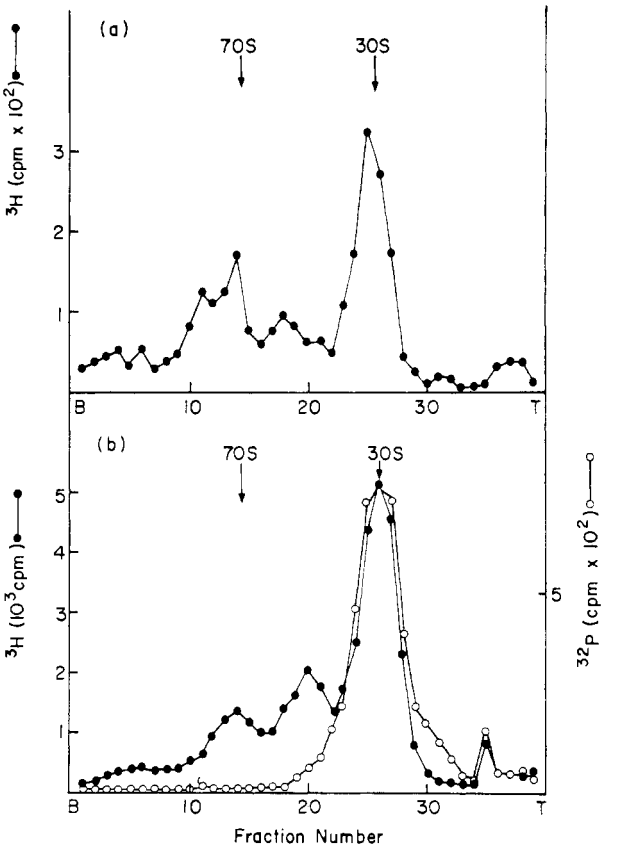

FIGURE 6: Zone sedimentation analysis of a poly(U)-directed poly(Phe) incorporating assay. Native 30S ribosomes or ribosomes reconstituted with a mixture of p16 RNA (^{32}P labeled) and m16 RNA (^{3}H labeled) were mixed with the components of the poly(U) system, employing [^{12}C]Phe. The incorporation mixtures were incubated at 37° for 30 min, then cooled, and layered onto 10–30% sucrose gradients in the buffer described by Jost *et al.* (1968). Centrifugation was carried out for 8 hr at 27,000 rpm. Part a shows the distribution of [^{3}H]uracil-labeled native 30S ribosomes, run as a control. Part b shows the reconstituted particles made with p16 RNA (○) and m16 RNA (●).

giarotti *et al.*, 1968). There is also some evidence to suggest that p16 RNA is trimmed to m16 RNA quite late in the biosynthetic process since some assembly mutants accumulate "30S" ribosomes containing p16 RNA (Bryant, 1973), and p16 RNA can be found in "30S" particles during a short exposure to ^{32}P. The incorporation of p16 RNA into 30S ribosomes by reconstitution does not result in the trimming to m16 RNA. This argues against the idea that one or more of the ribosomal proteins carries out the trimming step, unless the nuclease activity is lost during the LiCl–urea extraction of ribosomal proteins.

Since p16 RNA is incorporated into a rather homogeneous 30S particle, it is natural to ask whether or not these particles are active in peptide-bond formation the data show that particles which contain p16 RNA are inactive in the formation of poly(phenylalanine) (Table II), although they do bind poly(uridylic acid) (Table III). Any marginal activity which the particles exhibit can be accounted for by contaminating m16 RNA in the reconstitution mixture. The inactivity in polypeptide synthesis is apparently explained by the failure of p16 RNA containing particles to bind Phe-tRNA and 50S ribosomes to form an active complex.

The absence of protein S-3 (Table I) from p16 RNA containing particles, and the inability of these particles to bind Phe-tRNA, is consistent with an earlier finding of Nomura *et al.* (1969). They showed that ribosomes reconstituted with

TABLE IV: Poly(U)-Dependent Binding of Phe-tRNA by Particles Reconstituted with p16 and m16 RNA.

Expt No.[a]	Particle	Binding (10^4 cpm/A_{260})	% Bound
1 −50S	Native 30S	2.03	100
	Recon m(16)	1.16	57.2
	Recon (p16)	0.33	16.3
+50S	Native 30S	5.26	100
	Recon (m16)	2.71	51.4
	Recon (p16)	1.11	21.0
2 −50S	Native 30S	1.04	100
	Recon (m16)	0.71	68.8
	Recon (p16)	0.14	13.9
+50S	Native 30S	2.34	100
	Recon (m16)	1.82	77.7
	Recon (p16)	0.35	14.9

[a] Expt 1: Same particles used in Table IV, expt 1. The p16 RNA contained 21% m16 RNA. The standard binding reaction contained 50 μg of poly(U), 20 pmol of [^{3}H]Phe-tRNA (5×10^3 cpm/pmol) and 9–18 pmol of particles (0.2–0.4 A_{260} unit). A binding of 1×10^4 cpm/A_{260} unit represents 0.05 pmol of [^{3}H]Phe-tRNA bound per pmol of particles. The background of the assay (−30S particles) was 281 cpm without 50S ribosomes and 364 cpm with 0.87 A_{260} unit of 50S ribosomes. Expt 2: Particles used in this experiment are those shown in Figure 3. The p16 RNA contained 10% m16 RNA. The binding reaction was as described above with 10 pmol of [^{3}H]Phe-tRNA input and 4–10 pmol of particles (0.1–0.22 A_{260} unit). The background was 129 cpm without 50S ribosomes and 487 cpm with 0.6 A_{260} unit of 50S ribosomes.

m16 RNA and all proteins except S-3 resulted in particles with reduced activity in protein synthesis. These S-3-deficient particles also did not bind appreciable amounts of aminoacyl-tRNA. Also, Randall-Hazelbauer and Kurland (1972) identified protein S-3 as one of the three proteins required for A-site binding of Phe-tRNA (Kurland, 1972).

In the present case, there is no precise explanation for the failure of ribosomes made with p16 RNA to bind protein S-3 and, subsequently, their failure to bind aminoacyl-tRNA and 50S ribosomes. This precursor RNA differs from the mature form by containing excess nucleotides at both ends of the molecule and by being undermethylated (Brownlee and Cartwright, 1971; Hayes *et al.*, 1971; Lowry and Dahlberg, 1971; Sogin *et al.*, 1971). It is conceivable that either of these differences could result in a conformational distortion of the RNA and in the loss of biological activity. Although we have attempted to carry out controlled trimming of the RNA in the hope of producing active particles, we have been unsuccessful in obtaining the correct nucleolytic cleavages. These attempts have included the use of purified ribonuclease II, reported by others to be involved in rRNA maturation (Corte *et al.*, 1971; Yuki, 1971), and p16 RNA in the free and particle form. Although such requirements are unknown, it may be that specific methylations provide the specificity for trimming. The p16 RNA we used in this study is nonmethylated.

We initiated our studies with p16 RNA in an effort to more closely approximate the *in vivo* conditions of ribosome biosynthesis. It is clear from a number of studies (Lindahl, 1973; Rosset *et al.*, 1971) that ribosome assembly proceeds quite far before all the maturation steps (*i.e.*, trimming and methylation) are complete. However, we have obtained no evidence to support the idea that the p16 RNA used here, which is both nonmethylated and untrimmed, is a more effective substrate in the *in vitro* reconstituted system (Wireman and Sypherd, 1974). As shown here, a relatively homogeneous class of particles is formed with p16 RNA and ribosomal proteins. These particles, inactive in polypeptide synthesis and unable to form couples with 50S ribosomes, may be similar to late precursors found *in vivo*. It is not yet possible to define the basis for this inability to form couples, but it is possible that the *in vivo* precursors, like those formed by reconstitution with p16 RNA, are lacking a critical protein for couple formation. Of course, it is possible that complete methylation, and the trimming of excess oligonucleotides are also necessary to prepare the 30S particle for the initial steps of peptide synthesis.

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Biosynthesis of Viomycin. I. Origin of α,β-Diaminopropionic Acid and Serine†

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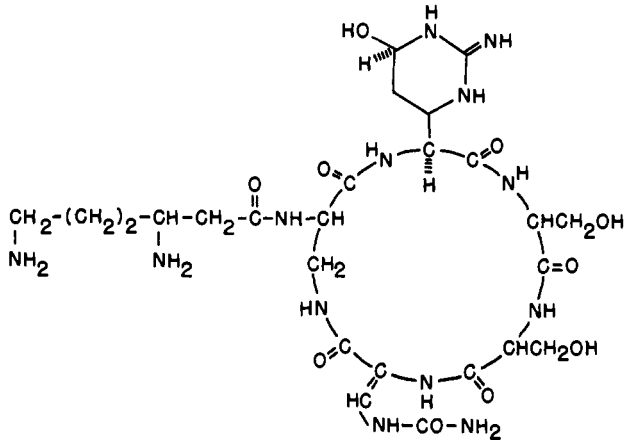
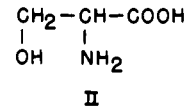
ABSTRACT: A study was made of the origin of the carbon skeletons of L-serine and L-α,β-diaminopropionic acid, two amino acids found in the polypeptide antibiotic viomycin. [^{14}C]Serine was incorporated into viomycin, and most of the radioactivity was present in those two amino acids. The labeling patterns of the serine and diaminopropionic acid from hydrolysates of viomycin produced using [1-^{14}C]- and [3-^{14}C]serine as precursors indicated that the carbon skeletons were both derived from serine without randomization. Radioactive glycine, formate, carbonate, and acetate were incorporated into viomycin, but the radioactivity was extensively randomized among all of the hydrolysis products. [U-^{14}C]Diaminopropionic acid was incorporated almost exclusively into the diaminopropionic acid residue of viomycin. The low amount of radioactivity in the serine residue indicated that the diaminopropionic acid was incorporated as such and did not involve prior conversion to serine.

Viomycin is a polypeptide antibiotic that has been used to a limited extent in the treatment of infections caused by *Mycobacterium tuberculosis* (Finlay *et al.*, 1951). The antibiotic is produced by several organisms including *Streptomyces puniceus* (Finlay *et al.*, 1951) and *S. floridae* (Bartz *et al.*, 1951). This latter organism is now referred to as *S. griseus* var. *purpureus* (J. Ehrlich, 1964, personal communication). Several structures have been proposed for viomycin (Bowie *et al.*, 1964; Dyer *et al.*, 1965; Lechowski, 1969; Bycroft *et al.*, 1968, 1971; Kitagawa *et al.*, 1968, 1972). Structure I was proposed by Bycroft *et al.* (1968).



I

The acid hydrolysis products of viomycin (Dyer *et al.*, 1964) are 2 equiv of serine (II) and 1 equiv each of L-α,β-diaminopropionic acid (III), L-3,6-diaminohexanoic acid (β-lysine)



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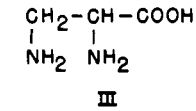

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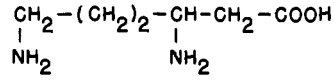
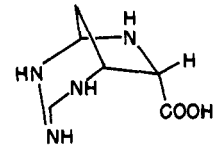


IV



V

(IV), 2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid (viomycidine) (V), ammonia, and carbon dioxide.[1] The structure of viomycidine was elucidated by X-ray crystallography (Floyd *et al.*, 1968; Bycroft *et al.*, 1968). This compound is not present as much in intact viomycin (Bycroft *et al.*, 1968) but rather represents a rearrangement product,

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[1] Urea has previously been observed to be a product of acid hydrolysis of viomycin (Bartz *et al.*, 1951), and we have observed that 1 equiv of urea was produced under these conditions (Dyer *et al.*, 1964). However, the commercial viomycin that we have used in recent years does *not* yield urea upon acid hydrolysis. Furthermore, the Streptomycete strain that we received from Charles Pfizer and Co. produced a viomycin that gave no urea upon acid hydrolysis. Also, from the 24 ^{14}C incorporation studies using this strain, no [^{14}C]urea was produced. These data suggest that the structure proposed for viomycin (Bycroft *et al.*, 1971; Kitagawa *et al.*, 1972), which include a ureido fragment, may be incorrect.