Kinetic and Thermodynamic Parameters of the Assembly in Vitro of the Large Subunit from *Escherichia coli* Ribosomes†

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ABSTRACT: When the 23S and 5S RNA and the total proteins from the large subunit of *E. coli* ribosomes are subjected to the two-step reconstitution procedure (4 mM Mg^{2+}, 44 °C, → 20 mM Mg^{2+}, 50 °C), fully active subunits are formed. Here we show that both the formation of the reconstitution intermediate RI_{50}*(1) which is the essential product of the first incubation and the formation of reconstituted 50S subunits from RI_{50}(2) particles during the second incubation can be treated as a first-order reaction. That is, alteration of the concentration of the reactants little influenced the kinetic parameters of the first step and did not change those of the second step. Therefore, the rate-limiting steps of the second incubation and most probably also that of the first one are unimolecular reactions. The temperature dependence of the rate constants of both partial reactions was determined and used to calculate the Arrhenius activation energy. When the RNA was isolated by the phenol method, we obtained a value of about 290 kJ/mol (70 kcal/mol) for the first step and 225 kJ/mol (54 kcal/mol) for the second one. However, when the RNA was prepared by an acetic acid method, a lower value of 225 kJ/mol (54 kcal/mol) for the first step was found, whereas the corresponding value for the second step remained unchanged. The positive activation entropy found in all cases indicates that the structure of a reconstituted intermediate must be first disordered before the more highly ordered structure of the subsequent reconstituted intermediate can be formed. Finally, we observed that varying the temperature of the first-step incubation led to a variation in the activities of the particles formed after a standard second-step incubation. Sucrose gradient analysis revealed that this variation in activity is directly related solely to the amount of RI_{50}*(1) particle formed during the first step at the different temperatures.

In the course of total reconstitution of the small subunit from *E. coli* ribosomes, the reconstitution intermediate RI_{30} is formed which undergoes a conformational change to the RI_{30}* particle. In spite of 22 reactants (21 proteins and the 16S RNA), the kinetics of 30S formation are those of a first-order reaction; i.e., the rate-limiting step of the reconstitution process is the conformational change. The Arrhenius activation energy was determined to be about 160 kJ/mol (38 kcal/mol) (Traub and Nomura, 1969).

Reconstitution of the large subunit from *E. coli* ribosomes requires a two-step incubation (Nierhaus and Dohme, 1974). The essential product of the first incubation is the RI_{50}*(1) particle, which is formed from RI_{50}(1) by a conformational change. The RI_{50}(1) particle contains 23S and 5S RNA and about 20 tightly bound proteins. The RI_{50}*(1) particle can add the remaining proteins in both the first and the second incubation step, thereby forming the RI_{50}(2) intermediate, whereas the conformational change from the RI_{50}(2) particle to the 50S subunit only proceeds during the second step (Dohme and Nierhaus, 1976).

In this paper, we describe the determination of the apparent reaction order and the molecularity of the rate-limiting step in both incubations. Furthermore, the Arrhenius activation energy is calculated from the dependence of the rate constants on temperature for both steps using RNA prepared in two different ways. Finally, we show that the temperature of the first step influences both the initial rate and the extent of RI_{50}*(1) formation.

Experimental Procedure

The 30S and 50S ribosomal subunits were isolated from *E. coli* K12, strain A19, as described (Schreiner and Nierhaus, 1973) with the modification of Dohme and Nierhaus (1976). The latter publication contains also the preparation procedure for the total proteins (TP50) and the phenolized RNA (23S and 5S) from the large subunit, the total reconstitution (two-step) procedure, and the poly(U) system. The only modifications introduced in the poly(U) system were the temperature and time of incubation: For poly(Phe) synthesis, the system was incubated for 60 min at 20 °C.

In addition to the phenol method for the isolation of RNA, a second method was used which followed essentially the procedure of Hochkeppel et al. (1976). After low-speed centrifugation (5 min at 12 000*g*), the 50S suspension was mixed with 1 volume of 8 M urea and stirred for 15 min at 6 °C. Crystalline magnesium acetate was added up to 0.8 M, and the solution was stirred again for 15 min at 6 °C. Next, 3 volumes of glacial acetic acid was added and the mixture was stirred for 1 h at 0 °C and centrifuged (30 min at 30 000*g*). The pellet was resuspended in 1 volume of a buffer containing 30 mM Tricine [*N*-tris(hydroxymethyl)methylglycine], pH 7.4, 400 mM KCl, 20 mM magnesium acetate, and 1 mM dithiothreitol. Crystalline magnesium acetate was added up to 1 M, and the solution was stirred for 15 min at 6 °C. Four volumes of glacial acetic acid was added and after stirring for 2 h at 0 °C the mixture was centrifuged 30 min at 30 000*g*. The pellet was washed twice with a buffer containing 30 mM Tricine, pH 8.0, and 20 mM magnesium acetate. The final pellet was treated into 1 volume of 30 mM Tricine, pH 8, and dissolved by dialysis against a 1000-fold volume of the same buffer for at least 6 h and then dialyzed against a 1000-fold volume of a buffer (10 mM Tris-HCl,[1] pH 7.8, 4 mM magnesium acetate) in a Spectrapor 3 (Spectrum Medical Industries, Los Angeles,

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[1] Abbreviations used are: TP50, total proteins isolated from the 50S subunits; A_{260} unit, that amount of material which gives an absorbance of 1 when dissolved in 1 mL of solvent when the light path is 1 cm; RNA^P, RNA isolated by standard phenol treatment; RNA^a, RNA isolated by the acetic acid method; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

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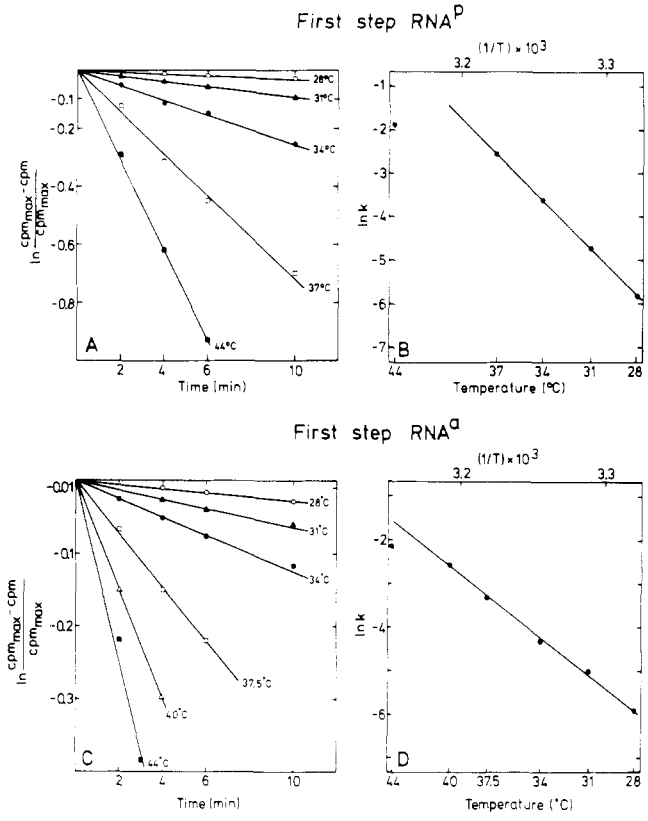

FIGURE 1: Kinetic analyses during the first incubation at various temperatures using TP50 and RNA as indicated. (A) Kinetics of the first step with phenol-treated RNA were followed by a standard second incubation (20 mM Mg^{2+}, 50 °C for 90 min), and the poly(Phe)-synthesizing activity was then measured. The activity of native 50S subunits was 13 700 cpm. The activity data (cpm) were plotted as ln $[(cpm_{max} - cpm)/cpm_{max}]$ vs. the time assuming a first-order law. The slopes of the resulting lines yield the rate constants k_{1p} °C (see text). (B) Plot according to the Arrhenius equation $k = Ae^{-Ea/RT}$ (k, rate constant; A, frequency factor; E_a, Arrhenius activation energy; R, gas constant; T, absolute temperature). The k_{1p} °C values were used for an Arrhenius plot (ln k_{1p} °C vs. $1/T$). The slope $-E_a/R$ leads to the Arrhenius activation energy. The value for 44 °C falls off the line. This point was not taken into consideration for the calculation of the activation energy. (C) An experiment with acetic acid treated RNA was performed as described under A. The activity of the native 50S subunits was 15 200 cpm. The data were plotted as in B assuming a first-order law. The slopes gave the rate constants $k_{1a}^{28} = 0.0028$ min^{-1}; $k_{1a}^{31} = 0.065$ min^{-1}; $k_{1a}^{34} = 0.0132$ min^{-1}; $k_{1a}^{37.5} = 0.0362$ min^{-1}; $k_{1a}^{40} = 0.076$ min^{-1}; and $k_{1a}^{44} = 0.12$ min^{-1}. (D) The k_{1a} °C values obtained in C were used in the Arrhenius plot.

Calif.) dialysis tube. The solution was centrifuged (5 min at 12 000g) and after determination of the absorbance at 260 nm was used directly without freezing. No traces of proteins were found, when 150 A_{260} units of RNA^a (tenfold amount of the optimal one used for 50S subunits) was prepared for the two-dimensional protein electrophoresis (Kaltschmidt and Wittmann, 1970; modified by Roth and Nierhaus, 1975).

For s-value determinations, sucrose gradient analyses were performed on a 14-mL linear sucrose gradient (5–20%) containing the ions present during the first reconstitution step (20 mM Tris-HCl, pH 7.5; 400 mM NH_4Cl; and 4 mM Mg^{2+}). Reconstituted particles (1.8 A_{260} units) or native 50S subunits were applied, and after centrifugation (5 h at 140 000g and 4 °C) the gradient profile at 260 nm was automatically recorded and the sucrose concentration determined with a refractometer. The area below each peak was measured with a compensation polar planimeter (OTT, Kempten, West Germany). The calculation of the s values of the peaks followed the method of McEwen (1967).

Particles formed during the first reconstitution step at various temperatures were isolated and tested as follows: The standard reconstitution mixture (Dohme and Nierhaus, 1976) was scaled up 25-, 50- and 75-fold for the incubations at 44 (7.5 min), 34 (15 min), and 31 °C (20 min), respectively. An aliquot (100 μL) was subjected to a standard second incubation and 1.6 A_{260} units of reconstituted particles was tested for poly(Phe) synthesis in the presence of 1 A_{260} unit of 30S subunits.

The remaining material after the first incubation was applied to a 38-mL linear sucrose gradient (5–20%) with the same ionic conditions as the first reconstitution step. After centrifugation for 18 h at 40 000g, the gradient profile at 260 nm was recorded and fractions containing RI_{50}*(1) particles (41 S) were collected. The particles were precipitated by the poly(ethylene glycol) method (Expert-Bezançon et al., 1974) and resuspended in a buffer with the same ionic conditions as that of the second reconstitution step. Two A_{260} units was incubated under the second-step conditions in the presence of TP50, and 1.6 A_{260} units of the reconstituted particles was assayed for poly(Phe) synthesis.

Results

Determination of the Apparent Reaction Order and the Activation Energy of the Assembly Process during the First and the Second Incubation. The formation of the RI_{50}*(1) particle during the first incubation of the two-step procedure (4 mM Mg^{2+}, 44 °C, → 20 mM Mg^{2+}, 50 °C) is a prerequisite for the formation of an active particle during total reconstitution (Dohme and Nierhaus, 1976). When the standard second incubation is performed, the poly(U) activity of the reconstituted particles thus reflects the extent of the RI_{50}*(1) formation during the first step (for a detailed explanation, see Discussion).

Following this principle, we analyzed the kinetics of the first incubation at various temperatures, and after a standard second incubation the poly(Phe)-synthesizing activity was measured. With TP50- and phenol-treated RNA (RNA^p), the activity data were plotted assuming a first-order law. Figure 1A demonstrates that the points fall on straight lines, indicating that the rate-limiting step of the reactions occurring during the first incubation does indeed follow a first-order law. From this figure the temperature-dependent rate constants k_{1p} °C of the rate-limiting step from the first incubation were determined to be: $k_{1p}^{28} = 0.003$ min^{-1}, $k_{1p}^{31} = 0.010$ min^{-1}, $k_{1p}^{34} = 0.027$ min^{-1}, $k_{1p}^{37} = 0.078$ min^{-1}, and $k_{1p}^{44} = 0.154$ min^{-1} (k_{1p} °C: I, first step; °C, incubation temperature; p, RNA^p was used). These values were plotted according to the Arrhenius equation (Figure 1B), and the activation energy was calculated from the slope of the resulting line (in this case, 280 kJ/mol; equivalent to 67 kcal/mol). From four determinations, we obtained a value for the Arrhenius activation energy of the rate-limiting step during the first incubation of $E_a(I) = 293 \pm 16$ kJ/mol (70 ± 3.8 kcal/mol).

When the same analysis was performed with TP50 and acetic acid treated RNA (RNA^a) significantly lower values for the activation energy were found. In four similar determinations of the Arrhenius activation energy of the first step using RNA^a, we obtained 225 ± 17 kJ/mol (54 ± 4 kcal/mol, see Figure 1C,D). In both Arrhenius plots (Figure 1B,D), the 44 °C points fall off the respective straight line. This is possibly due to the protein preparation. With some protein preparations, we found reproducibly a broader optimal temperature range (e.g., 41–44 °C) than with other preparations (43–44 °C). The broader the optimal temperature range, the more the 44 °C point deviates from the straight line.

When the RI_{50}*(1) particle is formed during the first-step

Energy Utilization and RNA Transport: Their Interdependence†

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ABSTRACT: The interdependence of RNA transport and the metabolism of nucleotide additives was investigated. Rat-liver RNA was radioactively labeled in vivo for 45 min before isolation of liver nuclei, and the concentration dependence of RNA transport on nucleotide additives was determined. In a parallel investigation, using nucleotide additives labeled in the base moiety, the distributions of label in the tri-, di-, and monophosphate forms were examined after various intervals of incubation. Analysis of results revealed that RNA transport was linearly related to the decline in energy charge of nucleotide additives, with high statistical correlation. Kinetic analysis of labeled-nucleotide metabolism led to a simple schematic model for pathways for the utilization of high-energy phosphate bonds, and predictions of the scheme were confirmed by studies examining the effects of nucleotide analogues upon RNA transport. Data concerning inhibitors and chelators intimated that multiple avenues of inhibition and stimulation may potentially influence RNA transport. On the basis of previous data and the results presented in this communication, we conclude that nucleocytoplasmic RNA transport is dependent upon high-energy phosphate-bond hydrolysis and that nucleotides do not stimulate RNA transport via a simple chelation mechanism.

Stimulation of in vitro RNA transport by nucleotide addition has been reported (Schneider, 1959; Ishikawa et al., 1969; Smuckler & Koplitz, 1974; Schumm & Webb, 1975), but the nature of phosphate ester involvement in the release process is unknown. Some investigators have viewed nucleotide participation in a physical sense, claiming that enhancement of RNA transport occurs by chelating divalent cations (Chatterjee & Weissbach, 1973; Sauermann, 1974) known to inhibit transport (Ishikawa et al., 1969). Others have considered nucleotide participation to be a biological function which supplies the energy necessary for the transport process (Ishikawa et al., 1969; Smuckler & Koplitz, 1974). We found that RNA transport in vitro had an activation energy (E_a)[1] of 13 kcal/mol (Clawson & Smuckler, 1978). Initial linear rates of RNA transport increased with temperature over the domain of 0–35 °C. The activation energy for RNA transport was shown to differ significantly from that for passive transfer of RNA, further indicating the energy-requiring nature of the process. Here we demonstrate with an in vitro rat-liver system that the utilization of the energy charge of nucleotide additives is linearly related to RNA transport, with a very high correlation. We propose a simple scheme for defining nucleotide participation in RNA transport in vitro.

Materials and Methods

Experimental Animals. Male pathogen-free Sprague-Dawley rats were obtained from the Charles River Breeding Laboratories and kept in our vivarium for at least 5 days before experimentation.

RNA Labeling. Preceding the experiments, the rats were starved overnight (15 h) and then given injections of [^{14}C]-orotic acid (New England Nuclear, 28 mCi/mmol) via the tail vein at a dosage of 3 μCi/100 g of body weight. The animals were sacrificed after 45 min of labeling.

Preparation of Nuclei. Livers were quickly taken from the rats and homogenized in sucrose buffer (0.25 M RNase-free sucrose, 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM mercaptoethanol) using Teflon–glass homogenizers, then mixed with 2 volumes of 2.3 M sucrose buffer (with the same additions), layered over a "cushion" of 2.3 M sucrose buffer, and centrifuged at 95 000g for 75 min at 5 °C with an SW27 rotor in a Beckman L5-65 ultracentrifuge (Blobel & Potter, 1966; Smuckler & Koplitz, 1974). The pelleted nuclei were resuspended in 0.88 M sucrose–TKM buffer (0.88 M RNase-free sucrose, 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 25 mM KCl) and diluted to a protein concentration of 7.4 mg/mL.

Transport Assay. Nuclei at a final protein concentration of 3.7 mg/mL were incubated with 0.88 M sucrose buffer, purified RNase inhibitor (Gagnon & de Lamirande, 1973), and concentrations of nucleotide as specified (e.g., see Figure 1). The nuclei were incubated in the different mixtures for 20 min at 20 °C (under these conditions, transport is linear). Reactions were terminated by the addition of ice-cold sucrose buffer, and the nuclei were sedimented by centrifugation at 800g for 8 min.

For assaying released labeled RNA, the supernatant was withdrawn and precipitated in 10% trichloroacetic acid. The precipitated material was collected by centrifugation at 800g for 30 min, the pellet was dissolved in 5 N NaOH, and radioactivity was measured by liquid scintillation counting. Supernatant radioactivity at 0 time was subtracted to determine the amount released—"facilitated transport" is the additional amount of transport obtained over that of preparations incubated without nucleotide. Total radioactivity was measured in an aliquot of nuclear suspension and was used here as a percentage standard. Total Cl_3AcOH-precipitable and soluble radioactivity was monitored throughout incubation. The amount of Cl_3AcOH-insoluble material (in the presence of RNase inhibitor) remained constant for 30 min.

Isolation and Analysis of Nucleotide Additives. For analysis of nucleotide metabolism in this in vitro RNA transport

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[1] Abbreviations used are: RNP, ribonucleoprotein; Cl_3AcOH, trichloroacetic acid; ATP, ADP, and AMP, adenosine tri-, di-, and monophosphates; AMPCP, methylene-blocked ADP; AMPCPP, α,β-methylene-blocked ATP; AMPPCP, β,γ-methylene-blocked ATP; EDTA, ethylenediaminetetraacetic acid; PCA, perchloric acid; E_a, activation energy; TKM buffer, 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$; ATPase, adenosine triphosphatase; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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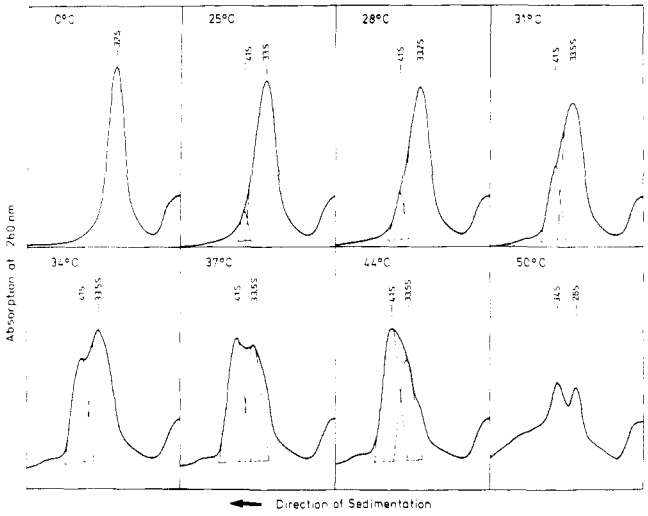

FIGURE 5: Sucrose gradient analysis of particles formed at various temperatures during the first incubation. The times chosen lie on the dashed line in Figure 5: 0 °C/30 min; 25 °C/30 min; 28 °C/25 min; 31 °C/20 min; 34 °C/15 min; 37 °C/10 min; 44 °C/7.5 min; and 50 °C/7.5 min. For details, see the Experimental Procedure.

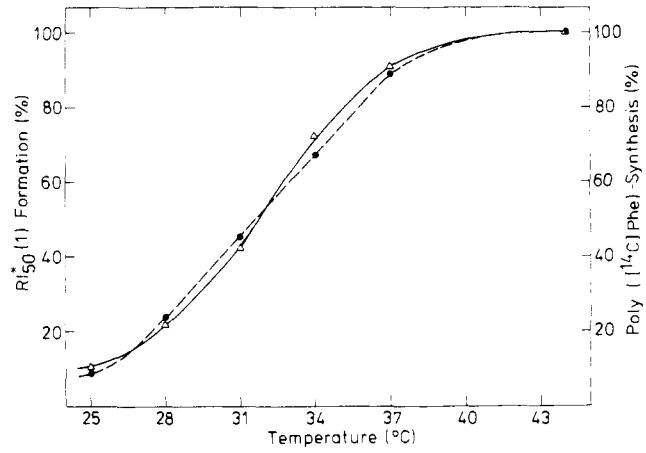

FIGURE 6: Correlation of the amounts of $RI_{50}^*(1)$ particles formed at various temperatures during the first incubation (areas below the 41S peaks in Figure 5) and the corresponding activities in the poly(U) system (Figure 4, points of intersection with dashed line). The 100% values are equivalent (a) to the amount of $RI_{50}^*(1)$ particles formed after 7.5 min at 44 °C in the first incubation and (b) to the activity of the particle (27 100 cpm) which was incubated for 7.5 min in the first step and subjected to the standard second incubation.

would be formed in the second one. A sucrose gradient analysis of the particles formed should enable us to distinguish between these two possibilities; for this purpose, the gradient analysis should be performed at times when the $RI_{50}^*(1)$ formation is as complete as possible, but when the subsequent $RI_{50}(2)$ formation is negligibly low. If the first hypothesis [quality of $RI_{50}^*(1)$ particles] is correct, about the same amount of $RI_{50}^*(1)$ particles or their equivalent should be found. In the second case [amount of normal $RI_{50}^*(1)$ particles], two main peaks corresponding to $RI_{50}(1)$ (33S) and $RI_{50}^*(1)$ (41S) particles would be expected, and the area under the $RI_{50}^*(1)$ peak should correlate with the "plateau" activity (Figure 4).

At an incubation temperature of 44 °C the $RI_{50}^*(1)$ formation is nearly complete at 7.5 min, whereas less than 10% of the particles are present in the $RI_{50}(2)$ state (Dohme and Nierhaus, 1976). At 7.5 min the activity of the 44 °C kinetic curve is just below the "plateau" activity (Figure 4). The corresponding times of the other kinetic curves lie roughly on a straight line (dashed line in Figure 4). The particles present at these times of the respective kinetic experiments were analyzed on a sucrose gradient. Figure 5 demonstrates that two main peaks exist, one at about 33S [$RI_{50}(1)$] and one at 41S [$RI_{50}^*(1)$]. The amount of the latter depends on the temperature. At 25 °C or below, practically no $RI_{50}^*(1)$ particles are found, whereas at 44 °C these particles form the main peak. The situation is different at temperatures above 44 °C in the first incubation; e.g., at 50 °C smaller products (26 and 34 S) are found (Figure 5, 50 °C).

When the amounts of $RI_{50}^*(1)$ particles formed at the various temperatures (area below the 41S peak; see Figure 5) are compared with the respective activities in the poly(U) system (Figure 4), an excellent correspondence between amount of $RI_{50}^*(1)$ particles and resultant activity is evident (Figure 6). This striking agreement suggests that the temperature of the first incubation influences the amount of normal $RI_{50}^*(1)$ formed. Therefore, the $RI_{50}^*(1)$ particles formed at various temperatures during the first incubation should be equally well convertible to active 50S subunits during a standard second incubation.

Accordingly, the $RI_{50}^*(1)$ particles were isolated on sucrose gradients after first incubations performed at 31, 34, and 44 °C, respectively. Table I demonstrates that the isolated particles could be processed to fully active ones, in contrast to the unfractionated mixture of reconstituted intermediates.

TABLE I: Activity of Particles Reconstituted at Various Temperatures during the First Incubation, Followed by a Second Incubation, after Fractionation on a Sucrose Gradient.[a]

temp of the 1st incubat (°C)	poly([^{14}C]Phe) synthesis (%) unfract reconstit particles	fract enriched with $RI_{50}^*(1)$ particles	values corr for 33S material
44	100 (105 900 cpm)	115	115
34	51	103	127
31	26	73	108

[a] Controls: Native 30S + 50S, 108 700 cpm; 30S, 840 cpm. After the first incubation performed at various temperatures, an aliquot of the reconstitution mixture was incubated under the standard conditions for the second incubation and tested in the poly(U) system (see column "unfractionated reconstituted particles"). The remaining material after the first incubation was subjected to a sucrose gradient centrifugation. The material sedimenting as $RI_{50}^*(1)$ particles (41S) was isolated and, after a standard second incubation in the presence of TP50, was also tested in the poly(U) system (see column "fraction enriched with $RI_{50}^*(1)$ particles"). This material was possibly contaminated with $RI_{50}(1)$ particles (33S). The amount of 33S particles present in the various preparations was determined by a further sucrose gradient analysis. These data were used to correct the activity values of column 3, the corrected values being shown in the last column.

The final activities of the unfractionated reconstituted particles obtained at 34 and 31 °C (51 and 26%, respectively) are lower than the corresponding ones shown in Figure 6 (64 and 46%, respectively). This difference is probably due to the large reconstitution volume in this experiment (5 and 7.5 mL, respectively) in contrast to the small volume (100 uL) used in the experiment for Figure 6. The large volume takes considerably longer to reach the incubation temperature than the small one.

When we correct the different $RI_{50}^*(1)$ preparations with respect to contamination with $RI_{50}(1)$, it becomes evident that all $RI_{50}^*(1)$ particles formed at the various temperatures are functionally equivalent (see last column in Table I). Thus, the

TABLE II: Scheme of in Vitro Assembly of the Large Ribosomal Subunit.[a]

		s value
1st incubat	(5S + 23S)RNA + proteins $\xrightarrow{0\ °C,\ 4\ mM\ Mg^{2+}}$ $RI_{50}(1)$	33
	$RI_{50}(1) \xrightarrow{44\ °C,\ 4\ mM\ Mg^{2+}} RI_{50}*(1)$	41
2nd incubat	$RI_{50}*(1)$ + proteins $\xrightarrow[50\ °C,\ 20\ mM\ Mg^{2+}]{44\ °C,\ 4\ mM\ Mg^{2+}\ or}$ $RI_{50}(2)$	48
	$RI_{50}(2) \xrightarrow{50\ °C,\ 20\ mM\ Mg^{2+}}$ rec. 50S	50

[a] See Dohme and Nierhaus (1976). The conversion $RI_{50}*(1) \rightarrow RI_{50}(2)$ can occur in both the first and the second incubations.

second of the two hypotheses mentioned above is the correct one.

Discussion

The Measurement of the Rate-Limiting Steps during the First and Second Incubations. Table II summarizes the assembly process of the 50S subunit in vitro (Dohme and Nierhaus, 1976).

The essential product of the first incubation is the $RI_{50}*(1)$ particle. This particle can neither be formed nor bypassed under the conditions of the second incubation; it is a prerequisite for the formation of an active particle during the second incubation (Dohme and Nierhaus, 1976; Spillmann et al., 1977). The essential step of the second incubation is the formation of active 50S subunits from inactive $RI_{50}(2)$ particles. Therefore, we have to measure the formation of $RI_{50}*(1)$ particles and reconstituted 50S subunits in the first and second incubations, respectively.

The measurement of the formation of reconstituted 50S subunits in the second step raises no difficulties. The appearance of reconstituted 50S subunits can be monitored by the increase of ribosomal elongation activity [poly(U) system].

The $RI_{50}*(1)$ particles are quantitatively converted to active 50S subunits in the second incubation. This became evident from recent studies (Dohme, 1976; Dohme and Nierhaus, 1976), and this contention is supported by the fact that the reconstituted particles attain 100% of the activity of native 50S subunits. Therefore, the elongation activity after the standard second incubation reflects the amount of $RI_{50}*(1)$ particles formed during the first incubation.

A serious objection to these arguments is the possibility that the reconstitution process could continue during the incubation involved in the poly(Phe) synthesis assay. To avoid such a continuation, we performed poly(U) system assays for 60 min at 20 °C, in the presence of 30 μM phenylalanine. After 60 min, the phenylalanine incorporation is still linear (data not shown). Under these conditions, no further reconstitution occurs as indicated by the following findings: (1) No elongation activity was measurable when RNA and TP50 were added directly to the poly(U) system. (2) No elongation activity was measured when, after a standard first incubation, the second one was omitted. (3) Less than 5% activity was found when the first incubation was omitted and only the second one performed. This low activity in the latter case is not due to the poly(U) assay conditions because a similar low activity near to background was found in the peptidyltransferase assay which is performed at 0 °C (Nierhaus and Dohme, 1974). The relatively high activity (up to 30% of that of native 50S subunits) in the poly(U) assay described previously when the first step was omitted (Nierhaus and Dohme, 1974) was caused by the limiting amount of phenylalanine (1.5 μM) present in the assay. When an excess of phenylalanine (≥30 μM) is used, as in the present study, values at or near background are found (Dohme and Nierhaus, 1976).

Thus, the reaction velocity can be directly determined in both reconstitution incubations by measuring the elongation activity. This has the important advantage, in contrast to the procedure of Traub and Nomura (1969) for the 30S subunits, that it was not necessary to isolate the reconstituted particles, thereby avoiding a possible inactivation by centrifugation, dialysis, etc.

Kinetic and Thermodynamic Parameters of the Rate-Limiting Steps of the First and Second Incubations. As shown above, the rate-limiting step follows the first-order law in both incubations (Figures 1 and 2, respectively). The rate-limiting step of the second incubation is a unimolecular reaction and that of the first incubation is most probably also unimolecular, as shown by the dilution experiments (Figure 3). These results are in excellent agreement with those of a previous qualitiative study (Dohme and Nierhaus, 1976) in which the conformational change $RI_{50}(1) \rightarrow RI_{50}*(1)$ was identified as the rate-limiting step of the first incubation, and the conformational change $RI_{50}(2) \rightarrow$ 50S as that of the second.

However, the dilution experiments indicate that the rate-limiting reaction taking place during the first incubation is more complicated than that of the second incubation. This interpretation is further supported by the surprising finding that not only the rate but also the extent of the $RI_{50}*(1)$ formation depends on the temperature of the first incubation.

After determination of the temperature dependence of the rate constants, the Arrhenius activation energy can be calculated. When the RNA moiety was isolated by the phenol method, we obtained a value of 293 ± 16 kJ/mol (70 ± 4 kcal/mol) for the first step and 225 ± 5 kJ/mol (54 ± 1.2 kcal/mol) for the second one. These values are of the same order as the energy of a covalent bond (about 400 kJ/mol, respectively, 100 kcal/mol) and are clearly higher than the activation energies of enzyme-catalyzed reactions (4–100 kJ/mol or 1–25 kcal/mol; Fruton and Simmonds, 1958). The corresponding value of 160 kJ/mol (38 kcal/mol) for the 30S subunit is lower than the values for the 50S subunits. Obviously, the larger number of components in the 50S particle generates many more possibilities to form binding sites and to reorientate them.

How is it possible that the rate-limiting step of an incubation at 44 °C for 20 min possesses a higher activation energy (293 kJ/mol) than that of an incubation at 50 °C for 90 min (225 kJ/mol)? The higher activation energy in the first incubation points to a multistep conformational change during this incubation. If, for example, the conformational change $RI_{50}(1)$

TABLE III: Thermodynamic Parameters of the Activation Process in Both Incubations (Using RNA^p).[a]

	1st incubat		2nd incubat	
	kJ/mol	(kcal/mol)	kJ/mol	(kcal/mol)
E_a	293	(70)	226	(54)
$\Delta H^{\ddagger}$	290	(69)	224	(53)
$\Delta G^{\ddagger}$	93	(22)	104	(25)
$\Delta S^{\ddagger}$	0.636	(0.152)	0.386	(0.092)

[a] $\Delta S^{\ddagger}$ is given in kJ mol^{-1} deg^{-1} (kcal mol^{-1} deg^{-1}). From transition-state theory (see Moore, 1966) the following equations can be obtained:

$$\Delta H^{\ddagger} \approx E_a - RT \quad (1)$$

$$k = \left(\frac{k_B T}{\hbar}\right) e^{-\Delta G^{\ddagger}/RT} \quad (2)$$

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger} - \Delta G^{\ddagger}}{T} \quad (3)$$

where k is the rate constant, R the gas constant, T the absolute temperature, k_B the Boltzmann constant, and $\hbar$ Planck's constant. The values listed in the table were calculated using the rate constants taken from Figures 1B and 2B, respectively, at 37 °C and the activation energies (E_a) for the rate-limiting steps from the first and second incubations (see Figures 1B and 2B, respectively).

→ RI_{50}*(1) proceeds in three steps each with 100 kJ/mol activation energy, a lower incubation temperature is required than for a single step with an activation energy of 225 kJ/mol. When the acetic acid method was used for the isolation of RNA (RNA^a), a lower activation energy was obtained in the first incubation than that formed with RNA^p (225 and 290 kJ/mol, respectively); in contrast, for the second incubation the same activation energy was found with both RNA preparations. The lower activation energy found with RNA^a in the first incubation indicates that the conformation of this RNA more closely resembles that of the RNA within the 50S subunit than does the conformation of RNA^p.

In this context, it makes no sense to assign the RNA^a a more "active" or "native" structure as compared to the RNA^p. Although the activation energy was lower with RNA^a, the maximal activities of the final reconstituted particles were always lower with RNA^a than with RNA^p [e.g., with RNA^a 60–85% of the RNA^p activity was obtained]. The relatively low activity obtained with RNA^a was not due to a reduced amount of 5S RNA (Sieber and Nierhaus, unpublished observations). It is possible that the RNA^a conformation allows simultaneously early and late assembly events, thus disturbing the ordered sequence of the assembly process. Therefore, RNA^p seems to be more suitable for studies on the assembly process in vitro than RNA^a.

The 23S RNA^a does not directly bind any additional proteins beyond those which bind to RNA^p under the conditions of the first step (Sieber and Roth, unpublished experiments). On the other hand, the 16S RNA^a binds seven proteins in addition to the six RNA-binding proteins originally found with 16S RNA^p (Hochkeppel et al., 1976).

In contrast to the conformational lability reported for the 16S RNA^a (Hochkeppel and Craven, 1976), the 23S RNA^a was stable with respect to the activation energy of the first step, even after several freeze–thaw cycles over a period of at least 1 week. Nevertheless, all experiments with RNA^a described in this paper were performed with freshly prepared, nonfrozen RNA^a.

When the rate constant and the activation energy of a reaction at a given temperature are known, we can calculate the activation enthalpy $\Delta H^{\ddagger}$, the free energy of activation $\Delta G^{\ddagger}$, and the activation entropy $\Delta S^{\ddagger}$ (transition-state theory; see Moore, 1966). Table III summarizes the respective data for the first and second incubation. Similar to the 30S particles (Traub and Nomura, 1969), we find positive values for the respective entropies of activation for both rate-limiting steps; i.e., the activation lowers the degree of order. Obviously, many noncovalent bonds are broken during the activation, leading to a loose complex at the start of the reaction. This interpretation is in agreement with the finding that the course of the in vitro assembly, especially during the first incubation (see the particularly high positive entropy of activation in Table III), is easily disturbed, and suggests the existence of labile intermediates during the conformational change $RI_{50}(1) \rightarrow RI_{50}$*(1).

Possible Mechanisms for Lowering the High Activation Energy in Vivo. The in vivo and in vitro assemblies of 50S subunits are similar in many respects. The three precursors (p_1 50 S, p_2 50 S, and p_3 50 S) of the in vivo assembly have s values (32, 43, and about 50 S, respectively; for review, see Schlessinger, 1974) very similar to those of the three in vitro reconstitution intermediates (33, 41, and 48 S, respectively; Dohme and Nierhaus, 1976). Furthermore, the five proteins L4, L13, L20, L22, and L24 which are essential for the RI_{50}*(1) formation (Spillmann et al., 1977) belong to the first proteins of the in vivo assembly process (Nierhaus et al., 1973; Pichon et al., 1975). However, in their later stages the in vivo and in vitro assembly process seem to diverge (see Spillmann et al., 1977).

Despite the similarities, the data and findings obtained from studies on in vitro assembly are not automatically applicable to the in vivo assembly. Even the rate-limiting steps could be different in vivo, for example, as a consequence of the limiting concentrations of essential proteins. The latter possibility would offer an efficient regulation mechanism for ribosomal biosynthesis.

The most striking difference between the in vivo and in vitro situations lies in the incubation requirements. The cell doubles its whole set of ribosomes in less than 30 min at 37 °C, when rich medium is supplied, whereas more then 120 min at much higher temperatures is required for the total reconstitution of the large subunit. Among the various possibilities for explaining this large difference, one finding is probably of major importance: In vivo the assembly parallels the transcription of the ribosomal RNA, leading to an "assembly gradient" along the RNA from the 5′ end toward the 3′ end (Spillmann et al., 1977). Due to this sequential process, the degree of disorder (measurable as entropy) never attains in vivo that maximal value which we generate in vitro by the simultaneous addition of 34 different proteins to two RNA molecules. Likewise, the disorder created by the activation steps during the reconstitution ($\Delta S^{\ddagger}$) will be clearly reduced in such an "assembly gradient".

If we assume for a moment that the rate constants of the rate-limiting steps at 37 °C are not different in vivo and in vitro, it follows from the eq 2 given in the legend of Table III that $\Delta G^{\ddagger}$ = constant and, therefore, $\Delta S^{\ddagger} \sim E_a$ (from eq 3). When $\Delta S^{\ddagger}$ is reduced at a given temperature (37 °C), the in vivo activation energy E_a will be diminished. However, the comparison of the doubling time in vivo with the incubation time in vitro (see above) indicates that the rate constants in vivo are larger than the corresponding ones in vitro. Thus, according to eq 2 $\Delta G^{\ddagger}$ will be smaller in vivo, leading to an additional decrease of the activation energy (see eq 3 with $\Delta H^{\ddagger} \approx E_a - RT$).

In addition to the "assembly gradient", other features specific for the in vivo assembly might lower the activation energy

and must be considered: (1) The RNA molecules of the precursor particle are longer than those in the mature particle (for review, see Fellner, 1974). (2) The 16S and 23S RNA are methylated during the assembly process (Fellner, 1974). (3) Specific assembly proteins may be found on the precursors but not on the mature subunits. Such proteins have been identified in the biosynthesis of eukaryotic cytoplasmic ribosomes (for review, see Hadjiolov and Nikolaev, 1976). Evidence for the existence of corresponding proteins in prokaryotic species has been reported for *Bacillus subtilis* (Guha et al., 1975) and is consistent with the results of a genetic approach (Bryant and Sypherd, 1973). (4) Some proteins are modified (methylated) during the assembly (Alix and Hayes, 1974; Chang and Chang, 1975). It is not clear up to now whether any or all of these features play a role and, if so, what importance they have in increasing the rate constants and, therefore, lowering the activation energy of the assembly process in vivo.

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