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Kinetics of Ribosome Dissociation and Subunit Association Studied in a Light-Scattering Stopped-Flow Apparatus†

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ABSTRACT: The association-dissociation kinetics of ribosomes from *Escherichia coli* have been studied under various conditions in a light-scattering stopped-flow apparatus. The dissociation reaction at 2 mg/ml at 25 °C, induced by lowering the $MgCl_2$ concentration from 18 to 3 mM, can best be described by three independent first-order processes with rate constants of 15 s^{-1}, 0.9 s^{-1}, and 3×10^{-2} s^{-1}, the slowest process comprising about 60% of the overall reaction. The fraction of ribosomes dissociating with the fastest rate (15 s^{-1}) is concentration dependent and becomes negligible at 0.1 mg/ml. Ribosomes treated with puromycin also show three dissociation rates with essentially the same rate constants as the nontreated samples. The dissociation induced by a high KCl concentration (0.85 M KCl, 18 mM MgCl) also shows three first-order phases with the same rate constants as for the dissociation induced by lowering the $MgCl_2$ concentration. The formation of 70S ribosomes from 30S and 50S subunits, induced by increasing the $MgCl_2$ concentration from 2 to 21 mM, follows second-order biphasic kinetics. A detailed analysis of the kinetic results shows that the two principal ribosomal forms must have one type of subunit in common. When the association data are analyzed assuming that the kinetic heterogeneity arises from two forms of only one subunit, the rate constants are found to be 6.4×10^6 and 1.05×10^6 M^{-1} s^{-1}. Sequential flow experiments show that the rapid and slow association species are to be identified, respectively, with phases II (0.9 s^{-1}) and III (0.03 s^{-1}) of dissociation. Relaxation measurements show that these correspond to type B ("loose") and A ("tight") ribosomes, respectively. Tight and loose ribosomes were isolated by sucrose density centrifugation, and dissociation and association kinetic studies confirmed the above assignments. Furthermore, the rate constants for these ribosomes agreed within experimental error with the rate constants derived from analysis of the multiphasic kinetic data. The association rate constants are for ribosomes dissociated by dilution with the appropriate buffer immediately before recording the kinetics of association. Ribosomes dissociated by dialysis overnight against 2 mM $MgCl_2$ show an association rate constant for the slower association reaction (type-A ribosome) that is about four times smaller, whereas the rate constant for the faster process is roughly the same. The activation energies of the dissociation reactions, whether induced by lowering the $MgCl_2$ concentration or increasing the KCl concentration, and the association reaction induced by increasing the $MgCl_2$ concentration are less than 3.5 kcal/mol. The rate constants of the dissociation at 3 mM $MgCl_2$ and of the association reaction at 21 mM $MgCl_2$ do not vary between pH 7.2 and 8.4. When 30S and 50S subunits are flowed against buffer containing 20 mM spermidine, the association process is *monophasic,* with an association constant $k = 6 \times 10^6$ M^{-1} s^{-1}. Approach-to-equilibrium studies show that k_{assoc} varies with $(Mg^{2+})^n$ and k_{dissoc} varies with $(Mg^{2+})^{-m}$ (n = 2.3-3.7, m = 2-3) for 4 mM < (Mg^{2+}) < 8 mM.

The magnesium ion-dependent association of *Escherichia coli* 30S and 50S subunits to form 70S ribosomes is known to be reversible (Tissieres et al., 1959) and Nomura and Lowry (1967) discussed the physiological importance of this association-dissociation process. Various aspects of the magnesium ion-dependent reversible association have been studied by ultracentrifugation methods (Belitsina and Spirin, 1970; Ball et al., 1973; Spirin, 1971; van Diggelen and Bosch, 1973; Noll et al., 1973a) and even polyacrylamide gel electrophoresis (Talens et al., 1970). Ball et al. (1973) demonstrated that 70S ribosomes are in equilibrium with their subunits. In an elegant study, Zitomer and Flaks (1972) determined the equilibrium constant of the magnesium ion-dependent reversible association and examined, also, the influence of temperature, monovalent cations, and polyamines upon the equilibrium by light-scattering experiments. Since light-scattering measurements involve a minimum perturbation of the system under

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investigation, this method is ideal for following the kinetics of association or dissociation of macromolecular species. A specially designed fluorescence and light-scattering stopped-flow apparatus allowed us to study, in detail, the magnesium ion-dependent reversible association of the ribosomal subunits under various conditions. Recently, Wolfe et al. (1973) determined relaxation times of the association reaction of ribosomal subunits following turbidity changes, but no actual rate constants were reported; Wishnia et al. (1975) reported magnesium-dependent rate constants for association–dissociation of type-A ribosomes; Debey et al. (1975) have reported equilibrium measurements for type-A and -B ribosomes (*E. coli* MRE600).

Materials and Methods

Chemicals and Organism. Alumina type 305, 2-mercaptoethanol, spermidine, and tris(hydroxymethyl)aminomethane (Tris[1]) as Trizma base were obtained from Sigma (St. Louis, Mo.), and deoxyribonuclease I, RNase free, was from Worthington (Freehold, N.J.). Reagent-grade sucrose was purchased from Merck (Rahway, N.J.) and polyvinyl chloride filters, BDWP 02500, 0.6 μm, were from Millipore (Bedford, Mass.). All other chemicals were of analytical reagent grade. *Escherichia coli* B was obtained from Grain Processing Co., Muscatine, Iowa, as a frozen wet paste, harvested at half-logarithmic growth phase.

Buffers. Buffer 1: 10 mM Tris, 50 mM KCl, 6 mM 2-mercaptoethanol, pH 7.5. Buffer 2: 10 mM Tris, 1 M NH_4Cl, 3 mM $MgCl_2$, 6 mM 2-mercaptoethanol, pH 7.5. Buffer 3: 10 mM Tris, 0.5 M NH_4Cl, 20 mM $MgCl_2$, 6 mM 2-mercaptoethanol, pH 7.5.

Preparation of Ribosomes. Ribosomes were prepared from *E. coli* B according to the procedure of Zamir et al. (1971) with slight modifications. All preparation steps were carried out at 4 °C. Forty grams of frozen *E. coli* cell paste was ground in a mortar with the same amount of alumina powder. The viscid paste was taken up in 100 ml of buffer 1, containing 20 mM MgCl, and then 3 μg of RNase-free DNase was added per ml of the resulting suspension. Alumina and cell debris were removed by centrifugation for 10 min at 40 000*g* and residual fine cell debris was removed at 80 000*g* for 35 min. The ribosomes were collected by centrifugation at 80 000*g* for 6 h and a small amount of viscous material on top of the ribosomal pellet was removed by gentle shaking with a few milliliters of buffer 1 containing 20 mM $MgCl_2$. The ribosomes were dissolved in 60 ml of buffer 2 and stored for 4–6 h. The suspension was centrifuged at 40 000*g* for 10 min to remove flocculent material and the ribosomes were collected by centrifugation at 80 000*g* for 15 h. Some denatured material on top of the precipitated ribosomes was removed by gentle shaking with a small volume of buffer 2 and the ribosomes were dissolved in 60 ml of buffer 3. After storage at 2 °C for 4–6 h, some aggregated material was removed by centrifugation at 40 000*g* for 10 min. The ribosomes were collected following centrifugation at 80 000*g* for 15 h. The pellet was washed gently with a few milliliters and then taken up in 5 ml of buffer 3 to make up a stock solution of 50–60 mg of ribosomes/ml. Some denatured material was removed by centrifugation at 40 000*g* for 10 min and the resulting preparation was stored at 4 °C. For some of the kinetic studies, tight (native) and loose (derived) ribosomes were isolated by sucrose density gradient centrifugation according to the procedures of van Diggelen and Bosch (1973).

Determination of Ribosome Concentrations. An absorbance of 0.145 at 260 nm for a 0.001% solution was used to determine the ribosome concentration. The molecular weights of the 70S ribosome and the 50S and 30S subunits were assumed to be 2.45×10^6, 1.55×10^6, and 0.90×10^6, respectively, as reported by Hill et al. (1969). The ratio of 30S to 50S subunits was determined by sucrose gradient centrifugation. Ribosomes were dialyzed overnight against 200 times the volume of buffer 1 with 2 mM $MgCl_2$. Samples of 100 μl with 80–100 μg of ribosomes were layered onto 5-ml linear sucrose gradients in the same buffer. Centrifugation was carried out at 4 °C and 155 000*g* for 100 min. The absorbance profiles of the gradients were obtained at 254 nm using an ISCO gradient analyzer (ISCO, Lincoln, Nebraska). The extinction coefficient at 260 nm is the same for the two ribosomal subunits (Hill et al., 1969) and it was assumed to be the same at 254 nm also. The ratio of 30S and 50S subunits was determined from the area under the respective peaks on the absorbance tracing.

Static Light Scattering Experiments. Static light-scattering experiments were performed in a Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation and emission monochromators were set to 400 nm and both excitation and emission slits were set for a 4-nm band-pass. Four-milliliter standard quartz fluorescence cuvettes with a path length of 10 mm were used and detection was at 90° to the incident beam. In all experiments, the transmittance at 400 nm was >98% and all solutions were filtered through polyvinyl chloride filters to remove dust particles. The measurements were performed at 25 °C and the temperatures of the samples were regulated to ±0.1 °C by means of a thermostated cuvette holder. To determine the dissociation of 70S ribosomes, a sample of the ribosome stock solution was dialyzed overnight against 200 volumes of buffer 1 containing 20 mM $MgCl_2$. After centrifugation at 20 000*g* and 4 °C for 10 min to remove some aggregated material, samples were diluted into a series of 4-ml solutions of buffer 1 containing concentrations of $MgCl_2$ from 2 to 20 mM. The final ribosome concentration was 0.04–0.2 μM. The reaction mixtures were allowed to equilibrate for several minutes at the experimental temperature and the relative light scattering was determined. Reassociation of ribosomal subunits was determined by diluting ribosome stock solution with buffer 1 to a $MgCl_2$ concentration of 2 mM. To samples of this ribosome solution, appropriate volumes of buffer 1 containing specific amounts of $MgCl_2$ were added to the desired $MgCl_2$ concentrations.

Kinetic Measurements. Preliminary kinetic experiments were performed in the fluorescence spectrophotometer. The dissociation reaction was induced by adding a small volume of a concentrated solution of associated ribosomes to 3 ml of buffer 1 containing 2 mM $MgCl_2$ by means of a special plunger, which allowed rapid mixing. The association reaction was initiated by adding a small volume of a concentrated $MgCl_2$ solution to a 3-ml sample of ribosomes in buffer 1 containing 2 mM $MgCl_2$. The mixing was done in the cuvette, seated in the cuvette holder of the instrument, and the change of the light scattering signal as a function of time was recorded on a strip chart recorder. Readings of the light-scattering signal could be taken about 5–7 s after addition of the ribosome or $MgCl_2$ samples to the cuvette. The scattering intensity of the sample at time $t = 0$ was determined from a reference sample which had been diluted into buffer 1 containing 20 mM $MgCl_2$.

The detailed kinetic measurements were performed in a specially designed fluorescence and light-scattering stopped-flow apparatus developed by two of us (L.J.P. and H.G.). The

[1] Abbreviation used: Tris, tris(hydroxymethyl)aminomethane.

instrument is also equipped with absorption optics, thus permitting absorption, fluorescence, and light-scattering measurements in the same instrument. The light source is a 150-W xenon arc lamp powered by a precision 1000-W current-regulated power supply (DeSa, 1970). The light passes through a Jarrell-Ash 0.25-m Ebert monochromator with 1-mm slits and is focused onto the observation quartz cuvette, which has an optical path length of 20 mm. The cuvette volume is 80 μl and 0.2–0.25 ml of each reagent is used per kinetic run. The temperature is monitored by a 1-mm diameter thermistor probe located 2 mm from the observation cuvette in the cuvette chamber. Temperatures are read from a digital voltmeter to 0.01 °C. Interchangeable variable diameter driving syringes allow the ratio of reactant volumes to vary from 1:1 to 1:7. Filters can be inserted between the reservoir syringes and the instrument to remove dust particles. The detection system and the data acquisition were described previously (Boelts and Parkhurst, 1971; LaGow and Parkhurst, 1972).

The dead time of the stopped-flow apparatus was determined to be 3–4 ms by following the combination of reduced whale myoglobin with varying concentrations of carbon monoxide at 430 nm (Gibson, 1969). Mixing is more than 99.5% complete at the point of observation both for the equal as well as the unequal driving syringes (as established by the disappearance of the absorption of an alkaline solution of phenolphthaleine at 552 nm when mixed with hydrochloric acid).

For kinetic measurements, samples of the ribosome stock solution were dialyzed overnight against 200 volumes of buffer 1 containing 20 mM $MgCl_2$ and were centrifuged afterwards at 20 000*g* and 4 °C for 10 min to remove some aggregated material. In the dissociation experiment where the concentration of $MgCl_2$ was lowered, driving syringes of unequal size with mixing ratios of 1:5 to 1:7 were used. The ribosomes in buffer 1 containing 18 mM $MgCl_2$ were in the smaller volume syringe and the larger syringe contained buffer 1. In the dissociation experiments induced by increasing the concentration of KCl and in the association experiments initiated by increasing the $MgCl_2$ or spermidine concentrations, driving syringes of equal size were used. Polyvinyl chloride filters with a pore size of 0.6 μm were inserted between the reservoir syringes and the instrument. The light-scattering measurements were carried out at 400 nm and for each sample a minimum of five kinetic runs was averaged by the computer, thus providing 1000 raw data points to generate a curve suitable for detailed fitting by various theoretical models. More than 25 separate ribosome preparations were used in these studies.

Data Fitting. For theoretical models which can be integrated in closed form, the minimum of the response function (the sum of squared residuals observed minus theoretical) was found by the Fletcher–Powell nonlinear minimization algorithm (Fletcher and Powell, 1963). For each apparent minimum, an estimate of the standard errors in the parameters was obtained by a statistical routine, which gives the variance–covariance matrix of the parameters (Draper and Smith, 1966). For theoretical models which could not be integrated in closed form or for which the integration resulted in functions which were difficult to evaluate, the Runge–Kutta method was used to integrate the differential equations (Margenau and Murphy, 1956). For each fit, a time-series analysis of the residuals was made (Swed and Eisenhart, 1943).

Results

Characterization of the Ribosome Preparation. The relative amounts of 30S and 50S subunits were determined by sucrose gradient centrifugation. The gradients were made in buffer 1 containing 2 mM $MgCl_2$ and the subunit distribution was determined with an ISCO gradient analyzer. The ratio of 30S to 50S subunits was found to be 0.9 ± 0.05. The fraction of ribosomal particles that were able to associate to 70S ribosomes was determined by sucrose centrifugation in buffer 1 containing 20 mM $MgCl_2$. From the particle distribution, it was estimated that 90 ± 5% were associated in fresh samples under these conditions. The fraction of 70S species was the same whether ribosomes which had not been dissociated after their isolation, or whether dissociated and reassociated ribosomes were used. Ribosomes which had been dissociated by dialysis overnight against buffer 1 containing 2 mM $MgCl_2$ associated to the same extent; however, after storage for several days, the fraction of ribosomes able to form 70S particles dropped to between 60 and 70%. All kinetic measurements were made on ribosomes which had been stored at 4 °C and used no later than the second day after isolation.

Activity of Ribosomes. A preparation of ribosomes on which kinetic measurements were made was assayed for activity in protein synthesis according to the procedure of Parisi et al. (1967) and was found to incorporate 1.86 nmol of phenylalanine/mg of ribosomal RNA. The native ribosomes, as well as those which had been dissociated for 1 h and then reassociated, incorporated 1.9 nmol of Phe/mg of ribosomal RNA.

Static Light-Scattering Measurements. Equation 1 gives the basic formula for light-scattering (Tanford, 1967):[2]

$$R_\theta = K' \times \sum_i c_i M_i \left(\frac{dn}{dc}\right)_i^2 \qquad (1)$$

where R_θ, c, M, and dn/dc are, respectively, Raleigh's ratio (a measure of the intensity of the scattered light), the concentration in g/ml, the molecular weight, and the refractive index increment, and K' is an instrumental constant.

The refractive index increment of *E. coli* ribosomes was determined by Scafati et al. (1971) to be 0.2 cm^3/g and was found to be the same for the 70S particles, as well as for their subunits, and the second virial coefficient is zero or at least less than 2×10^{-2} cm^3/g. Equation 1, therefore, can be written as:

$$R_\theta = K \times \sum_i c_i M_i \qquad (2)$$

where $K = K' (dn/dc)^2$ and is valid as long as no significant depolarization occurs. The molecular weights reported by Hill et al. (1969) were used to estimate the change of the light-scattering signal of the ribosome solutions. In predicting the relative intensities of the light-scattering signals from associated and dissociated ribosome solutions according to eq 2, the 10% excess of 50S over 30S subunits present in the preparation had to be taken into account.[3]

[2] Equation 1 is valid so long as the diameter of the molecules to be studied is smaller than a tenth of the wavelength of the incident light, the second virial coefficient can be neglected, and no significant depolarization occurs. The light-scattering experiments were performed at 400 nm. From electron microscopy data and data on the scattering of x rays in concentrated gels, the large diameter of 70S ribosomes is known to be about 290 Å (Spirin and Garrilova, 1969), which is considerably less than a tenth of the wavelength of the incident light.

[3] Furthermore, it was assumed that all ribosome particles were able to associate, although a small fraction of free 30S and 50S subunits were detected when centrifuged in 20 mM $MgCl_2$. Infante and Baierlein (1971) reported that hydrostatic pressure generated by centrifugation causes increased dissociation of sea urchin ribosomes. Zitomer and Flaks (1972) also found complete association of *E. coli* ribosomes at 20 mM $MgCl_2$, after fixation of the ribosome species in formaldehyde according to Spirin et al. (1970), while velocity sedimentation experiments with unfixed ribosome preparations always showed some dissociation.

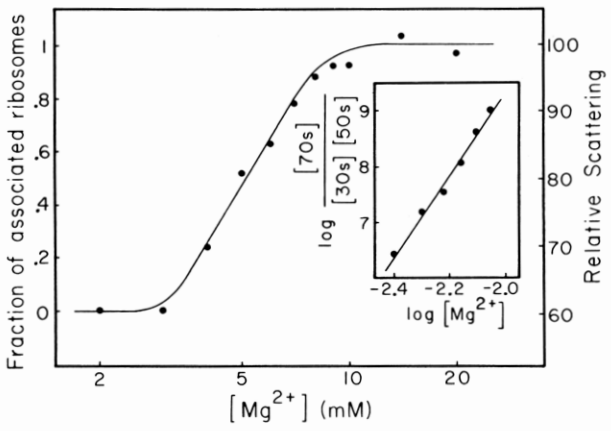

FIGURE 1: Static light-scattering measurement: equilibrium between 30S plus 50S subunits and 70S ribosomes as a function of $MgCl_2$. The ribosome concentration is 0.36 mg/ml; Temperature = 20 °C.

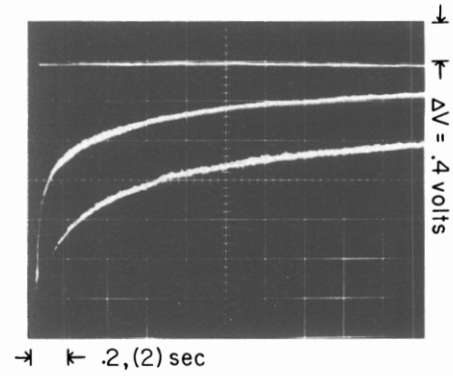

FIGURE 2: Reaction trace of ribosome subunit association. The final ribosome concentration is 2 mg/ml; the concentration of $MgCl_2$ is 21 mM; temperature = 20 °C. Upper trace, 2 s/div; bottom trace, 0.2 s/div.

The intensity of the light-scattering signal from the dissociated ribosome solution was, thus, calculated to be 58% of the signal of the associated sample, which is in good agreement with the experimental results. Figure 1 depicts the association equilibrium of ribosomes as a function of the $MgCl_2$ concentration over the entire transition range from 30S + 50S subunits to 70S particles. These measurements were made in the fluorescence spectrophotometer. It can easily be shown from eq 2 that for any Mg^{2+} concentration the difference between the signal of the respective sample and the signal of the completely dissociated sample, expressed as the fraction of the total possible signal change, represents the fraction of 70S ribosomes present. This was also verified experimentally by Zitomer and Flaks (1972) who studied the relative intensity change of a ribosome sample over the entire transition range and analyzed the distribution of 30S, 50S, and 70S particles after formaldehyde treatment.

The Mg^{2+}-dependent equilibrium between 30S and 50S subunits and 70S ribosomes can be represented by

$$30\,S + 50\,S + n\,Mg^{2+} \rightleftarrows 70S\,(Mg^{2+})_n$$

This equilibrium can be described by eq 3:

$$\ln \frac{(70S(Mg^{2+})_n)}{(30S)(50S)} = n \times \log (Mg^{2+}) + \log K_{assoc} \quad (3)$$

The insert in Figure 1 shows a plot of our data, where n was determined to be 6.4 compared to 5.3 (Debey et al., 1975) and 8.1 (Zitomer and Flaks, 1972).

Stopped-Flow Kinetic Measurements. The behavior of the stopped-flow instrument as a light-scattering photometer was tested with solutions of dissociated ribosomes in 2 mM $MgCl_2$ and the scattering signal was found to be a linear function of the ribosome concentration up to 2 mg/ml. Figure 2 depicts a typical oscilloscope trace of a $MgCl_2$-induced association reaction of 30S and 50S ribosomal particles forming 70S ribosomes. The signal to noise ratio is sufficiently high to warrant a detailed computer analysis of the kinetics of these subunit interactions. The dissociation and association reactions, unless otherwise specified, go to completion. For the dissociation process at low ribosome concentrations, the overall voltage change, ΔV_{max}, is therefore proportional to $(70\,S)_0$, the initial concentration of 70S ribosomes, and at any time, t, $\Delta V_{max} - \Delta V_t$ is proportional to $(70\,S)_t$. For the association reaction, $\Delta V_{max} - \Delta V_t$ is proportional to the concentration of dissociated subunits at time t.

At higher ribosome concentrations, the intensity of the incident beam was attenuated somewhat through the cuvette. Since this attenuation was different for associated and dissociated ribosomes, the scattered-light signals had to be corrected for this distortion. The turbidity (τ), defined as in eq 4

$$I = I_0\, e^{-\tau l} \quad (4)$$

of a ribosome solution with 2 mg of ribosomes/ml, was determined at 400 nm with a Cary 14 spectrophotometer using a sensitive slide wire (0–0.1 OD). The associated sample had a turbidity of 0.034 and the dissociated one 0.019. The turbidity ratio is, roughly, what is calculated from the ratio of the Rayleigh scattering intensities according to eq 2. This result also shows that the absorption by ribosomes at 400 nm is negligible. The scattered light of a turbid solution is the difference of the incident and the transmitted light and using the definition of turbidity in eq 4 this equals:

$$I_s = I_0\,(1 - e^{-\tau l}) \quad (5)$$

Equation 5 can be expanded and, with the turbidity known for the associated and dissociated states of the ribosome sample, i.e., for the beginning and the end of the reaction, the true turbidity at each time interval can be calculated from the light-scattering signal. The turbidity thus calculated is proportional to the concentration of the starting material in the same way as outlined above for the scattered signal. Only reactions at ribosome concentrations of 2 and 1 mg/ml were corrected, using a series expansion of eq 5 through second order. For the dissociation reactions at 0.5 mg of ribosomes/ml and lower, no correction was made.

Kinetics of Dissociation. The dissociation of 70S ribosomal particles in the stopped-flow apparatus was induced by a jump of the Mg^{2+} concentration from 18 to 3 mM. The reaction was defined to have gone to completion after 2.5 min, but corrections determined from experiments with the fluorescence spectrophotometer were made for the very slow process.[4,5] This very slow phase will be neglected in the following discussion.

As depicted in Figure 3, the dissociation reaction at a concentration of 2 mg of ribosomes/ml after mixing (starting with a concentration of 12 mg/ml) is multiphasic. A simple model

[4] In the spectrofluorimeter, a fourth very slow phase could be detected with rate constant ca. 0.0023 s^{-1} (3 mM Mg^{2+}) and with amplitude corresponding to about 18% of the total dissociation reaction

[5] In the spectrofluorimeter, a third very slow phase with a half-life of ca. 6 min is observed (21 mM Mg^{2+}, 1 mg/ml of ribosome), and amplitude about 8–15% of the total association reaction.

TABLE I: Dissociation of 70S Ribosomes Under Different Conditions.[a]

Fraction		Rate Constants (s^{-1})
(1) Ribosomes, dissociated by decreasing $MgCl_2$		
0.22 ± 6%	k_0	14.9 ± 15%
0.18 ± 7.5%	k_2	0.87 ± 14%
0.59 ± 3%	k_4	0.033 ± 5%
(2) Ribosomes (puromycin), dissociated by decreasing $MgCl_2$		
0.3 ± 10%	k_0	25 ± 30%
0.13 ± 9%	k_2	0.95 ± 28%
0.57 ± 4%	k_4	0.030 ± 9%
(3) Ribosome dissociated by increasing KCl		
0.18 ± 8%	k_0	13 ± 16%
0.18 ± 9%	k_2	0.83 ± 20%
0.64 ± 4%	k_4	0.031 ± 6%

[a] Final ribosome concentration after mixing is 2 mg/ml. 1 and 2: dissociation is induced by a shift of the $MgCl_2$ concentration from 18 to 3 mM. 3: at a $MgCl_2$ concentration of 18 mM the dissociation is induced by a shift of the KCl concentration from 0.05 to 0.85 M. Temperature = 20 °C.

assuming two types of 70S ribosomes, which dissociate with different reaction rates, does not fit the experimental data very well, since the residuals show an improbable distribution. Model I assumes three different types of 70S ribosomes, each dissociating with its own specific reaction rate.

MODEL I.

$$70\,S \xrightarrow{k_0} 30\,S + 50\,S$$

$$70\,S' \xrightarrow{k_2} 30\,S + 50\,S \qquad (6)$$

$$70\,S'' \xrightarrow{k_4} 30\,S + 50\,S$$

$$(70\,S)^{total} = (70\,S) + (70\,S') + (70\,S'') = (70\,S)_0\, e^{-k_0 t} + (70\,S')_0\, e^{-k_2 t} + (70\,S'')_0\, e^{-k_4 t} \qquad (7)$$

This model[6] leads to a decrease in the sum of squares and gives a more probable distribution of the residuals, as is shown in Figure 3. The three computed first-order rate constants and the fractions of ribosomes reacting with the respective rates are listed in Table I. The slowest process amounts to 60% of the total reaction.

Belitsina and Spirin (1970) reported the preparation of 50S particles containing polyphenylalanyl-tRNA, which were isolated in the presence of 0.5 M NH_4Cl. To test whether one of the observed dissociation processes was due to 70S ribosomes containing nascent protein, a ribosome sample was treated with puromycin in the crude cell extract before subjecting it to the salt-wash purification procedure to remove peptidyl-tRNA (Pestka, 1971). The dissociation reaction of the puromycin-treated ribosomes, again, is best described by three first-order rates. As shown in Table I, the rate constants of the two slower processes agree within experimental error with the rate constants of the ribosome sample not treated with puromycin,

[6] Equations 6 and 7 are to be regarded as formal models used to fit the data. As discussed later, the first phase is probably 100S → 2(70S) dissociation. Since the first phase is more than ten times faster than the second, k_0 is virtually unaffected by the approximations implicit in eq 7.

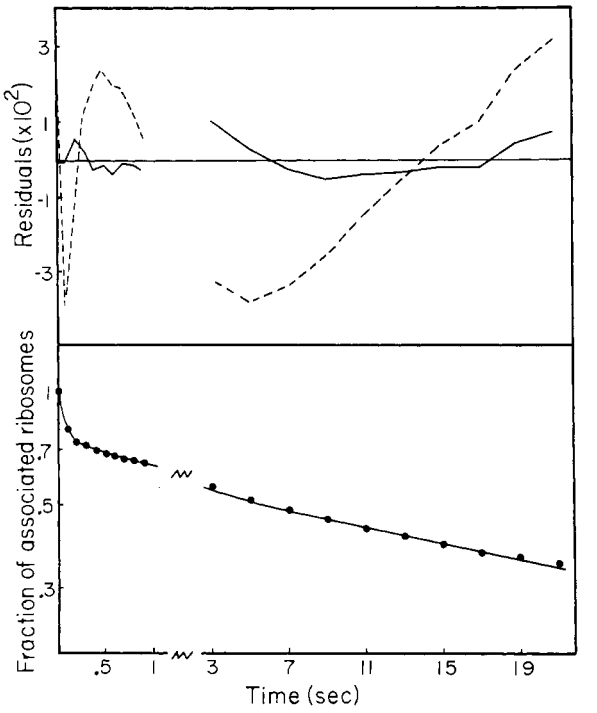

FIGURE 3: Dissociation of 70S ribosomes induced by a shift of the $MgCl_2$ concentration from 18 to 3 mM. The final ribosome concentration is 2 mg/ml; Temperature = 20 °C. Dotted line, residuals for two exponential decays; solid lines, three exponential decays.

while the rate constant of the fast is only slightly different. The fractions of ribosomes reacting with their respective rates also agree for the two different samples, the slowest process contributing about 60% to the overall reaction.

Monovalent cations are known to favor dissociation of 70S ribosomes to 30S and 50S subunits (Tissieres et al., 1959). Zitomer and Flaks (1972) studied the effect of high potassium chloride concentrations on the equilibrium constant of the dissociation-association process. The kinetics of ribosome dissociation, induced by a shift of potassium chloride from 0.05 to 0.85 M KCl, were determined at a $MgCl_2$ concentration of 18 mM. Under these conditions, the reaction proceeds from the completely associated state to 95% dissociation. Again, the overall reaction is best described by three first-order processes with rate constants and amplitudes given in Table I. The rate constants agree within experimental error with the respective rate constants for the dissociation induced by a shift of the $MgCl_2$ concentration and, again, the largest fraction of the ribosomes reacts with the slow rate.

The dissociation reaction induced by lowering the concentration of $MgCl_2$ was studied at different ribosome concentrations. With decreasing concentrations of ribosomes the fraction of the fastest reaction decreases, as is shown in Table II, while the decrease in the overall signal change is somewhat more than proportional to the ribosome concentration. At a concentration of 0.1 mg of ribosomes/ml, the fraction of the fastest reacting species is negligible and a model assuming only two types of ribosomes fits the data with a good residual distribution. Such a fit also gives within experimental error the same rate constants as does the fit at higher concentrations for the two slower processes using a model with three exponential decays. When concentrated ribosome solutions are mixed with buffer 1 containing 20 mM $MgCl_2$, conditions for which it is known from the static equilibrium measurements at low ribosome concentrations that no dissociation of 70S ribosomes

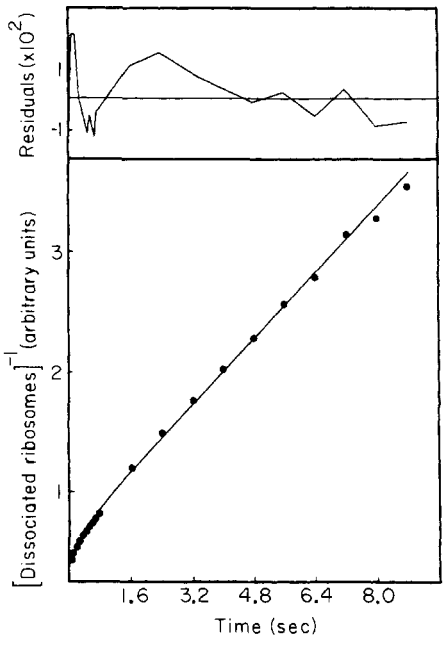

FIGURE 4: Association of 30S and 50S subunits induced by a shift of the $MgCl_2$ concentration from 2 to 21 mM; Temperature = 20 °C. The final ribosome concentration is 1.8 mg/ml. The experimental data are fit according to Model II, heterogeneity in only one subunit.

TABLE II: Dissociation of 70S Ribosomes as a Function of Concentration.[a]

Final Ribosome Concn (mg/ml)		Fraction		Rate Constant (s^{-1})
2		0.24 ± 7%	k_0	14.5 ± 15%
		0.18 ± 8%	k_2	0.87 ± 16%
		0.57 ± 3%	k_4	0.029 ± 5%
0.5		0.12 ± 15%	k_0	14.9 ± 30%
		0.19 ± 9%	k_2	0.86 ± 19%
		0.69 ± 3%	k_4	0.030 ± 6%
0.1	(1)	0.07 ± 30%	k_0	14.3 ± 60%
		0.18 ± 16%	k_2	0.66 ± 20% Type B ("loose")
		0.75 ± 4%	k_4	0.030 ± 7% Type A ("tight")
	(2)	0.2 ± 11%	k_2	1.1 ± 20%
		0.79 ± 4%	k_4	0.032 ± 7%

[a] The dissociation is induced by a shift of the $MgCl_2$ concentration from 18 to 3 mM. At a ribosome concentration of 0.1 mg/ml, the data are fit: (1) according to Model I, (2) with only two first-order reactions. Temperature = 20 °C.

into subunits occurs, a dissociation process is observed, which shows, roughly, the amplitude of the fast step in the overall dissociation reaction. At low ribosome concentrations, no such effect can be detected.

Kinetics of Association. The association of the 30S and 50S subunits to 70S particles was induced by shifting the Mg^{2+} concentration from 2 to 21 mM.

Figure 4 shows the association reaction of 30S and 50S subunits. The ribosomes were dialyzed overnight against buffer 1 with 20 mM $MgCl_2$ and diluted to a final concentration of 2 mM $MgCl_2$ immediately before the kinetic measurements. The association kinetics were multiphasic.

Two models were used to fit the experimental data. Model II assumed that one of the subunits was heterogeneous. The two forms are designated as A_1 and A_2, each of which reacts with the second subunit B forming two types of 70S particles:

TABLE III: Association of 30S and 50S Subunits.[a]

Final Ribosome Concn (mg/ml)	Fraction	Rate Constant ($M^{-1} s^{-1}$)
(A) 1.8	0.26 ± 6%	k_1 6.4×10^6 ± 5% Type B ("loose")
	0.73 ± 5%	k_3 1.05×10^6 ± 8% Type A ("tight")
0.9	0.28 ± 7%	k_1 6.7×10^6 ± 6%
	0.72 ± 5%	k_3 1.1×10^6 ± 7%
(B) 2	0.41 ± 6%	k_1 8.5×10^6 ± 10%
	0.59 ± 7%	k_3 2.5×10^5 ± 8%
1	0.42 ± 5%	k_1 9.8×10^6 ± 12%
	0.58 ± 6%	k_3 2.76×10^5 ± 7%

[a] See Model II. The association reaction was induced by a shift of the $MgCl_2$ concentration from 2 to 21 mM. The listed rate constants are (A) for a sample of ribosomes that had been diluted into standard buffer containing 2 mM $MgCl_2$ just before studying the kinetics; (B) for a sample of ribosomes dialyzed overnight against standard buffer containing 2 mM $MgCl_2$. Temperature = 20 °C.

MODEL II:

$$A_1 + B \xrightarrow{k_1} C_1$$

$$A_2 + B \xrightarrow{k_3} C_2$$

The rate of formation of 70S ribosome particles ($C_1 + C_2$) is the same as the rate of disappearance of 30S particles (B). The differential equations were integrated numerically to calculate the concentrations of A_1, A_2, and B as a function of time.

Model III assumed that both subunits were heterogeneous and one kind of subunit could react only with the corresponding kind of other subunit.

MODEL III:

$$A_1 + B_1 \xrightarrow{k_1} C_1$$

$$A_2 + B_2 \xrightarrow{k_3} C_2$$

The concentrations of A_1 and B_1, as well as of A_2 and B_2, were considered to be equal at time $t = 0$. This model represents simply the sum of two independent second-order reactions. The Fletcher–Powell minimization procedure (Fletcher and Powell, 1963) was used to fit the data. A fit of the experimental data with Model II is indicated by the solid line in Figure 4. A favorable residual distribution is shown in the upper portion of Figure 4. Part A of Table III lists the computed rate constants and the respective fractions of fast and slow reacting particles for the association reaction studied at two different ribosome concentrations. The rate constants found and the fractions of fast and slow reacting species agree within experimental error for the two concentrations.

Figure 5 shows the association reaction at two ribosome concentrations and the solid lines represent the progress of the reaction according to Model II using the *mean* values of the rate constants from part A of Table III. The figure shows that Model II describes the rate of the reaction very well, even for the different ribosome concentrations.

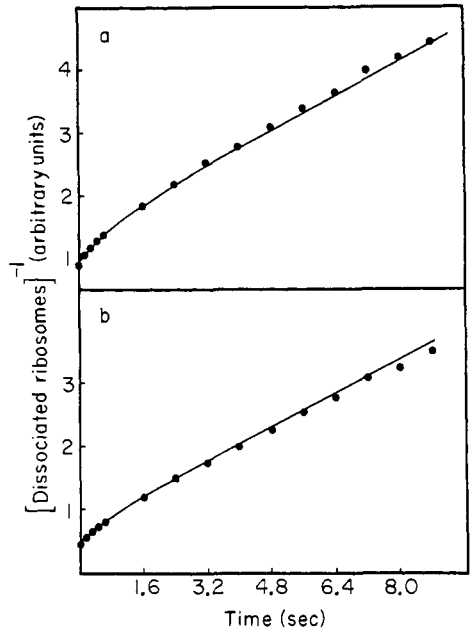

FIGURE 5: Association of 30S and 50S subunits induced by a shift of the $MgCl_2$ concentration from 2 to 21 mM. The final ribosome concentration is (a) 0.9 mg/ml and (b) 1.8 mg/ml. The solid lines represent the computed progress curves of the reactions using the rate constants $k_1 = 6.55 \times 10^6$ and $k_3 = 1.075 \times 10^6$ M^{-1} s^{-1} with 0.27 and 0.725 as the respective fractions of "fast" and "slowly reacting" ribosomes; Temperature = 20 °C.

The association reaction of ribosomes that had been dialyzed overnight against buffer 1 containing 2 mM $MgCl_2$ could also be fit by Model II. Part B of Table III gives the computed rate constants for two association reactions performed at different ribosome concentrations. Again, the rate constants and the respective fractions of fast and slow reacting species agree very well within experimental error; however, the rate constant for the slower phase is about one-fourth that for undialyzed samples.

When the association experiments were fit with Model III, a good fit could be obtained for the individual curves. Model III also shows a probable residual distribution; however, as shown in Table IV, the computed rate constants do not agree well for the experiments with different ribosome concentrations.

Spermidine-Induced Association. It has been shown (Pestka, 1971; Cohn and Lichtenstein, 1960) that addition of polyamines, especially spermidine, to solutions of 30S and 50S ribosomal subunits promotes association to give 70S ribosomes. In order to study the kinetics of this process, solutions of 30S and 50S subunits (4 mg/ml, 2 mM $MgCl_2$) were flowed against spermidine solutions (10–40 mM), and the formation of 70S ribosomes followed in the light-scattering stopped-flow apparatus. Results are shown in Figure 6.

There was some variability with different ribosome preparations. In one preparation of the five studied, a small initial lag phase was observed. At high spermidine concentrations, the rate constants for various preparations did not differ by more than a factor of two, even for the preparation showing a lag phase. Unlike the Mg^{2+}-induced association, the spermidine-induced association did not appear to be biphasic. Three theoretical models were considered for fitting four curves for concentrations of spermidine 5, 7.5, 10, and 20 mM after mixing. Although it is well known (Chien, 1948) that the equations for these mechanisms can be solved exactly (in terms

TABLE IV: Association of 30S and 50S Subunits.[a]

Final Ribosome Concn (mg/ml)	Fraction	Rate Constant (M^{-1} s^{-1})
(A) 1.8	0.45 ± 8%	$k_1 = 11.5 \pm 10^6 \pm 24\%$
	0.54 ± 7%	$k_3 = 1.17 \pm 10^6 \pm 5\%$
0.9	0.41 ± 9%	$k_1 = 22 \pm 10^6 \pm 26\%$
	0.59 ± 7%	$k_3 = 1.25 \pm 10^6 \pm 6\%$
(B) 2	0.57 ± 4%	$k_1 = 9.35 \times 10^6 \pm 12\%$
	0.42 ± 5%	$k_3 = 2 \times 10^5 \pm 8\%$
1	0.55 ± 5%	$k_1 = 15.2 \times 10^6 \pm 20\%$
	0.45 ± 4%	$k_3 = 2.4 \times 10^5 \pm 10\%$

[a] See Model III. The association reaction was induced by a shift of the $MgCl_2$ concentration from 2 to 21 mM. The listed rate constants are (A) for a sample of ribosomes that had been diluted into standard buffer containing 2 mM $MgCl_2$ just before studying the kinetics; (B) for a sample of ribosomes dialyzed overnight against standard buffer containing 2 mM $MgCl_2$. Temperature = 20 °C.

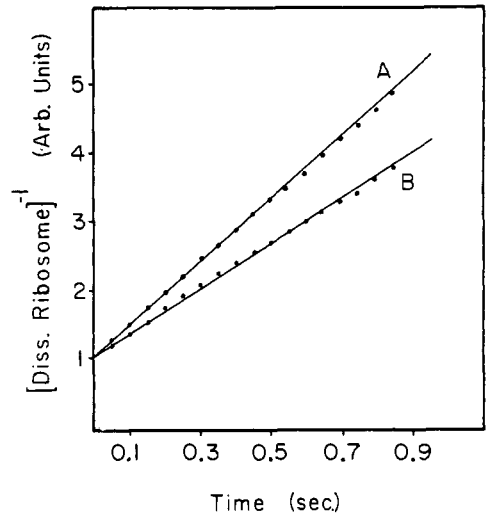

FIGURE 6: Spermidine-induced association of ribosomal subunits at 20 °C. Final ribosome concentration was 2 mg/ml for both curves (buffer 1, 2 mM $MgCl_2$). Final spermidine concentration was: (a) 7.5 mM, (b) 5 mM.

of Bessel functions and incomplete Γ functions), it proved more rapid to fit the data to equations solved by a Runge-Kutta numerical integration program written for the Super-Nova on-line computer.

Each of the models involved a pseudo-first-order spermidine binding step since, in all cases, the concentration of spermidine was severalfold greater than the dissociation equilibrium constant for spermidine binding. Each model involved the spermidine concentration in a subunit association step, since, experimentally, the overall rate of the association reaction increased with increasing spermidine over the range 5–20 mM, but the overall extent of association remained unchanged.

Model III is the sum of Models I (steps 1, 2, and 3) and II (steps 1, 2, 4, and 5).

(1) 30 S + spermidine $\xrightarrow{k_1}$ 30 S-spermidine

(2) 50 S + spermidine $\xrightarrow{k_2}$ 50 S-spermidine

(3) 30 S-spermidine + 50 S-spermidine $\xrightarrow{k_3}$ 70 S-spermidine

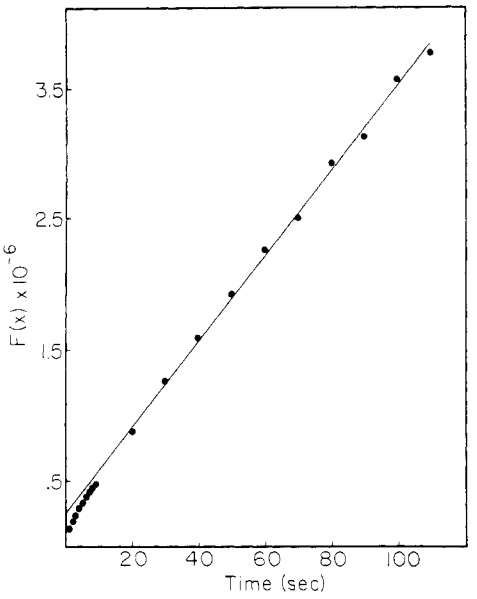

FIGURE 7: Association of 30S and 50S subunits towards an equilibrium state induced by shifting the concentration of $MgCl_2$ from 2 to 5 mM. The final ribosome concentration is 1 mg/ml; Temperature = 20 °C. The ordinate $F(x)$ represents the expression given in eq 6. For details, see text.

(4) 30 S-spermidine + 50 S $\xrightarrow{k_4}$ 70 S-spermidine

(5) 50 S-spermidine + 30 S $\xrightarrow{k_5}$ 70 S-spermidine

Model III did not appear to improve significantly the fit over Model I (best sum of squares Model I = 0.076, best sum of squares Model III = 0.0777). Model II gave a very poor fit to the data (sum of squares 0.369, normalized data, four curves, 20 points/curve).

All of these models neglect the possibility of heterogeneity of the ribosomes, but, since at high spermidine concentrations the curves are not biphasic, this probably is not a bad assumption. The models also assume an average spermidine binding rate constant and neglect any site-site interaction terms. The data did not appear to warrant consideration of more detailed mechanisms. Both Models I and III give a rate constant for subparticle association of 6.4×10^6 M^{-1} s^{-1} and a rate constant for spermidine binding to the subparticles of about 5.0×10^4 M^{-1} s^{-1}. It is important to note that, although steps 4 and 5 do not appear to be significant in accounting for our results, we do not wish to imply, in general, that association of 50S and 30S subunits requires prior spermidine binding to both subunits (step 3). The insignificance of steps 4 and 5 appears to arise, rather, from the fact that the binding of spermidine to the subunits rapidly depletes the concentration of spermidine-free subunits. We *can* conclude, however, that it is not necessary to have one subunit free from spermidine (Model II) for association.

Activation Energy. The dissociation and the association reactions were also studied as a function of temperature at constant pH. The rate constants of all processes increase slightly with increasing temperature; however, the activation energies are all less than 3.5 kcal/mol.

pH Dependence. The dissociation reaction at 3 mM $MgCl_2$ and the association reaction at 21 mM $MgCl_2$ were both studied as a function of pH between pH 7.2 and 8.4. No effect on the rate constants could be detected. This is consistent with the results of Ball et al. (1973) that the equilibrium between 30S–50S subunits and 70S ribosomes is not sensitive to changes in pH between 6 and 8.

TABLE V: Association of 30S and 50S Subunits Towards Equilibrium at 5 mM $MgCl_2$.[a]

Fraction	Rate Constant of Association (M^{-1} s^{-1})	Rate Constant of Dissociation (s^{-1})
("loose") 0.23 ± 6%[b]	$k_1 = 2.6 \times 10^5 \pm 8\%$	$k_2 = 0.2 \pm 17\%$
("tight") 0.77 ± 2%[b]	$k_3 = 3.9 \times 10^4 \pm 9\%$	$k_4 = 5.5 \times 10^{-3} \pm 10\%$
0.39 ± 8%[c]	$k_1 = 3.2 \times 10^5 \pm 12\%$	$k_2 = 6.53 \times 10^{-2} \pm 15\%$
0.61 ± 5%[c]	$k_3 = 5.9 \times 10^4 \pm 9\%$	$k_4 = 3.8 \times 10^{-3} \pm 9\%$

[a] The association reaction was induced by a shift of the $MgCl_2$ concentration from 2 to 5 mM. The final ribosome concentration is 1 mg/ml. Temperature = 20 °C. [b] See Model IIa. [c] See Model IIIa.

Approach to Equilibrium. The association of 30S and 50S subunits was also studied at a $MgCl_2$ concentration of 5 mM, which is close to physiological conditions (Lusk et al., 1968). At this $MgCl_2$ concentration and a ribosome concentration of 1 mg/ml, the reaction proceeds towards an equilibrium of roughly 50% free 30S and 50S subunits and 50% 70S ribosomes. For a reaction of the type

$$30\,S + 50\,S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} 70\,S$$

under conditions where the concentration of 30S subunits is equal to the concentration of 50S subunits and the reaction proceeds towards equilibrium, the rate constants for both association and dissociation can be determined. As discussed by Capellos and Bielski (1972, p 45, eq 14–18), the rate equation for such a process can be written as:

$$F(x) = \frac{x_e}{((30\,S)^2 - x_e^2)} \ln\left[\frac{x_e((30\,S)_0^2 - x_e x)}{(30\,S)_0^2 (x_e - x)}\right] = k_f t \quad (8)$$

where x and x_e denote the concentration of free 30S subunits at time t and at equilibrium, respectively. A plot of $F(x)$ vs. time should give a straight line, with the slope equal to the association rate constant. Figure 7 shows such a plot, which is heterogeneous. When the slow part of the reaction is analyzed, an apparent association rate constant of 3.25×10^4 M^{-1} s^{-1} is obtained. The apparent dissociation rate constant can be obtained from the equation

$$k_r = \frac{k_f((30\,S)_0 - x_e)^2}{x_e} \quad (9)$$

and is found to be 6.7×10^{-3} s^{-1}.

Models II and III used to fit the overall association experiments were modified by inclusion of the reverse reaction (and denoted as IIa and IIIa) and were used to fit the data for the approach to equilibrium studies.

MODEL IIA.

$$A_1 + B \underset{k_1}{\overset{k_2}{\rightleftharpoons}} C_1$$

$$A_2 + B \underset{k_3}{\overset{k_4}{\rightleftharpoons}} C_2$$

The rate constants computed according to Models IIa and

IIIa are listed in Table V. Comparing the fractions of ribosomes reacting with the faster and slower rates from part A of Table III with Model IIa of Table V shows that for Model II about 25% of the ribosome population is "fast reacting", and about 75% is "slow reacting" whether the association proceeds to completion or towards equilibrium. Model III also gives the same fraction of "fast-reacting" ribosomes (40%) and "slow reacting" (60%) in both experiments. A comparison of the rate constants of the association reaction at 21 and 5 mM $MgCl_2$ reveals that the association rate constant falls off by a factor of 25 with the decrease in the $MgCl_2$ concentration. The dissociation rate constants (Table II, k_2 and k_4) also are influenced by the $MgCl_2$ concentration. An increase of the Mg^{2+} concentration from 3 to 5 mM decreases the rate constant of dissociation by a factor of about 5 for the rapid (4.4) as well as for the slow part (5.3) of the reaction, according to Model IIa. The association reaction was also studied in three sets of experiments, where the final concentration of Mg^{2+} varied from 4.5 to 8 mM for each set and the curves for four different Mg^{2+} concentrations were fit by Model IIa using Runge-Kutta numerical integration. The association and dissociation rate constants for both phases varied with the concentration of Mg^{2+}. The association constants in this region varied with $(Mg^{2+})^n$, whereas the dissociation rate constants varied with $(Mg^{2+})^{-m}$. For the faster phase of the reaction, $n = 3.4 \pm 0.3$, $m = 2.3 \pm 0.6$; for the slower phase (type A or "tight ribosome"), $n = 2.7 \pm 0.4$, $m = 2.3 \pm 0.3$. Figure 8 depicts the association reaction towards equilibrium and illustrates the fit obtained with Model IIa. A fit with Model IIIa shows a residual distribution similar to that for IIa. Also indicated in Figure 8 is the computed progress of the reaction and the final equilibrium state using the rate constants of the association reaction at 21 mM and the dissociation reaction at 3 mM $MgCl_2$. This dotted curve shows clearly that the rate constants vary with (Mg^{2+}).

Sample Variation. The standard deviations for the rate constants listed in Tables I–IV are computed from the Fletcher-Powell program for a single experiment in which at least four to five runs were averaged. For the same preparation, the rate constants computed from different experiments of four to five runs each agree within these stated standard deviations. For reactions fitted by the Runge-Kutta procedure, the standard deviations of the rate constants for different experiments were calculated directly. Larger deviations in the rate constants were found for experiments in which different sample preparations were used. The standard deviation in the association as well as the dissociation rate constants amounted to ±25% of the given rate constant when averages were taken over all of the different preparations.

Discussion

The association and dissociation reactions have rate constants which show a magnesium dependence of approximately third order in the transition region near 5 mM Mg^{2+}. Since the individual reactions are nonaccelerating and, with respect to ribosome concentration, follow second or first-order kinetics (for the association and dissociation processes, respectively), a prior rapid equilibrium with Mg^{2+} is suggested:

$$30\,S + 50\,S \overset{3\,Mg^{2+}}{\rightleftharpoons} 30\,S\text{-}(Mg^{2+})_x + 50\,S\text{-}(Mg^{2+})_{3-x}$$

$$\underset{k_d}{\overset{k_a}{\rightleftharpoons}} 70\,S\text{-}(Mg^{2+})_3 \overset{3\,Mg^{2+}}{\rightleftharpoons} 70\,S\text{-}(Mg^{2+})_6$$

(Mg^{2+} stoichiometry approximate)

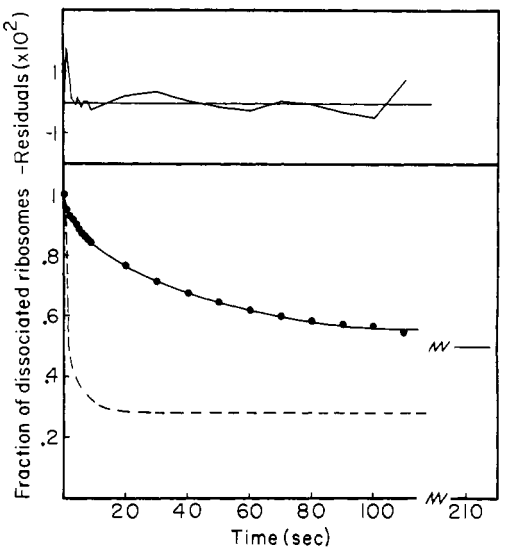

FIGURE 8: Association of 30S and 50S subunits towards equilibrium induced by a shift of the $MgCl_2$ concentration from 2 to 5 mM. The final ribosome concentration is 1 mg/ml; Temperature = 20 °C. The solid line represents the fit of the experimental data by Model IIa. The dashed line represents the progress curve computed according to Model IIa but using the rate constants of the association reaction at 21 mM $MgCl_2$ (6.55×10^6 and 1.075×10^6 M^{-1} s^{-1}) and the rate constants of the dissociation reaction at 3 mM $MgCl_2$ (0.90 and 3.0×10^{-2} s^{-1}) with the respective fractions 0.23 and 0.77.

where k_a and k_d are the rate constants deduced from kinetic light-scattering measurements.

Conformational changes have been reported to occur in both 30S and 50S subunits upon the association or dissociation reaction (Sherman and Simpson, 1969; Teraoka and Tanako, 1972; Ball et al., 1973; Chang, 1973). In the proposed scheme, no conformational transition has been considered, but the association process proceeds as a second-order reaction and, thus, a conformational change cannot be the rate-limiting step in the formation of 70S ribosomes. Although the reaction curves give no direct evidence of a sequential process, the curves, nevertheless, are heterogeneous.

At ribosome concentrations of 1–2 mg/ml (starting with a concentration of 6–12 mg/ml before mixing), the dissociation reaction can be described by three independent first-order processes, which implies that three different types of particles exist, each one dissociating with its own specific rate. The rate constants of the three reactions are 15, 0.9, and 3×10^{-2} s^{-1}. The three different types of ribosomes are not artifacts generated by shearing forces in the mixing device of the stopped-flow apparatus, since heterogeneity was also observed in preliminary dissociation experiments performed in the fluorescence spectrophotometer, where the ribosomes were mixed very gently with the appropriate buffer to cause dissociation.[4] Ribosomes (70S) which have been treated with puromycin to release peptidyl-tRNA also show three dissociation reactions, the rate constants of which agree with the dissociation reactions of untreated ribosomes. When the dissociation of 70S particles is induced by a high concentration of KCl, the process again reveals three first-order reactions, with rate constants and amplitudes in agreement with the dissociation induced by lowering the $MgCl_2$ concentration. Additional support for the existence of three different dissociation processes derives from the observation that the fraction of the fast reaction (rate constant of 15 s^{-1}) decreases with decreasing ribosome concentration. At a final ribosome concentration of 0.1 mg/ml, its contribution to the overall process is negligible (less than

4%). The origin of this fast dissociation step appears to arise from aggregated forms of ribosomes (in equilibrium with 70S forms), which are known to be present in concentrated ribosome solutions (Zitomer and Flaks, 1972) and have been observed to form even in more dilute solutions (Hocker et al., 1973). Only the two slower processes observed in the stopped-flow apparatus with rate constants of 0.9 and 3×10^{-2} s^{-1}, thus, will be attributed to the dissociation of 70S ribosomes into 30S and 50S subunits. The association reaction shows two phases in the stopped-flow apparatus[5] and detailed analysis of the curves as the ribosome concentration is varied shows that Model II (but not Model III) can fit the data (Tables III and IV). The experiments were performed at two ribosome concentrations (differing by a factor of 2) and, although single experiments can be fit with both models very well, only for model II are the computed rate constants of the fast process independent of concentration. Model II also gives the same fractions of "fast" and "slow reacting" ribosomes as are found in the dissociation studies. This suggests that the ribosome kinetic heterogeneity arises from structural difference in only one of the two subunits.[7] Recent nonkinetic work by others suggests that it is the 50S subunit that is altered (van Diggelen and Bosch, 1973; Noll et al., 1973b).

It is well known (van Diggelen and Bosch, 1973; van Diggelen et al., 1973) that ribosomes are heterogeneous with respect to association–dissociation equilibrium and Wishnia et al. (1975) have reported kinetic measurements on type A ("tight") ribosomes. Their association constant at 8 mM Mg^{2+} is about two to three times what we have consistently found for the most rapid phase for unfractionated ribosomes. Their dissociation rate constant is closest to that which we observe in the spectrofluorimeter for the final phase (half-time 5 min). Our approach-to-equilibrium studies, however, show that we must pair the rapid association and dissociation rates to fit the observed kinetics. In sequential flow experiments, stopped-flow association kinetics were identical, whether the ribosomes had been dissociated in the first flow experiment for 2 min or for 30 min, showing that a dissociation reaction with rate constant 0.003 s^{-1} at 3 mM Mg^{2+} cannot be paired with any of the stopped-flow association rate constants, but must be paired with the slowest association rate.[5]

Furthermore, our studies on isolated tight and loose ribosomes gave rate constants which agreed within experimental error with those obtained from analyses of the multiphasic kinetic data. Thus, the dissociation rate constant at 3 mM Mg^{2+} was 0.03 s^{-1} for tight ribosomes (some tenfold larger than that reported by Wishnia et al. (1975)), whereas the association rate constant we measured for tight ribosomes (1×10^6 M^{-1} s^{-1}) was less than 1/20 of that reported by Wishnia et al. (1975). The association reaction at 5 mM $MgCl_2$, a concentration close to physiological conditions (Lusk et al., 1968), proceeds towards an equilibrium and the rate constants for association and dissociation were determined. Models IIa and IIIa both gave a good fit to the experimental data, but model IIa (only one subunit heterogeneous) gave the same ratio of rapidly and slowly reacting material as found in the dissociation reaction. At 5 mM $MgCl_2$ according to Model IIa, the rate constants for association were 2.6×10^5 and 3.9×10^4 M^{-1} s^{-1}, which are about 25 times smaller than at 21 mM $MgCl_2$. The dissociation rate constants (5 mM $MgCl_2$) were 0.2 and 5.5×10^{-3} s^{-1}, which are about five times smaller than at 3 mM $MgCl_2$. The dissociation and association rate constants, therefore, show a strong dependence on the $MgCl_2$ concentration and, moreover, the fast and the slow phases for each process are influenced in the same way and by the same magnitude. Equilibrium constants calculated at 5 mM Mg^{2+} from the association and dissociation rate constants show that the major fraction of the ribosomes are of the "A" or "tight" form.

It is of interest to note that spermidine-induced association appears to give monophasic kinetics with an associate rate constant essentially equal to that of the fastest phase (6×10^6 M^{-1} s^{-1}) (B type) for the 21 mM Mg^{2+} experiments. The rate constants of about 10^6 M^{-1} s^{-1} for the association reaction of 30S and 50S subunits can be compared with the second-order rate constants for protein–protein associations. The formation of the actomyosin complex studied by Finlayson et al. (1969) shows a rate constant of 1.4×10^5 M^{-1} s^{-1}, the reaction of antibodies with heme proteins proceeds with a rate constant of 10^6 M^{-1} s^{-1} (Noble et al., 1969), and the rate constant of the dimer–tetramer aggregation of hemoglobin was found to be 4.3×10^5 M^{-1} s^{-1} (Kellett and Gutfreund, 1970). The only example reported with a rate constant larger than 10^6 M^{-1} s^{-1} is the interaction of peroxidase with cytochrome *c* (Chance, 1951) for which a rate constant of 1.2×10^8 M^{-1} s^{-1} was reported.

The low activation energies (<3–5 kcal/mol) we find may suggest that the association as well as the dissociation reactions are diffusion limited. To explain the existence of fast and slow processes, it may be further assumed that the different types of subunits and ribosomes differ in their net charge. Since nothing is known of the subunit area involved in binding, the net charge of the particles, and, more importantly, the charge distribution at the binding sites, further considerations are mere speculation at this time.

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[7] A more general model with heterogeneity for both subunits and permitting all four association reactions would include models II and III as limiting cases and with four adjustable rate constants would surely fit the data.

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