Analysis of the *Escherichia coli* Ribosome-Ribosomal Subunit Equilibrium Using Pressure-induced Dissociation*

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Hydrostatic pressure can be used to perturb the ribosome-ribosomal subunit equilibrium. We have used glutaraldehyde fixation and subsequent sucrose gradient analysis to determine the equilibrium concentrations of *Escherichia coli* 70 S, 50 S, and 30 S particles at pressures from 1 to 1400 atm. This method is shown to be sufficiently rapid and free of interfering ribosomal aggregation artifacts when performed at Mg"+ concentrations below 8 mM. We show directly that the *E. coli* ribosome is in equilibrium with its subunits and that the pressure-sensitive reaction is appropriately described by the expression: ln Kp = ln Ks + (PΔV/RT), where Kp and Ks are the equilibrium constants at pressure P and 1 atm, respectively, and ΔV is the change in molecular volume that occurs during the reaction. The method provides values for Ks under different conditions, and the effects of Mg"+ ion can be readily ascertained. Ks and ΔV were also estimated by a method of fitting computer-generated sucrose gradient profiles to experimental profiles. Determination of ΔH", ΔS", and ΔV" at 5 mM Mg"+ are presented. The results are discussed in the context of previous thermodynamic studies of the *E. coli* ribosome.

Since the initiation of protein synthesis is thought to require free ribosomal subunits (see, for example, Ochoa and Mazumder, 1974; Grunberg-Manago and Gros, 1977), the dissociation of the ribosome may be a critical point in the regulation of the synthesis of proteins. Of late, considerable attention has been given to both the kinetics (Wolfe et al., 1973; Wishnia et al., 1975; Görisch et al., 1976; Schulz et al., 1976a; Noll and Noll, 1976; Chaires et al., 1977; Wishnia and Boussett, 1977; Godefroy-Colburn et al., 1978; Chaires et al., 1979; Goss and Parkhurst, 1980) and thermodynamics (Zitomer and Flaks, 1972; Ball et al., 1973; Deby et al., 1975; Guernant et al., 1974; Schulz et al., 1976b; Noll and Noll, 1976; Wishnia et al., 1979; Infante and Baierlein, 1971) of ribosomal subunit association, in hopes of understanding this fundamental process in more detail. Such studies provide information toward an understanding of the exact molecular mechanism of association and the mechanism by which such factors as Mg"+ and the initiation factors (IF1, IF2, and IF3) influence the state of association. Further, they may provide information on the forces that hold the ribosomal subunits together.

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We have previously used temperature and pressure-jump relaxation kinetics to study the association of *E. coli* ribosomes (Chaires et al., 1977; Chaires et al., 1979), and have found that the association process is complex, with the mechanism:

\[
30S + 50S \xrightarrow{k_{12}} 70S \xrightarrow{k_{23}} 70S
\]

most consistent with our data. Both "tight" and "loose" couples appear to follow this mechanism, although the 70 S state is more favored in the case of loose couples. For both tight and loose couples, the forward rate constant, k_{12}, approaches the value expected for a diffusion controlled encounter.

To extend these studies, we use in the present report the pressure fixation method of Baierlein and Infante (1974) to study the *Escherichia coli* ribosome-ribosomal subunit equilibrium. The utility of the method is demonstrated, and the reversibility of the pressure-induced dissociation is established. From equilibrium data, values of ΔG", ΔH", ΔS", and ΔV" are determined for the overall association process. Matching of computer-generated sedimentation profiles with experimental sucrose density gradients provides an independent verification of the observed equilibrium values.

**MATERIALS AND METHODS**

**Solutions and Chemicals—** Buffers designated as TKM, D contain 10 mM Tris-HCl, 60 mM KCl, x mM Mg(OAc)₂, as indicated, and 1 mM diithothreitol. In some cases diithothreitol was omitted, and these buffers are identified as TKM. The pH of the solutions was adjusted to 7.75 at the temperature used in the experiment. In order to obtain reproducible ribosome preparations, we found it necessary to autoclave all glassware and solutions, probably to eliminate RNAse contamination. Sucrose solutions were made using autoclaved water.

The chemicals used were: sucrose (RNAse free) from Schwarz-Mann; glutaraldehyde (Union Carbide) was a 50% solution; lysisyme (Warlington Biochemical) was at 11,000 units/mg; deoxycholate (Na salt) was from Matheson (Norwood, OH); and bovine serum albumin was from Miles Research Products (Kankakee, IL).

**Preparations of Ribosomes—** *E. coli* of strain MRE600 were grown to approximately midlog phase in 4.5-liter batches at 37°C as described by Chaires et al. (1977). The cells were slowly cooled to about 12°C and kept for 15-30 min at this temperature. After harvesting at 0-4°C, the cellular pellets were quick-frozen and stored at -70°C for up to 4 months. Disruption of the cells followed the procedure of Keller and Davis (1971). The frozen pellet (from the equivalent of a 1-liter culture) was suspended in 7-7.5 ml of a buffer containing 10 mM Tris-HCl (pH 7.8 at 4°C), 10 mM Mg(OAc)₂, and 2 mM lysozyme at 1 mg/ml. This suspension was frozen in an acetone/dry ice bath and

1. "Tight" and "loose" ribosomes are operationally defined according to Noll and Noll (1976), and Van Diggelen and Bosch (1973). If ribosomes are sedimented in sucrose gradients containing 5 mM Mg²⁺, loose couples are totally dissociated and will sediment as subunits, while tight couples are largely associated.
2. The abbreviations used are: Ac, acetate; BSA, bovine serum albumin.
Association of E. coli Ribosomal Subunits

then thawed slowly. The freeze-thaw cycle was repeated and 0.21 ml of 10% deoxycholate was added. The lysate thus formed was kept on ice for 3 min and then centrifuged at 30,000 × g for 10 min. The supernatant (S300) was centrifuged at 30,000 × g and then immediately layered onto 35.5-m1 15–30% sucrose gradients in TKM12 or TKM14. Although not examined in detail, 70 S ribosomes prepared using 0.14 M KCl, 50 mM Tris-Cl at this stage of purification did not aggregate. The 70 S ribosome mixture was isolated from the gradients after a 4-h centrifugation at 26,000 rpm in a Spinco SW 27 rotor. The ribosome fractions from several gradients were pooled and the ribosomes were collected by pelleting at 49,000 rpm in a Spinco Ti 50 rotor for 2–4 h. Ribosome pellets were suspended in 1 ml TKM12 or TKM14. Unwanted particles were removed by centrifugation at 30,000 × g for 20 min. The supernatant was made 50% in glycerol in TKM14D and stored at −20°C for up to 4 weeks prior to use. This solution contained 600–900 AU units of ribosomes per ml and was used directly after dilution with the appropriate buffers.

**Fixation of Ribosome-Subunit Equilibrium Mixtures at Various Hydrostatic Pressures**—The method used was essentially as described in detail by Baierlein and Infante (1974) and has been used to analyze the effect of hydrostatic pressure on sea urchin ribosomes. Briefly, a 1-ml plastic disposable syringe is sealed shut at one end and is kept at a vertical position with the syringe head being the bottom of the tube thus formed is added in order: a small, sterile glass bead; then 0.1 ml of a solution containing 14% glutaraldehyde in 25% sucrose is added directly to the bottom, avoiding contaminating the upper portion of the tube. This glutaraldehyde solution was a dilution of a 25% glutaraldehyde solution made by adding 9 ml of 0.12 M Tris-HCl, 1 M Tri-HCl and 1 ml of 15% sucrose. All glutaraldehyde solutions were used immediately, because a precipitate forms upon standing (Subramanian, 1972). We found that such neutralization was essential for E. coli ribosomes in order to obtain resolvable ribosome-subunit sedimentation patterns. Such a precaution is not necessary with sea urchin ribosomes. Above the 0.1 ml of glutaraldehyde solution in the tube is layered 0.1 ml of 15% sucrose containing 2.1 mg/ml bovine serum albumin. Subramanian (1972) suggested the use of BSA as a “protector-protein” to reduce glutaraldehyde-induced ribosomal aggregation. Although BSA was used in our studies, we did not observe any differences between preparations with BSA and those without. The final addition to the tube is 0.5 ml of the ribosome sample, which is layered on top of the 15% sucrose layer. The buffer conditions are the same in these two layers and can be varied, but in most of the studies TKM13D was used. The tubes are then sealed by inserting the syringe plunger as described elsewhere (Baierlein and Infante, 1974). Inversion and shaking of the sealed syringe results in the mixing of the layers, which is facilitated by the glass bead, thus fixing the ribosomes and subunits. The final concentration of glutaraldehyde is 2% and that of BSA is 0.3 mg/ml. The entire 0.7-ml mixture is layered on top of a 20% sucrose cushion. The tubes are centrifuged at 49,000 rpm in a Spinco SW 27 rotor for 2 h at 4 °C. When the ribosomes are to be subjected to hydrostatic pressure prior to fixation, the sealed syringe is placed in a pressure cell which is full of water at the desired temperature; up to six tubes can be accommodated by the cell. Hydrostatic pressure is applied to the contents of the pressure cell using a hydraulic pump. After the desired pressure is achieved (which requires less than 10 s of manual pumping), the cell is sealed, disconnected from the pump, and completely submerged in a large water bath at the temperature of the experiment. Although the ribosome-subunit equilibrium adjusts rapidly to the new pressure (Chaires et al., 1977), we routinely kept the sample at the high pressure for 5 min. After this period of time, the contents of the sample tube (syringe) are mixed by repeated inversions of the pressure cell (still charged with hydrostatic pressure). Two minutes after fixation, the pressure is released, and the sample tube is removed from the cell and cooled at 0 °C prior to layering the fixed ribosome mixture on sucrose gradients.

The concentration of ribosomes used was always at 5 AU units/ml (0.12 μM) or less. At concentrations above 10 AU units/ml, we found significant formation of dimers and various other multimers, in agreement with Guernert et al. (1974).

**Computer Simulations of Zone Sedimentation Patterns**—Sedimentation patterns were simulated using the counterdiffusion distribution analogue developed by Bethune and Kegeles (1961), with a computer program adapted and apparent to the original program (Chaires, 1978; Chaires and Kegeles, 1977).

**Stopped Flow Experiments**—Stopped flow experiments were performed on a commercial Durham-Gibson apparatus. Ribosome solutions were prepared by diluting a concentrated stock solution (+1000 AU units/ml) into the desired buffer just prior to the experiment. The course of the reaction was followed by monitoring changes in 90° light scattering at a wavelength of 436 nm.

**RESULTS**

**Efficacy of Glutaraldehyde Fixation of Ribosome-subunit Equilibrium Mixtures**—The use of a pressure cell to examine the thermodynamic parameters of the ribosome-subunit equilibrium requires the fixation of the reacting components at a given pressure and subsequent analysis of the ribosome-subunit equilibrium mixture on sucrose gradients. We have found that glutaraldehyde is an effective agent in these analyses. For the fixation procedure we have used to be quantitatively valid, two criteria must be met. First, the fixation procedure must not, by itself, promote ribosome dissociation or subunit association. Second, the rate of fixation must be fast relative to the rate of dissociation, lest the ribosome-subunit equilibria be perturbed, following Le Chatelier’s principle, during the course of fixation. That the first criterion is met is not an easy matter to establish; we have used sucrose density gradient sedimentation to convince ourselves that the assumption is valid for our experiments. First, when unfixed E. coli ribosomes are sedimented in 5 mM Mg2+, a profile such as shown in Fig. 1B (dashed line) is observed. The mass seen sedimenting as 30 S and 50 S zones arises from two sources, loose couples (or unreactive subunits) and a contribution from 30 S material that dissociates as tight couples during sedimentation (Chaires and Kegeles, 1977). Since the association constant, K+, for tight couple subunit association is in the order of 10^7 M^-1, this latter contribution is expected to be slight. When ribosomes are fixed prior to sedimentation, the pattern seen in Fig. 1B (solid line) is observed. Note that the mass in the 30 S zone is roughly equal to that seen in the unfixed sample. The mass seen in the fixed sample now represents, again, loose (or unreactive) 30 S material and a contribution from the small amount of dissociated tight 30 S material present in the original equilibrium mixture. We argue that the near equality of the mass of the 30 S zone seen in these two profiles indicates that glutaraldehyde is not promoting any gross levels of dissociation. At these salt conditions, however, tight couples are largely associated, and we would have a difficult time detecting small perturbations caused by glutaraldehyde. We thus are content to argue that, at least for these salt conditions, no gross perturbations are evident, and that the first criterion mentioned above is met. More rigorous and quantitative studies on this point are in progress (Crossin et al., 1981).

Previous hand-mixing experiments (Baierlein and Infante, 1974) indicated that the glutaraldehyde fixation of sea urchin ribosomes was complete within 30 s. Fig. 2 shows a stopped flow experiment that refines this estimate and indicates that fixation is complete in less than a few seconds. Ribosomes were dissociated by decreasing the total Mg2+ concentration from 6 to 1.5 mM by the addition of EDTA, and the kinetics of the subsequent dissociation was determined by monitoring changes in 90° light scattering. As is seen in Fig. 2, the dissociation is slow, characterized by a t1/2 of 38 s, and, as judged from linear semilog plots of amplitude versus time, appears to be first order with a rate constant k = 0.018 s^-1.
Influence of Mg$^{2+}$ concentration and glutaraldehyde fixation on ribosome sedimentation behavior. Sucrose gradient profiles of 70 S ribosomes (A and B) or 30 S subunits (C) were obtained after centrifugation on 15-30% sucrose gradients at 25 °C in a Spinco SW 41 rotor as described under "Materials and Methods." In all cases, 0.5 ml of sample was layered on each gradient. In A, 70 S ribosomes isolated from sucrose gradients as described under "Materials and Methods" were fixed with glutaraldehyde (2%) in TKM buffer containing 10 mM Mg$^{2+}$. B and C, identical with A, except that fixation was in TKM buffer containing 5 mM Mg$^{2+}$. ---, same 70 S ribosomes sedimented on a parallel gradient without prior fixation. In C, 30 S subunits were isolated from low Mg$^{2+}$ (1 mM) gradients in which 70 S ribosomes were centrifuged at 40,000 rpm for 4 h. The 30 S fraction was used directly for resedimentation in sucrose gradients after dilution to reduce the sucrose concentration. Profiles of three gradients run in parallel are shown. ---, 30 S subunits adjusted to 10 mM Mg$^{2+}$ and fixed with glutaraldehyde (2%). ----, identical with solid line except that fixation of 30 S subunits was at 5 mM Mg$^{2+}$. ----, 30 S subunits at 5 mM Mg$^{2+}$ without glutaraldehyde fixation.

However, if glutaraldehyde is added simultaneously with the EDTA, there is no change in the 90° light scattering, as is seen in Fig. 2. Thus, the glutaraldehyde appears to react rapidly with the 70 S ribosome, preventing subsequent dissociation. The rate of glutaraldehyde fixation may be as fast as the mixing time of the stopped flow apparatus and is certainly completed in less than 1 s. We emphasize, however, that this rate will depend on the concentrations of both ribosomes and glutaraldehyde, and the rate observed here applies only to this particular set of conditions. In any case, the rate of fixation is clearly fast relative to the dissociation process, as required for our experiments. Parenthetically, the rate constant we obtain from these experiments, $k_2 = 0.018 \text{s}^{-1}$, is in good agreement with the values we obtained earlier using pressure-jump methods, $k_2 = 0.015 \text{s}^{-1}$ at 2.5 mM Mg$^{2+}$ (Chaires et al., 1977), and with the value reported by Wishnia et al. (1975) at 1.5 mM Mg$^{2+}$, $k_d = 0.03 \text{s}^{-1}$.

**Mg$^{2+}$ Concentration Effects on the Analysis of Ribosome-subunit Mixtures**—The influence of Mg$^{2+}$ on the ribosome-subunit equilibrium required that we consider the effects of this ion in detail. We have found that the range of permissible Mg$^{2+}$ concentrations for these studies is limited to below 10 mM. For example, we found that at 10 mM Mg$^{2+}$, the sedimentation of both the 70 S and 30 S species is greatly affected.

Fig. 1A shows that when 70 S ribosomes are isolated and then fixed with glutaraldehyde in the presence of 10 mM Mg$^{2+}$ and recentrifuged on sucrose gradients, about 10-20% of the 70 S material sediments much more rapidly, near 100 S. We can not say whether the glutaraldehyde is causing the formation of these aggregates or simply stabilizing a natural component in ribosome preparations at this Mg$^{2+}$ concentration. We believe that the latter is the case, because some aggregates are observed even in the absence of glutaraldehyde when the 70 S ribosomes are sedimented in 10 mM Mg$^{2+}$ (not shown). The sedimentation patterns of unfixed and glutaraldehyde-fixed 70 S ribosomes in 5 mM Mg$^{2+}$ are given in Fig. 1B. At 5 mM Mg$^{2+}$, no 100 S dimers are observed.

A similar problem is present for the 30 S subunit. When fixed with glutaraldehyde at 10 mM Mg$^{2+}$, almost one-third of a 30 S subunit preparation sediments as an aggregate to a position slightly less than that of the 50 S subunit. Aggregates of approximately 70 S are also formed. This is shown in Fig. 1C along with the sedimentation profiles of 30 S subunits which were either fixed with glutaraldehyde at 5 mM Mg$^{2+}$ or were sedimented in 5 mM Mg$^{2+}$ without prior fixation. The two latter conditions show no significant formation of multimers. The aggregate forms at 10 mM Mg$^{2+}$ are also present when glutaraldehyde is absent, so they appear to be due primarily to the presence of 10 mM Mg$^{2+}$. We find that at 8 mM Mg$^{2+}$ and lower there is no evidence of aggregation of either the 30 S nor the 70 S ribosomes, but at Mg$^{2+}$ concentrations higher than 10 mM, more extensive aggregation occurs. The sedimentation of the 50 S subunit appears to be much less affected by Mg$^{2+}$ concentrations up to 20 mM, where about 20% of the subunits form apparent dimers and trimers. All of these observations are in good general agreement with the results presented by Guermant et al. (1974). Of particular importance, they show that glutaraldehyde fixation does not result in aggregate formation in the experiments reported here where 5 mM Mg$^{2+}$ has been the primary concentration used.
Effect of Pressure on Ribosome Dissociation—Fig. 3 shows the sedimentation profiles of ribosomes that have been subjected to various hydrostatic pressures. In each case, the ribosomes have been fixed with glutaraldehyde at the pressures indicated. This series, which was done at 5 mM Mg\(^{2+}\) and 10 °C, shows that the 70 S species is the predominant form at pressures of 8000 p.s.i. and lower. There is a steady increase in the proportion of 30 S and 50 S subunits as pressure is increased. By integrating the subunit and 70 S areas in sedimentation profiles such as illustrated in Fig. 3, it is possible to determine the equilibrium constant at each pressure. From these determinations, the relationships between pressure and the equilibrium constant for the ribosome-subunit equilibrium under a variety of conditions may be determined. The following equation describes the relationship of pressure and \(K_p\) for a pressure-sensitive reaction in which the ribosome is in equilibrium with its subunits:

\[
\ln K_p = \ln K_0 + \left( \frac{P \Delta V}{RT} \right)
\]

Here, \(P\) is the pressure in our pressure cell in atmospheres, and \(K_p\) and \(K_0\) are the dissociation constants at pressure \(P\) and at zero (1 atm) pressure, respectively. \(\Delta V\) is the change in molecular volume during the reaction given in milliliters/mol, \(R\) is the gas constant, and \(T\) is the absolute temperature.

\[
\Delta V = (\text{volume } 70 \text{ S}) - (\text{volume } 30 \text{ S} + \text{volume } 50 \text{ S})
\]

It is important to point out that \(\Delta V\) does not necessarily denote an actual change in size of the subunits per se. Electrostatic or hydrophobic effects on the solvent molecule may also produce changes in the effective volume of the reactants. Such changes in the interactions between the reactants and the solvent have a pressure dependence that is also correctly described by Equation 2 (Baierlein and Infante, 1974).

Fig. 4 presents the relationship between pressure and the dissociation constant for the ribosome-subunit equilibrium at 0 °C and two concentrations of Mg\(^{2+}\). The ordinate intercepts, determined by extrapolation, give directly values of \(\log K_0\), the equilibrium constant at zero pressure (for the pressure ranges used, this is equal to the atmospheric constant, within experimental error). The data for one of the series presented (closed symbols) were obtained at a Mg\(^{2+}\) concentration of 2 mM. Since there is considerable dissociation of the ribosome under these conditions, it is possible to estimate the dissociation...
The equilibrium constant for ribosomes at various pressures was calculated as described in the legend to Fig. 4. The values at 10 °C; 20 °C; 30 °C, were obtained by a van't Hoff plots for log Kd at 12,000 p.s.i. (●) and 20,000 p.s.i. (●).

### Table I

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>ΔV (ml/mol)</th>
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<tr>
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<td>30</td>
<td>190</td>
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### Table II

<table>
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<th>Pressure (p.s.i.)</th>
<th>ΔG° at 0 °C (kcal/mol)</th>
<th>ΔH° (kcal/mol)</th>
<th>ΔS° (entropy units)</th>
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</tr>
<tr>
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<td>-9.9</td>
<td>-17.2</td>
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</tr>
<tr>
<td>20,000</td>
<td>-8.8</td>
<td>-24.9</td>
<td>-99.1</td>
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Table III
Position of leading zone as a function of $K_e$ and $\Delta V$ from computer-simulated patterns

The final peak positions of the leading ribosomal zone on sucrose gradients are given as the fractional distance, which equals distance from top of gradient/total length of gradient. Experimental values were determined using an SW 41 rotor at 40,000 rpm for 4 h in 15-30% sucrose gradients containing TKM Buffer at 5 or 2 mM Mg$^{2+}$. Under these conditions, the glutaraldehyde-fixed 70 S ribosome sediments to a fractional distance of 0.64. At 2 mM Mg$^{2+}$, the unfixed “70 S” position is 0.53–0.54. At 5 mM Mg$^{2+}$, the “70 S” position is 0.59–0.60. At 10 mM Mg$^{2+}$, the “70 S” position is 0.60–0.63. The underscored values indicate the fractional distances of the leading zone in the computer-generated pattern which coincide with the 5 mM Mg$^{2+}$ experimental values. The values in boxes are the values where the computer data coincide with the 2 mM Mg$^{2+}$ actual gradients.

<table>
<thead>
<tr>
<th>$\Delta V$ (m$^3$)</th>
<th>$K_e$ (m$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x $10^{-10}$</td>
<td>0.65</td>
</tr>
<tr>
<td>5 x $10^{-10}$</td>
<td>0.60</td>
</tr>
<tr>
<td>5 x $10^{-10}$</td>
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</tr>
<tr>
<td>2.5 x $10^{-10}$</td>
<td>0.61</td>
</tr>
<tr>
<td>1 x $10^{-10}$</td>
<td>0.59</td>
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of the pattern. Table III shows that values of $K_e = 0.25-0.5 \times 10^5$ m$^{-1}$ and $\Delta V = 108-142$ ml/mol provide the best fit to the experimental gradients run at 5 mM Mg$^{2+}$. This agrees reasonably well with our values of $K_e = 5 \times 10^5$ m$^{-1}$ and $\Delta V = 114$ ml/mol measured at 0 °C using the pressure-glutaraldehyde fixation procedure. Further, at 2 mM Mg$^{2+}$, an upper limit of $K_e = 1 \times 10^5$, and $\Delta V = 142-191$ ml/mol are obtained with the computer simulation approach (Table III), which are in fair agreement with our values of $K_e = 1.9 \times 10^5$ and $\Delta V = 204$ ml/mol (at 0 °C) obtained above. These results suggest that our values are correct at least in order of magnitude.

**DISCUSSION**

A comparison of the thermodynamic values obtained in this study with those reported in the literature for similar ionic conditions is made in Table IV. As is seen in Table IV, the equilibrium constants seem to fall into two classes, one class with values of $10^5-10^6$ m$^{-1}$, and the other with a value of $10^6$ m$^{-1}$. As shown by Debey et al. (1975), these classes correspond to what have been termed loose and tight couples, respectively. These terms are operationally defined: if ribosomes are sedimented in sucrose gradients containing 5 mM Mg$^{2+}$, loose couples will be totally dissociated and sediment as subunits, while tight couples will be largely associated. This behavior is readily explained by the equilibrium constants given in Table IV, as shown in a computer simulation study of the zone sedimentation of *E. coli* ribosomes (Chaires and Kegeles, 1977). Inspection of the equilibrium constants shows that our value of $2.0 \times 10^5$ m$^{-1}$ (25 °C) is within the range of values of 9.92–$25 \times 10^5$ m$^{-1}$ reported for the association of tight couples.

Few values $\Delta H^\prime$ are available for tight couple association. Noll and Noll (1976) report that $\Delta H$ is Mg$^{2+}$-dependent, increasing to a value of $-20$ kcal/mol at $2.6$ mM Mg$^{2+}$, which is the highest Mg$^{2+}$ concentration for which they report a value. Our measured enthalpy (~6.2 kcal/mol) is low in magnitude relative to this value and to the values of $-70$ to $-85$ kcal/mol for loose couples seen in Table IV. This enthalpy is, however, consistent with our experience from temperature-jump relaxation kinetics experiments on this system at 2.5 mM Mg$^{2+}$ (Chaires et al., 1977), in which low relaxation amplitudes were consistently observed, considerably lower than would be expected for $\Delta H$ of $-20$ kcal/mol. Further, sedimentation patterns of unfixed ribosomes at 5 mM Mg$^{2+}$ at 2.5 and 25 °C are essentially identical in both shape and in peak positions relative to fixed markers. This is consistent with our low enthalpy change and is contrary to what may be predicted if $\Delta H = -20$ kcal/mol. The lowering of $K_e$ in going from 2.5 to 25 °C for ribosomes having a $\Delta H$ of $-20$ kcal/mol leads to clear differences in the simulated profiles, namely a slowing of the leading zone and an increase in the mass of the trailing zone as may be assessed directly in computer-simulated patterns (see Table III; Chaires and Kegeles, 1977). Thus, our $\Delta H^\prime$ value is qualitatively consistent with these observations. Direct measurement of $\Delta H^\prime$ using alternate procedures, such as calorimetry, would be useful.

Disparate values for $\Delta S^\circ$ are also apparent in Table IV, apparently reflecting some difference between tight and loose couples. Our value of 21.4 entropy units is grossly different from enormous values of 120–196 entropy units reported by others, which most likely refer to loose couples. This may reflect real differences in the properties of the two types of particles. Taking the values of the entropy as a measure of available conformational states, the results suggest that loose couples are more "flexible" at a given position at least in accord with our previous kinetic study which indicated that loose couples undergo the 70 S–70 S transition more readily than do tight couples (Chaires et al., 1977).

Our value for $\Delta V$ at 5 mM Mg$^{2+}$ is quite small and represents a slight expansion of the system upon subunit association. Relative to the molar volume of a ribosome solution, the $\Delta V$ represents an expansion of only about 0.01%. Estimates for the volume of reaction, $\Delta V$, of subunit association are sparse, and our value agrees well (in order of magnitude) with the two values that have been reported. We note, however, that the value of $\Delta V$ for the dissociation of sea urchin ribosomes is about $240$ ml/mol (Baierlein and Infante, 1974), and that for *Artemia* is $200$ to $300$ ml/mol (Nieuwenhuyzen et al., 1980).

We now discuss the sources and magnitudes of error in our measurements, in particular to assess the significance of the apparent Mg$^{2+}$ dependence of $\Delta V$, and of the trends seen in Tables I and II. For three replicate determinations at 5 mM Mg$^{2+}$ and 25 °C, for example, we have observed a range of $K_e$ of 2–4 $\times 10^5$ m$^{-1}$ and in $\Delta V$ of 155–184 ml/mol, with an error of 10–20%. For four replicate determinations at 5 mM Mg$^{2+}$ and 0 °C, a range of 110–123 ml/mol was observed for $\Delta V$, with an error of ±5–10%. Given the complexity and lability of the ribosome system, we regard these errors as tolerable and the variations in $\Delta V$ with Mg$^{2+}$ and temperature real. Further, our values for $K_e$ and $\Delta V$ at 2 and 5 mM Mg$^{2+}$ may be used to simulate accurately the zonal sedimentation patterns of these ribosomes (Table III), giving us considerable confidence in their credibility. We are aware, however, that *E. coli* ribosomes may be rapidly inactivated, and have, in fact, proposed a simple method for the detection of inactive (or loose) subunits (Chaires and Kegeles, 1977). Such inactive material may be an insidious source of error in our measurements.

1 A. A. Infante, B. Demple, and J. B. Chaires, unpublished results.
identify two types of inactive material: (i) inactive subunits, incapable of associating at 5 mM Mg\textsuperscript{2+}; and (ii) undissociable 70 S ribosomes. inactive subunits, as judged by the presence of a clear 50 S zone in sucrose gradients containing 5 mM Mg\textsuperscript{2+} (Chaires and Kegeles, 1977), are present to a variable extent in our preparations. Some preparations have no detectable levels of this inactive material, while others show 5-15\%, even though, in all cases, we isolated our 70 S ribosomes from a sucrose density gradient. We estimate that the lowest amount of inactive subunits detectable by our method is 5\%. Undissociable 70 S particles are seen in some preparations, and are identified as material that will not dissociate at high (22,000 p.s.i.) pressure. We have observed a maximum contribution of 15\% from this material. Properly, when the equilibrium constant,

$$K_0 = \frac{[70 S]}{[30 S][50 S]}$$

is calculated, this inactive material should be subtracted from the equilibrium ribosome and subunit concentrations. The qualitative effects of this correction are as follows: inactive subunits would increase $K_0$, while undissociable 70 S particles will decrease $K_0$. If both types of inactive material are present, these effects on $K_0$ tend to cancel, although not exactly, since the subunit term is squared in the denominator. At low levels of dissociation, small amounts of inactive material can drastically increase $K_0$. For example, in Fig. 4, we determine, at 5 mM Mg\textsuperscript{2+}, 200 atm, and 0 °C, $K_0 = 2 \times 10^9$ M\textsuperscript{-1}. If only 3\% of the total ribosome concentration is inactive subunits, $K_0$ is increased to 8.7 to 10\,10 M\textsuperscript{-1}, which propagates to increase $\Delta V$ as well, to a value of 172 ml/mole. This would diminish the apparent Mg\textsuperscript{2+} dependence of $\Delta V$. However, in order to abolish the apparent differences in $\Delta V$ at 2 and 5.0 mM Mg\textsuperscript{2+} (208 and 114 ml/mole, respectively), we would have to accept the rather extreme view that virtually all of the subunits observed at 5 mM Mg\textsuperscript{2+} and 200 atm were, in fact, inactive, which is not consistent with our observations (compare Fig. 3a and 3b). We feel, then, that the data in Tables I and II, and the Mg\textsuperscript{2+} dependence of $\Delta V$, while perhaps not rigorously quantitative, signify valid trends. We note that Wishnia et al. (1979) reported an increase of $\Delta V$ with decreasing Mg\textsuperscript{2+}, although the authors cautioned against taking the trend too seriously.

With these reservations, we now consider the trends that appear in Tables I and II. First, from Table I, $\Delta V$ appears to increase with temperature. This means that the coefficient of thermal expansion, $\Delta a$, is positive:

$$\Delta a = \frac{1}{V} \left( \frac{\delta \Delta V}{\delta T} \right)_P$$

From Table II, both the entropy and enthalpy are seen to decrease with pressure. This is to be expected, since, from Maxwell’s relations,

$$\left( \frac{\delta \Delta V}{\delta T} \right)_P = - \left( \frac{\delta \Delta S}{\delta P} \right)_T$$

A further relationship may be derived:

$$\frac{\delta \Delta H}{\delta P} = V - \left( \frac{\delta \Delta V}{\delta T} \right)_P$$

While the precise molecular meaning of these trends for the case at hand is beyond our grasp, we regard these trends as an indication of the internal consistency of our data. From Table I, we calculate $(\delta \Delta V/\delta T)_P = 2.4$ ml mol\textsuperscript{-1} deg\textsuperscript{-1}. From Table II, after appropriate conversions to the same units, $(\delta \Delta S/\delta P)_T = -2.5$ ml mol\textsuperscript{-1} deg\textsuperscript{-1}, in excellent agreement with the requirements of Maxwell’s relation, as stated above. Since these values are derived from separate series of experiments, their close agreement lends considerable confidence to the consistency of our data and to the validity of our approach. Values of the same sign and order of magnitude have been reported for the pressure denaturation of chymotrypsinogen (1.32 ml deg/mol) (Hawley, 1971) and ribonuclease (0.25 ml deg/mol) (Brandts et al., 1970). Also, Zipp and Kauzmann (1973) report a value of 2.4 ml deg/mol for the pressure denaturation of metmyoglobin.

Finally, from our equilibrium constants at 2 and 5 mM Mg\textsuperscript{2+}, we estimate $(d \log (K)/d \log [\text{Mg}^{2+}]) = 5.3$, in good agreement with values of 5–10 that have been reported for tight couples (Deby et al., 1975; Noll and Noll, 1970). This quantity represents the change in number of magnesium ions “bound” when subunits associate, and suggests that ~5 ions are released upon 70 S ribosome formation. Whether these ions are released from specific sites or are simply released from non-specific, electrostatically bound magnesium is a matter of some debate, and has been discussed lucidly and in detail by Wishnia (Wishnia et al., 1975; Wishnia and Boussert, 1977).

Subunit association, at around room temperature is, according to our data, driven almost equally by enthalpic and entropic contributions. The signs and magnitudes of the enthalpy and entropy suggest that the association is ionic in nature, as opposed to hydrophobic. While thermodynamics, in general, provides a powerful tool for probing the molecular details of a particular reaction, its application to the present problem of defining the forces that hold the ribosomal subunits together is difficult. To interpret the thermodynamic parameters in terms of particular molecular events is at best hazardous, given the extreme complexity of the association process and of the ribosome structure. For example, since base-pairing between the 16 S and 23 S rRNA has been strongly implicated in the association of subunits (Chapman and Noller, 1977; Herr and Noller, 1979), it is tempting to use the value of 1–2 ml/mole (Chapman & Stutevant, 1969; No-guchi et al., 1971) for the volume of reaction for the formation of an A-U base pair to calculate the number of base pairs involved in association from our overall $\Delta V$. This would amount to about 50–100 base pairs, which is quite reasonable; but this term also includes other contributions, from, say, the exposure of hydrophobic residues resulting from conformational rearrangements (Acharya & Moore, 1973). Thus, the
information of interest lies buried in the overall thermodynamic constants, and may be extracted only by the careful elucidation of all the steps in the process and then assessing their relative contribution to the observed value.

The pressure fixation method used here should complement data obtained from recent light-scattering studies. The method does offer several advantages over light scattering. Apart from its simplicity and relatively inexpensive instrumentation, the method enables one to visualize precisely the reactants and products involved in the equilibria and to visualize the presence of unsuspected species. For example, in the case of ribosomes, small amounts of 100 S particles may easily go undetected in a typical light-scattering experiment unless particular care is taken; however, the observed weight-average molecular weight is strongly influenced by the presence of such higher aggregates, leading to an overestimate of the apparent equilibrium constant. Such aggregates are, however, readily identified during the zone sedimentation step of our method and may be readily taken into account. The pressure method used here may be easily adapted to study the interaction between ribosomes and various ligands.

Finally, knowledge of \( K \) and \( \Delta V \) enables us to simulate accurately the zonal sedimentation of ribosomes, assisting in the unambiguous interpretation of often complex patterns (Chaires and Kegeles, 1977). This has already proven useful in the interpretation of chloroplast ribosome sedimentation patterns (Margulies and Tiffany, 1979).

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