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Pressure-Induced Dissociation of Sedimenting Ribosomes: Effect on Sedimentation Patterns

(ultracentrifuge/sea urchin/*Strongylocentrotus*/ribosomal conformation)

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ABSTRACT **Anomalous sedimentation patterns arise when free ribosomes from sea urchin eggs are centrifuged at high speeds. Pressure-induced dissociation of the ribosomes during sedimentation can explain the peculiar behavior; the assumption of such dissociation also yields estimates of the equilibrium constant (as a function of KCl concentration) and the change in molecular volume (500 ± 100 ml/mol) in the reaction: subunits ⇄ ribosome. Such dissociation during centrifugation may explain many experiments in which apparent reduced sedimentation coefficients for ribosomes, and increased coefficients for the subunits, have been ascribed to conformational changes.**

Anomalies in the sedimentation characteristics of ribosomes and subunits under various ionic conditions have been reported in many studies. These results have been generally interpreted in terms of conformational changes in the ribosomes and ribosomal subunits (1–7). However, a recent study (8) showed that the sedimentation pattern of free ribosomes from sea urchins is strongly dependent not only on the ionic conditions but also on the gravitational field and the concentration of ribosomes. When these factors are appropriately varied, the ribosomes display an apparently reduced sedimentation velocity or dissociate into subunits of apparently increased velocities. The results were interpreted in terms of (*a*) the dissociation of ribosomes at a rather distinct point during ultracentrifugation, and (*b*) the notion that the dissociation is due to a shift in the equilibrium between subunits and ribosomes in the sucrose gradient.

The cause of this dissociation is suggested by the results of studies of protein dissociation, which have shown that the high pressure experienced by particles in the analytical ultracentrifuge can induce dissociation of multimers into subunits if the molecular volume decreases upon dissociation (for a general treatment of this subject, see refs. 9 and 10). Some of these ideas were formulated by Josephs and Harrington (11, 12) and were used to explain the anomalous sedimentation of myosin. In this report, we examine the effect of hydrostatic pressure on the dissociation of ribosomes during zonal centrifugation in sucrose gradients. We conclude that pressure does induce the 75S free ribosomes to dissociate into their 56S and 35S subunits, and that this behavior can explain various peculiar sedimentation patterns of both eukaryotic and prokaryotic ribosomes that had previously been ascribed to conformational changes.

MATERIALS AND METHODS

Unfertilized eggs of the sea urchin, *Strongylocentrotus purpuratus*, were used in these studies, since the bulk (>95%) of the ribosomes from this source are not complexed with mRNA and peptidyl-tRNA (8). The procedures used to obtain cell-free homogenates are described in detail elsewhere (13, 8). The eggs were homogenized in TM buffer containing 0.25 M sucrose and the indicated concentrations of KCl. (TM buffer contains 5 mM $MgCl_2$–50 mM triethanolamine, pH 7.8.) A 15,000 × *g* supernatant (S15) was prepared and analyzed on 15–30% linear sucrose gradients at 5°C in the Spinco SW 41 rotor. Although the results with other rotors are similar to those reported here, the longer length of the SW 41 tubes allows for greater separation of the subunits. The positions of ribosomal subunits as markers under the various conditions of centrifugation were determined with isolated subunits prepared from primary gradients in 0.5 M

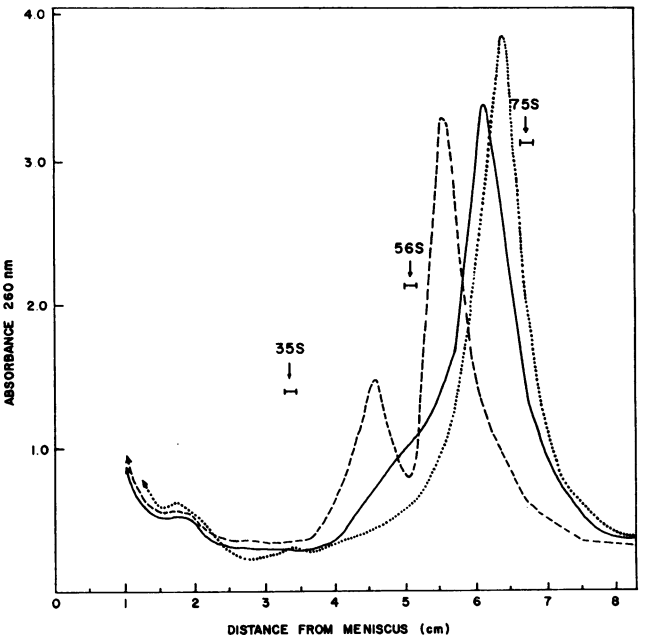

FIG. 1. How rotor speed changes the sedimentation patterns of sea urchin ribosomes. S15 extract was layered onto a 15–30% sucrose gradient in TM buffer containing 0.24 M KCl. Centrifugation in the SW 41 rotor was at: (····) 24,000 rpm for 14 hr; (——) 30,000 rpm for 9.25 hr; (– – –) 41,000 rpm for 5 hr. The ranges of the positions attained in parallel gradients by the isolated small (35 S) and large (56 S) subunits, and by 75S ribosomes are indicated. The ribosome range was determined in 0.05 M KCl, where no dissociation is perceptible. The *abscissa* gives distance from the meniscus, which itself is 7 cm from the rotor axis.

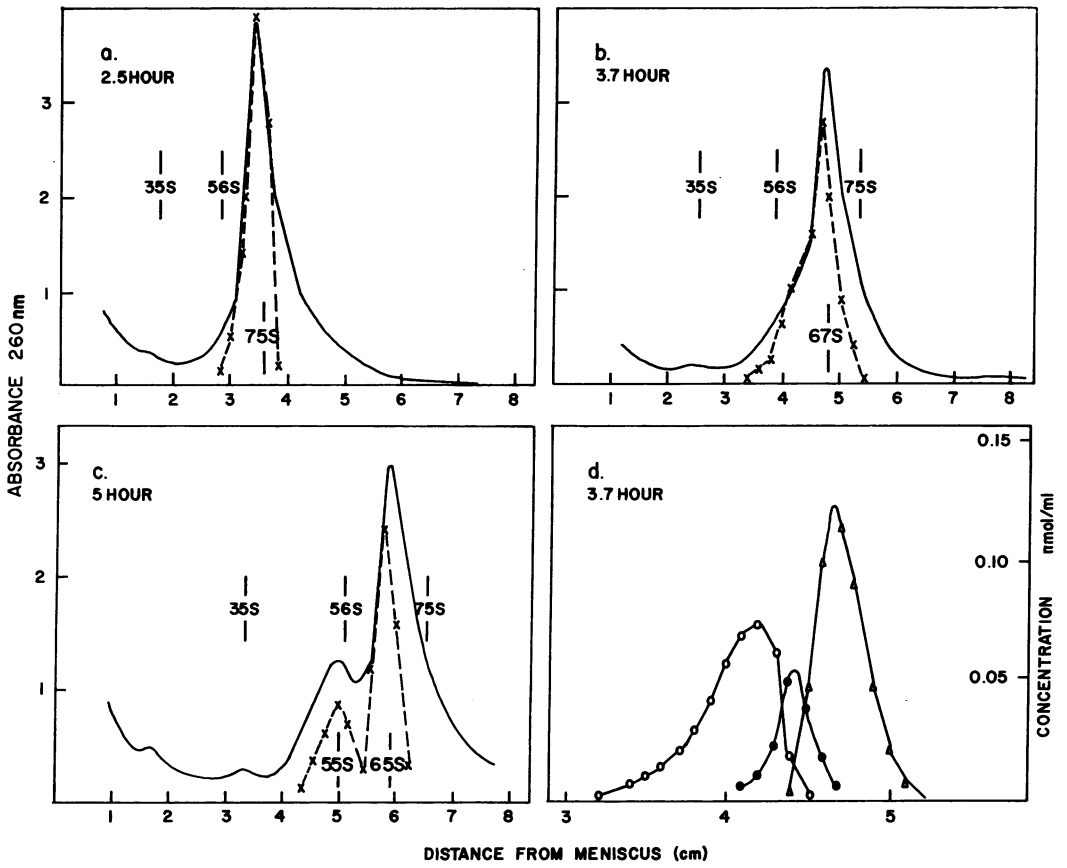

FIG. 2. Effect of length of centrifugation on sedimentation pattern of ribosomes. An S15 extract was layered onto 15–30% sucrose gradients in TM buffer containing 0.20 M KCl and centrifuged at 41,000 rpm for the indicated times. In *a–c*, the continuous curves are the experimentally determined sedimentation patterns; the computer-generated patterns are described by the values of (x). The computer patterns were calculated with the parameters ΔV = 500 ml/mol and $K(x_0) = 10^{-12}$, determined by prior fit to an array of 15 patterns as described in the text. The initial concentration of ribosomes equaled 0.6 mg/ml. In *d* the computer-generated pattern for the 3.7-hr centrifugation shows the composition in nmol/ml of the total absorbance profile given in *b*: 35S subunit, (○); 56S subunit, (△); and the 75S ribosome (●); the ribosomes would be formed from the overlapping subunit peaks only after the pressure was reduced (see ext).

KCl. The positions of ribosomal markers (75 S) were determined in gradients containing a very low concentration of KCl (0.05 M), where no dissociation is perceptible (8). The sedimentation patterns were determined by passing the gradients through a flow cell and continuously monitoring the absorbance at 260 nm (13).

RESULTS

Dependence of sedimentation patterns on rotor speed and on duration of run

The sedimentation patterns in Figs. 1 and 2 set the problem strikingly. In Fig. 1, ribosomes in the same homogenate (S15) were centrifuged in separate sucrose gradients (containing 0.24 M KCl) at different rotor speeds. As the rotor speed was increased, the time of centrifugation was decreased such that the product (rotor speed)^2 × (elapsed time) remained constant, at least to a good approximation. Because sedimentation velocity is proportional to (rotor speed)^2, one would, perhaps naively, expect to find the same pattern in the same location down the tube at all three speeds tested. Yet, at 24,000 rpm there is only a single peak, with a sharp trailing edge; at 30,000 rpm, there is a single, slower peak with a shoulder on the trailing edge; and at 41,000 rpm, there are two distinct peaks. The latter peaks, although at 48 S and 61 S, are in fact the small (35S) and large (56S) subunits, as shown by their RNA size, reassociation, and *in vitro* activity with poly(U) when reassociated (8). Therefore, as the rotor speed increases—and, with speed, the hydrostatic pressure—there is a clear shift toward dissociation.

Fig. 2 illustrates how the time of centrifugation affects the sedimentation patterns of sea urchin ribosomes. In these experiments rotor speed and ionic conditions were kept constant. At the end of a short centrifugation (2.5 hr, Fig. 2*a*) the ribosomal material has sedimented as though it was composed of purely 75S particles. Longer centrifugation (3.7 hr, Fig. 2*b*) broadens the ribosomal zone and produces an apparent reduction in the sedimentation coefficient, to about 67 S. After 5 hr (Fig. 2*c*) two well-separated peaks, corresponding to sedimentation coefficients of 55 S and 65 S, appear.

Pressure-induced dissociation provides an explanation for these results (as will be shown more conclusively in the next section). When the ribosomal material starts to sediment it is predominantly in the form of monoribosomes moving at about 75 S. As the material reaches higher pressures, the equilibrium (ribosomes $\rightleftarrows$ subunits) shifts continuously toward further dissociation. The subunits move more slowly than the ribosome, and so, as they become a larger fraction

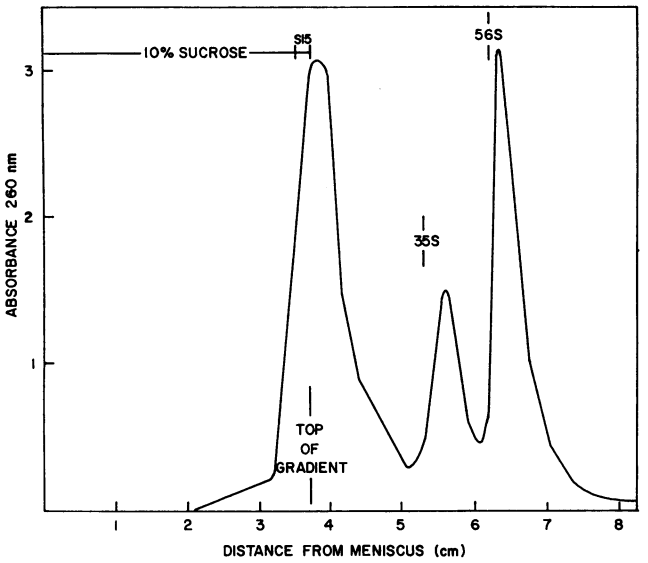

FIG. 3. Dissociation is related to position in centrifuge tube. 0.4 ml of an S15 extract was placed on a 6.7-ml, linear 15–30% sucrose gradient (0.20 M KCl), and then 5 ml of 10% sucrose was layered above the sample. Centrifugation was for 2 hr at 41,000 rpm. The reference points (35 S and 56 S) are the positions reached by separated subunits run in parallel in similar abbreviated gradients. The absorbance at the top of the gradient is nonribosomal material in the extract.

of the total material, the absorbance peak moves more slowly than 75S. *This gives the appearance of a reduction in the sedimentation coefficient of ribosomes.* Ultimately, the difference between the sedimentation velocities of the two subunits makes itself felt, and two separated peaks emerge from one broad, slow peak. This process yields subunits with apparently *increased* rates of sedimentation.

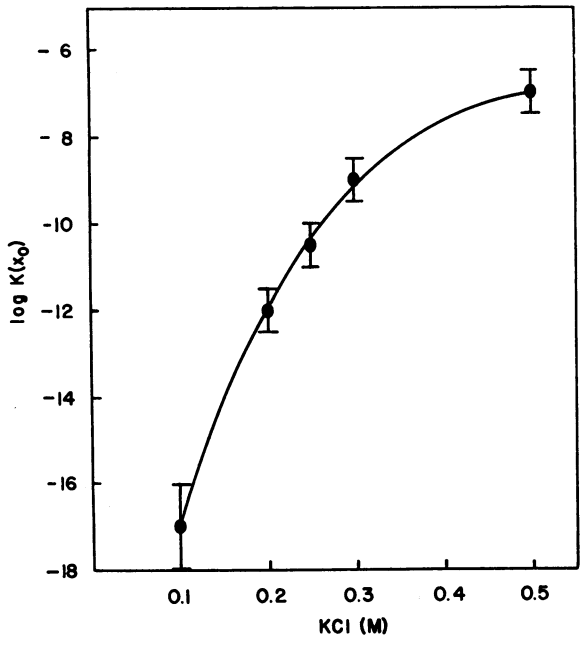

FIG. 4. Values for log $K(x_0)$ as a function of KCl concentration, together with estimated uncertainties, given that ΔV = 500 ml/mol. Rigorously, $K(x_0)$ is the equilibrium constant at zero pressure; the value at 1 atm is about 2% larger. The units of $K(x_0)$ are in mol/liter.

Theoretical sedimentation patterns based on this model are also given in Fig. 2. The bases for these calculated patterns are provided below. First, we present direct experimental evidence that pressure is inducing dissociation.

Importance of position in the centrifuge tube

If pressure is the crucial parameter, then starting at high pressure should produce early dissociation. To test this, ribosomes (in an S15 extract) were layered onto an abbreviated sucrose gradient; then the tube was filled to the top with sucrose of lower density, such that the ribosomes began sedimenting at a position (and pressure) that they would normally reach after some 2.8 hr of centrifugation at 41,000 rpm. After only 2.0 hr of centrifugation, there were two distinct peaks, which had sedimented only a little farther than had isolated subunits run in parallel (Fig. 3). In contrast, as shown in Fig. 2*a*, when the ribosomes are layered onto the top of the usual gradient under the same ionic conditions there is little or no dissociation after 2.5 hr. It is evident that the dissociation depends on the position of the ribosomes in the hydrostatic gradient rather than on the sucrose concentration, separation from smaller molecules in the sample, or the time of exposure to a given salt concentration.

It seems appropriate to develop more fully the mechanism of pressure-induced dissociation and some of its implications.

Dependence of equilibrium constant on pressure

Two basic requirements for pressure-induced dissociation are (*a*) that there exists a dynamic equilibrium between single ribosomes and subunits:

$$\text{ribosomes} \rightleftarrows \text{small subunit } (SS) + \text{large subunit } (LS),$$

and (*b*) that the molecular volume of the ribosome exceeds the sum of the volumes of the two subunits:

$$\Delta V \equiv V_{\text{ribosome}} - (V_{LS} + V_{SS}) > 0. \qquad [1]$$

If the volume change ΔV is indeed positive, the subunits have to elbow aside solution in order to reassociate. This requires energy, in an amount equal to the work $P(x)\Delta V$ done, where $P(x)$ is the hydrostatic pressure at the position x in the centrifuge tube. Thus, an increase in pressure will increase the energy required for reassociation, and so will push the ribosome–subunits equilibrium in the direction of further dissociation. The energy here is in some ways analogous to an extra term in the energy of chemical binding, and so one may expect the law of mass action for the reacting system to have the form:

$$\frac{[SS]\,[LS]}{[\text{ribosome}]} = K(x_0)e^{P(x)\Delta V/RT}. \qquad [2]$$

Here $K(x_0)$ is the equilibrium constant at the meniscus, where the pressure is zero (because the rotor spins in a vacuum), and RT is the product of the gas constant and the absolute temperature. The entire expression on the right is the equilibrium constant at position x, denoted subsequently by $K(x)$. Provided ΔV is itself unaffected by pressure and, hence, has the same value for all locations within the tube, the equation is indeed correct, as earlier, rigorous derivations by Josephs and Harrington (12) and by TenEyck and Kauzmann (14) demonstrated. The implication is that the equilibrium constant depends, through $P(x)$, on both rotor speed and position down the tube. The pressure is proportional to

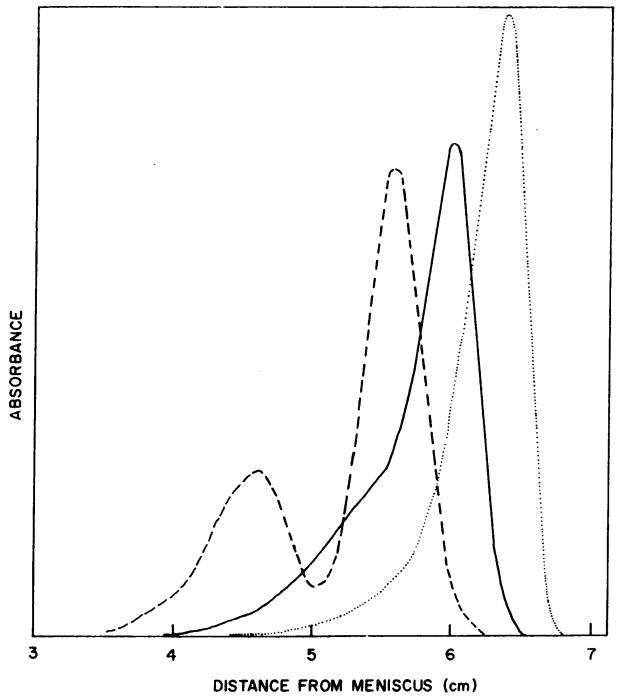

FIG. 5. Computer-generated sedimentation patterns for 0.24 M KCl. ΔV = 500 ml/mol; $K(x_0) = 10^{-10.5}$ mol/liter. The initial concentration of ribosomal material is 0.6 mg/ml. (····) 24,000 rpm for 14.6 hr; (——) 30,000 rpm for 9.35 hr; (– – –) 41,000 rpm for 5 hr. Absorbance is on an arbitrary scale.

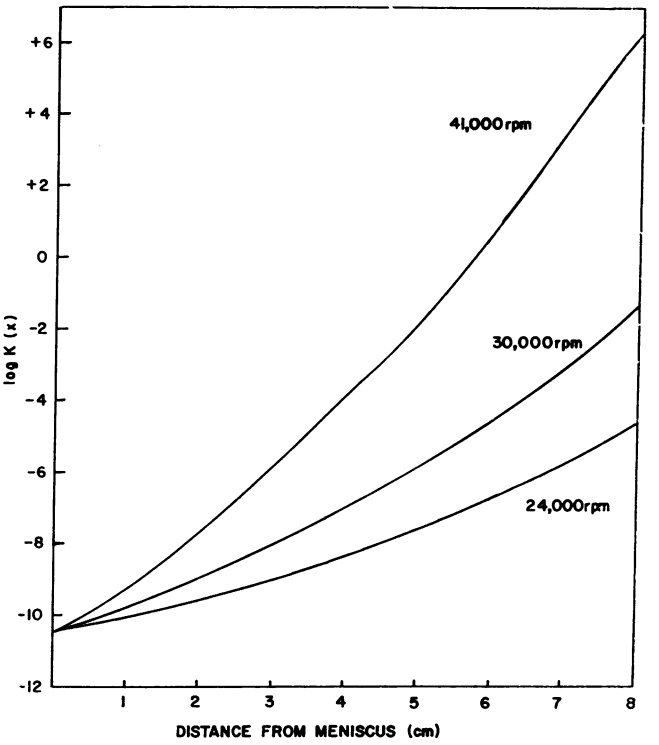

FIG. 6. How the equilibrium constant varies with position and rotor speed. The values are those used to simulate 0.24 KCl. The curvature arises because both centrifugal field and sucrose density increase with distance.

(rotor speed)^2 and increases in a roughly linear manner with distance (within a typical centrifuge tube): thus, the equilibrium constant itself grows at an extremely rapid rate with both rotor speed and position (11).

To construct a simple theory that will reproduce the essential features of the sedimentation patterns, one needs only the following elements.

a. The equilibrium constant at the meniscus, $K(x_0)$, is assumed to be a function of salt concentration. (In these studies we varied the KCl concentration but kept the Mg concentration at 5 mM.)

b. The variation of the equilibrium constant $K(x)$ is given by Eq. [2], where ΔV is taken to be independent of both pressure and salt concentration.

c. As the particles sediment, reversible reactions change the local concentrations so that equilibrium at the local value of $K(x)$ is achieved.

d. Diffusion is negligible.

These elements are readily incorporated into a computer program. The computer lets each species drift at its own sedimentation rate for a short time (such that the particles drift at most a few millimeters), then assesses local concentrations and reequilibrates at the local value of the equilibrium constant; it then reiterates. To calculate absorbance at 260 nm, we weight the concentrations of ribosome, large subunit, and small subunit by their absorbance per mole, taken to be in the ratio 3:2:1, that is, in the ratio of their approximate molecular weights.

To compute the local values of the sedimentation velocities, we start with the published sedimentation coefficients and particle densities (13), together with the local density and viscosity in the sucrose gradient (at 1 atm pressure, and thus without correction for increases due to high pressure). Next, we test the velocities so calculated by comparing computed positions with experimental ones: for each subunit, we use the position of the isolated subunit when run for 5 hr at 41,000 rpm; for the ribosome, we use the position of the single peak after 14.6 hr at 24,000 rpm in 0.1 M KCl, where the effects of pressure and salt are minimal. The computed positions agree with these experimental ones to within 4%. Finally, to improve the calculated local rates, we apply a separate, fixed factor to the initially-calculated speed for each species so that the positional agreement will be exact for the runs just described.

Computed sedimentation patterns

Experimental data at five different salt concentrations (0.1, 0.2, 0.24, 0.3, and 0.5 M KCl) and three rotor speeds (24,000, 30,000, and 41,000 rpm) provide an array of 15 sedimentation patterns. To fit these patterns with the theory, we have six adjustable parameters: a value of $K(x_0)$ for each salt concentration and the single value of ΔV. A good fit is provided by ΔV = 500 ml/mol, together with the log $K(x_0)$ values displayed in Fig. 4. Computer runs at 300 and 800 gave distinctly poorer fits; one is hard put to choose between 500 and 600, and thus we estimate an uncertainty of the order of $\pm$100 ml/mol for ΔV.

Computer-generated patterns for 0.24 M KCl are displayed in Fig. 5. The time of centrifugation was adjusted to hold constant the product (rotor speed)^2 $\times$ (elapsed time), as in Fig. 1. An increase in the rotor speed, and hence the pressure, changes the pattern dramatically. The good agreement between this figure and the experimental data presented in

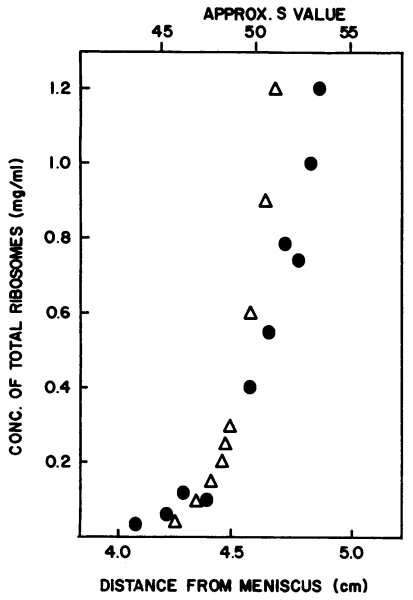

FIG. 7. Concentration dependence of apparent sedimentation velocities. The values indicate the position (or S value) of the 35S subunit derived from ribosomes during sedimentation in 0.24 M KCl. Both the experimental (●) and the computer (△) runs were for 5 hr at 41,000 rpm; the theoretical parameters are the same as in Fig. 5. The large subunit behaves similarly.

Fig. 1 supports the thesis that *pressure-induced dissociation can explain the patterns.* It also provides a simple method for determining both the equilibrium constant of ribosome dissociation and the change in molecular volume.

To illustrate how the equilibrium constant grows with increasing pressure, Fig. 6 presents the values of log $K(x)$ that we used to simulate sedimentation patterns in 0.24 M KCl. Though $P(x)$ may seem insignificant as it appears in Eq. [2], its growth with both rotor speed and position has an extreme effect on $K(x)$. Indeed, we were forced to plot the logarithm because $K(x)$ itself grows too rapidly to permit a meaningful graph.

The relationship between the sedimentation characteristics of ribosomes and the duration of centrifugation shown in Fig. 2 was examined on the bases of the model of pressure-induced dissociation. The patterns generated by the computer, also displayed in Fig. 2, agree well with the data, and thus demonstrate that a *continuous* shift toward dissociation can have unforeseen *cumulative* effects. The computations indicate that the slow peak in Fig. 2*b*—the "67S ribosome"—is a composite: the leading edge contains only the large subunits; the trailing edge, only the small subunit; and ribosome is present only in the central region, where there is overlap. This calculated distribution, shown in Fig. 2*d*, holds after the rotor has stopped and reequilibration at atmospheric pressure has taken place; during centrifugation there will be very much less ribosome. To test this predicted distribution, material from the leading half of the peak was isolated and examined: its density (1.60 g/ml) and RNA size (28 S) were those characteristic of the large subunit (13). Virtually no small subunit was found.

Influence of ribosomal concentration: Evidence for an equilibrium

The initial concentration of ribosomes influences their dissociation during centrifugation (8). Fig. 7 shows that, with constant time of sedimentation, the apparent S-value of the small subunit decreases (i.e., nearly total dissociation occurs earlier in the run) with decreasing ribosome concentration. Such behavior follows directly from the law of mass action as presented in Eq. [2]. Quite directly, lowering the concentration reduces the probability that two subunits will meet and so pushes the equilibrium in the direction of further dissociation. Computed values are also shown in Fig. 7 and agree rather well with the experimental values. These experiments support the assumption that dissociation is at least partially reversible.

DISCUSSION

Extensive evidence is presented elsewhere that describes the anomalous dissociation of sea urchin ribosomes into active subunits *during* sedimentation in sucrose gradients (8). The present report provides a mechanism for this dissociation; namely, the action of hydrostatic pressure imposed on the sedimenting ribosome. Some remarks about the elements of the theory are in order. The size of ΔV may well be affected by both pressure and salt concentration. At this stage, however, one does not have good reason to select any single, specific mode of variation with pressure or salt concentration, and so it seems best to see how far one can go with a fixed value of ΔV. Also, a positive ΔV need not mean a literal increase in molecular volume, nor does it necessarily signify a conformational change. For example, if charged groups partially neutralize one another when the subunits associate, the reduced electrostriction of the aqueous surroundings can produce a volume change whose influence would be correctly described by Eq. [2].

The small diffusion constants of the ribosomal particles and the high sucrose viscosity indicate that diffusion should be negligible, of the order of 1 mm in 15 hr. The very sharp rise of the trailing edge at low salt, low rotor speed (Fig. 1) supports this conclusion experimentally. The broad leading edge present at medium and high initial ribosomal concentrations seems to be the nondiffusive, still-debated phenomenon discussed by Brakke (15) and Schumaker (16).

The computer-generated patterns shown here are based on the theory that pressure induces a shift in a chemical equilibrium (Eq. [2]). The good agreement between the theoretical patterns, based on this model, and the experimental data provides a compelling argument for the existence of a dynamic equilibrium between ribosomes and subunits. This is supported by results of Falvey and Staehelin that demonstrate a slow exchange *in vitro* of free subunits with "runoff" 80S mammalian ribosomes (17). Moreover, subunit exchange of bacterial ribosomes occurred when density-labeled and unlabeled polysomes were allowed to complete protein synthesis separately, and the resulting free 70S ribosomes were then mixed (A. R. Subramanian and B. D. Davis, personal communication). A rapid equilibration could also explain the subunit exchange observed by Kaempfer (18) when density-labeled and unlabeled polysomes "ran-off" together. However, this mechanism cannot account for the difference in dissociation of freshly "run off" ribosomes and those isolated from sucrose gradients (18).

The importance of an equilibrium has not been seriously considered heretofore and its presence would influence details of current models for the role of single ribosomes in protein synthesis (18, 19). Complexed ribosomes are very likely not

in reversible equilibrium with subunits, since monosomes derived from polysomes by brief digestion with RNase and the polysomes themselves are not dissociated by either high salt or high pressure (20, 21).

Our results highlight the danger inherent in assigning sedimentation coefficients to ribosomal particles without taking into account possible effects of pressure on the equilibrium between ribosomes and subunits. Some otherwise enigmatic observations with ribosomes (e.g., refs. 21 and 22) may well be explained by pressure-induced dissociation during centrifugation. Suggestions of conformational alterations in both bacterial and eukaryotic ribosomes (2–7, 23), advanced to explain apparently reduced sedimentation coefficients, must be reevaluated in the light of our present results. Finally, pressure-induced dissociation also provides a probe that should prove useful for obtaining thermodynamic information about the equilibrium between ribosomes and subunits.

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