STUDIES OF RIBOSOMAL DIFFUSION COEFFICIENTS USING LASER LIGHT-SCATTERING SPECTROSCOPY

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ABSTRACT Using an optical beating technique, the diffusion coefficients and relative scattered intensity of *Escherichia coli* 70S, 50S, and 30S ribosomes are measured as a function of temperature and Mg^{2+} concentration. For solutions at 10 mM Mg^{2+} and between 0°C and about 40°C, the values of $D_{20,w}$ obtained are 1.7, 1.9, and $\approx 2.1 \times 10^{-7}$ cm^2/s, respectively. Preparative procedures drastically affect these values and equivalent hydrodynamic ellipsoids of revolution models give large axial ratios indicating extensive hydration or a deviation from the assumed shape. Calculations also indicate that the subunits expand upon dissociation. Measurements of $D_{20,w}$ vs. temperature indicate that 70S particles undergo a conformational change prior to dissociation and can be heat dissociated at 30–32°C at low concentrations. Treatment of 70S ribosomes with EDTA causes a biphasic dissociation reaction. Addition of Mg^{2+} after dissociation with EDTA shows that longer waiting times yield fewer 70S particles and that even short waiting times may yield ribosomes differing from the native conformation. Addition of *p*-chloromercuribenzoic acid (PCMB) is shown to dissociate 70S particles, but to a lesser extent than ethylenediaminetetraacetic acid (EDTA).

INTRODUCTION

Ribosomes are subcellular particles that are intimately involved in protein synthesis. Ever since the initial characterization of their physical structure (Tissiéres et al., 1959), they have been the focus of intense biochemical research in an effort to better understand protein synthesis. This study was undertaken to better elucidate the physical characteristics of ribosomes and, in particular, to probe some conformational changes associated with them. The ribosomes of the bacteria *Escherichia coli* are composed of two subunits with sedimentation coefficients 30S and 50S, which are constructed from one and two chains of RNA, respectively, and a wide assortment of proteins. For further biochemical characteristics of ribosomes, consult the reviews by Lengyel and Söll (1969) and Nomura (1970) and the monograph by Spirin and Gavrilova (1969).

Even though ribosomes have been studied for some time now, there is still considerable discrepancy in many of their measured physical parameters which may be caused by ribosomal complexity, structural heterogeneity, or mode of preparation. One physical parameter which has not been studied in much detail to date is the diffu-

sion coefficient. The importance of the translational diffusion coefficient, D_t, lies in the fact that it can be related, through hydrodynamic parameters, to such fundamental molecular quantities as molecular weight, size, and shape. Classically, D_t is measured by constructing an artificial concentration gradient and observing its motion as a function of time. Because ribosomes are so large and their diffusion coefficients so low, long waiting times are required for the measurement. Precise experimental conditions become difficult to maintain and such complex molecules also often tend to undergo spontaneous changes.

An alternative procedure to obtain D_t is to use the technique of self beat spectroscopy to determine the half-width of the spectrum of the light scattered by a solution of macromolecules (for reviews, see Ford, 1972; Ford et al., 1973; and Dubin, 1972). This technique eliminates the need to set up an artificial gradient and measurements can be made on solutions in macroscopic equilibrium. Compared with classical means, measurements made in this way are fast, efficient, and require small amounts of sample. Thus, in trying to further understand the physical structure of the ribosome and its role in protein synthesis through measurements of the diffusion coefficient, the self beat technique is a natural selection.

MATERIALS AND METHODS

70S Ribosome Preparation

E. coli strain A19 (obtained from Dr. A. Reiner of the Microbiology Dept.) was either grown in minimal salts media (Davis and Minigoli, 1950) or purchased in frozen 1-in cubes from General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio. In both cases the bacteria were stored frozen and small samples taken as needed. Ribosomes were prepared in a manner patterned after that of Tissiéres et al. (1959). Approximately 30 g wet wt of bacteria were mixed with 60 g of alumina (Bacteriological Grade A-305; Alcoa Chemical Div., Pittsburgh, Pa.) and ground with a precooled mortar and pestle for about 10 min until the mixture became pasty, indicating cell breakage. 70–80 ml of buffer A (10 mM Tris, 10 mM magnesium acetate [$MgAc_2$],[1] 160 mM NH_4Cl, 1 mM dithiothreitol [DTT], pH 7.4) was then added and the mixture stirred until it was evenly suspended. DNase (bovine pancrease I; Sigma Chemical Co., St. Louis, Mo.) was added and the mixture allowed to incubate for several hours to degrade the viscous DNA. Two low-speed centrifugations (10,000 rpm for 30 min in a Sorvall RC2-B centrifuge; Ivan Sorvall, Inc., Newtown, Conn.) removed the alumina and cell fragments. A high-speed centrifugation (45,000 rpm, 1 h in a 50 Ti Spinco rotor, L2-65B centrifuge; Beckman Instruments, Spinco Div., Palo Alto, Calif.) pelleted the ribosomes, which were then resuspended in buffer B (10 mM Tris, 10 mM $MgAc_2$, 1 M NH_4Cl, 1 mM DTT, pH 7.4) and stored overnight. Further washings were performed in a similar manner with the pellet in the final high-speed spin being resuspended in buffer A. The ribosomes were then layered on a linear 5–40% sucrose gradient using buffer A as solvent and spun in a SW 25.2 rotor (Beckman L2-65B centrifuge; Beckman Instruments, Inc., Fullerton, Calif.) for 14½ h at 4°C. The tubes were then tapped and the contents collected dropwise in test tubes (6 or 7 drops/tube) and assayed by measuring the absorbance at 2,600 Å. The desired fractions were then pooled and dialyzed 3 or 4 days vs. buffer A to remove the sucrose. The ribosomes were then con-

[1] *Abbreviations used in this paper:* DDT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $MgAc_2$, magnesium acetate; PCMB, *p*-chloromercuribenzoic acid.

centrated with an Amicon model 202 filtration unit (Amicon Corp., Lexington, Mass.) using UM10 filters. 1 ml samples of a 1% solution were then stored at −70°C or at liquid nitrogen temperatures until needed. All isolation steps were carried out at 4°C.

Preparation of 50S + 30S Subunits

Preparation of subunits was similar to that above with the exception that after the final high-speed spin, the ribosomes were resuspended in buffer A containing only 1 mM $MgAc_2$, and the corresponding sucrose gradient also contained a reduced concentration of $MgAc_2$. The dissociated ribosomes were then layered on the sucrose gradient and spun, and the subunits were isolated by collecting and pooling fractions from the two resulting peaks. Once frozen, sample aliquots were thawed, diluted, and examined in a Spinco Model E Analytical Ultracentrifuge to check for purity. Concentrations were determined spectrophotometrically using the extinction coefficients measured by Hill, Rossetti, and van Holde (1969).

Sample Preparation for Light Scattering

Ribosome samples were thawed and diluted with buffer A to the desired concentration. Stock solutions diluted 10:1 into clean buffer were found to be sufficiently clean for light scattering and this procedure eliminated much of the need for messy filtering techniques. The buffer A used for dilution had been made using ultrapure reagents where possible and specially distilled water. The buffer had been filtered two or three times through an Amicon UM2 filter with a mean pore size of 10–15 Å into a clean flask. Buffers were always freshly made the day before use. The diluted sample was then filtered through a Millipore filter (Millipore Corp., Bedford, Mass.) of 0.22 μm pore size directly into a clean scattering cuvette. Alternatively, the ribosomes were diluted and placed in a Spinco SW56 swinging bucket rotor and spun for 1½ h at 20,000 rpm and the desired sample extracted from the center of the tube with a clean pipette to be placed directly in the scattering cuvette without filtering. This procedure was used when dilutions of 10:1 from stock could be made. The final check for cleanliness was made by placing the scattering cuvette in the apparatus and viewing the scattered beam through a telescope at a 90° scattering angle. If clean, only a diffuse beam would be seen against a dark background.

The specially distilled water used for preparing samples was house distilled, deionized through one charcoal and 2 resin filters (Continental Water, Ridgefield, N.J.), filtered through two 0.22 μm and one 0.025 μm Millipore filters and finally redistilled through a still modeled after that of Timasheff and Townend (1970) which was especially designed to eliminate bubbles and particulate matter. The still is slightly modified from the model of the above authors and is designed to run continuously, being fed from a reservoir that is pressurized with N_2. With proper maintenance, it can consistently produce the high quality water needed. The hose from the storage reservoir is of Teflon. All water referred to as "distilled" water has been prepared in this manner. All glassware used in preparing samples was washed in distilled, deionized water with a low metal ion concentration detergent (Acationox, Scientific Products Div., American Hospital Supply Corp., Evanston, Ill.) and rinsed five times with distilled water and allowed to air dry.

Scattering cells were regular, 1 cm square cross-section fluorometric cells of optical glass having four faces and the bottom polished, and the top fitted with a Teflon stopper. They were initially soaked in nitric acid or glass-cleaning solution and then washed with detergent and water and finally rinsed with "distilled" water. To check on a cell's cleanliness, it was carefully filled with "distilled" water, placed in the scattering apparatus, and viewed at 90° through a telescope. If dirt or bubbles were present, they would appear as "stars" against an otherwise dark background. Once clean, the cells were inverted, covered with a beaker, and allowed to air dry. After use, cells were also rinsed 10–15 times with "distilled" water. Using these procedures, cells could be routinely cleaned in 15–30 min.

When additions were made to the scattering solution, the Teflon cell top was very carefully removed so as not to change the position of the cell in the scattering apparatus. A measured amount of filtered additive was placed on the horizontal platform of a clean Teflon plumper. The plumper was initially lowered to the bottom of the scattering cell and then raised and lowered 10 times to insure proper mixing. The plumper was then carefully removed and the Teflon cap replaced. Control experiments adding buffer to the scattering solution showed the addition process itself changed subsequent measurements of D_t by less than 1%.

Light-Scattering Apparatus

The light-scattering apparatus used is essentially the same as that described previously (Ford et al., 1973). With this apparatus a 0.1% ribosomal solution will typically allow the diffusion coefficient to be obtained in 30–60 s with an accuracy of 1–2%.

The correlation computer used to determine the diffusion coefficient also counts the number of scattered photons during an experiment which in turn can be used as a measure of the relative scattered intensity. Since this measurement of relative intensity must be carried out simultaneously with measurements of the correlation function for periods up to several minutes, it is a value that has been averaged over this time. To determine changes in intensity for several-second intervals, a computer of average transients (CAT 400B, Technical Measurement Corp., North Haven, Conn.) was also connected directly to the photomultiplier pulse amplifier to count pulses for time periods that could be externally selected, thus allowing a finer monitoring of relative intensity. Periods of 2.5 or 5 s were typically used. Both types of intensity measurements are given here. All measurements reported here were made at 90° to the incident light path.

THEORY

The theory describing the spectrum of the light scattered from a solution of molecules has been presented in detail in several reviews (i.e., Ford, 1972; Ford et al., 1973; and Dubin, 1972) and hence it will be dealt with only briefly here. Since it is impossible to directly measure the frequency spread in the scattered light because it is too small, the corresponding spread in the beat frequencies present is measured. Alternative to measuring the beat frequency spread directly, it is possible to consider the statistical nature of the scattered photons as they impinge on the photomultiplier tube by calculating the autocorrelation function, $c(\tau)$, which is the Fourier transform of the spectrum. It can be shown that for scattering from a monodisperse solution, $c(\tau)$ for the output current of the photomultiplier tube is given by

$$c(\tau) = 1 + \text{constant}\exp(-2\Gamma\tau), \tag{1}$$

where $\Gamma = D_t(4\pi n/\lambda_0)^2 \sin^2(\theta/2)$ and λ_0 is the incident wavelength, n is the index of refraction, and θ is the scattering angle.

In the situation where several distinct particle types are present in solution, the autocorrelation function becomes more complex. For the specific case of two components

$$C(\tau) = A + B(C_1 M_1 e^{-\Gamma_1\tau} + C_2 M_2 e^{-\Gamma_2\tau})^2, \tag{2}$$

where A and B are constants of the experiment, C_1 the weight concentration, and M_1 the molecular weight of component one; Γ_1 and Γ_2 are given by Eq. 1 for the isolated

particles. In the case where data from a two-component system is fit to only a single exponential, (constant $e^{-2\Gamma\tau}$), the time constant Γ is an effective one and may be related to Γ_1 and Γ_2 by a least squares analysis giving

$$\Gamma = \frac{\frac{1}{2}\left[\frac{(C_1M_1)^2}{(\Gamma_1+\Gamma)} + \frac{4(C_1M_1)(C_2M_2)}{(\Gamma_1+\Gamma_2+2\Gamma)} + \frac{(C_2M_2)^2}{(\Gamma_2+\Gamma)}\right]}{\left[\frac{(C_1M_1)^2}{(\Gamma_1+\Gamma)^2} + \frac{8(C_1M_1)(C_2M_2)}{(\Gamma_1+\Gamma_2+2\Gamma)^2} + \frac{(C_2M_2)^2}{(\Gamma_2+\Gamma)^2}\right]}. \tag{3}$$

The hydrodynamic dimensions of a diffusing molecule may be related to D_t via the Stokes-Einstein equation

$$D_t = k_B T/f, \tag{4}$$

where k_B is Boltzmann's constant, T is absolute temperature, and f is the frictional coefficient. For spherical particles $f = 6\pi\eta r$, where η is the viscosity of the solvent and r the effective hydrodynamic radius. For prolate and oblate ellipsoids of revolution, Perrin (1936) and Herzog et al., (1934) have given the appropriate form of f (Tanford, 1961).

RESULTS AND DISCUSSION

The experimental results are presented in Figs. 1–8. Table I presents a summary of various determinations of D_t and related parameters. Fig. 1 is a plot of diffusion coefficient corrected to standard conditions, $D_{20,w}$, against temperature for unwashed, once washed, and three-times washed 70S ribosomes. In all cases the plots show the same general character, with the once washed being almost indistinguishable from the three times at lower temperatures. The strong decrease in $D_{20,w}$ at higher temperatures indicating larger particle formation is interpreted to be a manifestation of molecular aggregation which has been previously observed in ribosomal solutions (Zubay and Wilkins, 1960; Tamaoki and Miyazawa, 1966; McLaughlin et al., 1968), although the aggregation we observe commences at significantly lower temperatures than that of McLaughlin et al. Although not shown, the relative scattered intensity increases rapidly as aggregation progresses. If the solution is subsequently cooled, either slowly or rapidly, the lower value of D_t persists, indicating that larger particles are still present, so the process appears to be irreversible in this respect. The number of washings also markedly affects the temperature at which aggregation begins. Apparently the aggregation is a strong function of the number of washings and, once started, proceeds quickly. It is interesting to note that unwashed and once washed ribosomes begin aggregating below physiological temperatures. It should also be pointed out that from our data it is impossible to tell unambiguously whether the ribosomes themselves are participating in this process or if protein is flaking off to subsequently aggregate. Tal (1969) showed that aggregation could be reduced with a low ionic strength buffer and low Mg^{2+}, but these conditions are far from physiological.

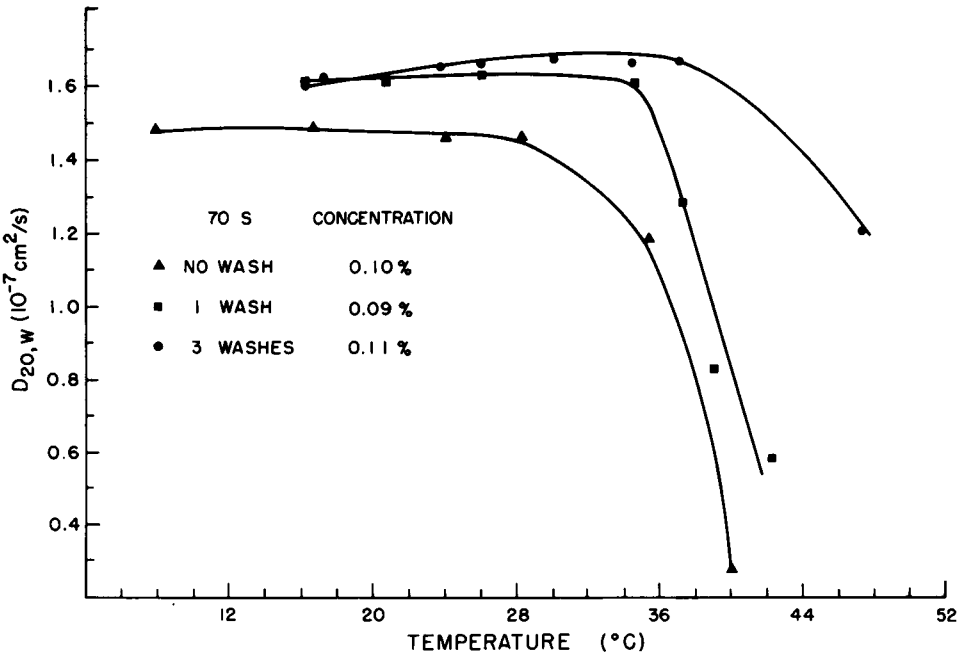

FIGURE 1 Diffusion coefficient reduced to standard conditions, $D_{20,w}$ against temperature for unwashed, once washed, and three-times washed 70S ribosomes. Unwashed solutions are a mixture of 70S and 50S particles (see text).

Analysis in the Spinco Model E Analytical Ultracentrifuge shows that for a typical preparation of unwashed ribosomes, there is roughly an equal concentration of 50S and 70S particles present, and the unwashed values of $D_{20,w}$ in Fig. 1 are for such a solution. If it is assumed that the ratio of D_{70S}/D_{50S} for unwashed particles is the same as for washed particles, Eq. 3 may be used to find D_{70S} (unwashed) = 1.43 and D_{50S} (unwashed) = $1.6 \cdot 10^{-7}$ cm^2/s. Using an equivalent hydrodynamic sphere model and the above values, it is possible to calculate that approximately 150,000 daltons of material is removed in the first washing; for this calculation the inverse partial specific volume was assumed equal to the particle density. This is in fairly good agreement with the value of 200,000 daltons obtained by Hill et al. (1970).

Following Tanford (1961), the minimum frictional coefficient, or the corresponding maximum D_t, may be obtained from

$$f_{min} = k_B T/D^0_{max} = 6\pi\eta[3M\bar{V}/4\pi N_0]^{1/3}$$

where $\bar{V}$ is partial specific volume, M is molecular weight, and N_0 is Avogadro's number. Comparison of D^0_{max} with the actual observed value then gives an indication of a particle's asymmetry with a ratio of 1 indicating a spherical shape. Using the values of $\bar{V}$ and molecular weights given by Hill, Rossetti, and van Holde (1969), values of f_{obs}/f_{min} are calculated and are given in Table I. These values of f_{obs}/f_{min} are signifi-

TABLE I

DETERMINATIONS OF $D_{20,w}$ AND RELATED PARAMETERS

	70S(unwashed)	70S(washed)	50S	30S	Reference*
$D_{20,w}(10^{-7}\ cm^2/s)$	1.83		1.91	2.95	1
		1.71	1.90	2.18	2
	1.43	1.7	1.9	~2.1	3
f_{obs}/f_{min}	1.7	1.47	1.58	1.77	3
R_{sphere} (Å)	149	126	113	102	3
Axial ratio					
Prolate ellipsoid	13	8	11	15	3
Oblate ellipsoid	16	10	13	19	3
Partial specific volume		0.596	0.585	0.59	4
Extinction coefficient ($E_{1\%}^{260nm}$)		145	145	148	4
Molecular weight (10^6 daltons)					
	2.6		1.8	0.7	1
		2.65	1.55	0.9	4
		2.9	1.7	1.0	5

*(1) Tissiéres et al. (1959), (2) Koppel (1973), (3) this work, (4) Hill et al. (1969), (5) Scafati et al. (1971).

cantly higher than typical values given by Tanford for globular proteins. If it is assumed that these values are due entirely to asymmetry, and not a hydration layer, the axial ratios of equivalent hydrodynamic ellipsoids of revolution may be calculated using the appropriate equations (Tanford, 1961). The radius of an equivalent sphere and the axial ratios for prolate and oblate ellipsoids are given in Table I. These axial ratios are much larger than those obtained from X-ray scattering (Hill, Thompson, and Anderegg, 1969) and electron microscopy (Hart, 1962). Using R_{sphere} to compute a hydrodynamic volume for washed 70S particles, we get a value some 48% larger than that obtained when the dimensions of Hill, Thompson, and Anderegg (1969) are used. If the excess hydrodynamic volume is assumed to be entirely due to hydration, then this layer of water would have to be some 15 Å thick. Although X-ray scattering is not as sensitive to hydration layers as are diffusion measurements, it is thought this difference could not entirely account for the large hydrodynamic volume. These values could also be indicating that various portions of protein or RNA are extended out from the particle in a tentacle-like fashion, thus enlarging its domain, or the ribosome deviates significantly from the assumed ellipsoidal shape. These results might also be caused by a small amount of stable aggregates or some dimerization as Hill et al. (1969) suspected in their work.

At 30°C, in the concentration range of 0.11–0.02%, 70S ribosomes washed three times give $D_{20,w} = 1.7 \cdot 10^{-7}\ cm^2/s$ and show very little or no apparent dependence on concentration (graph not shown) and hence, values of D_t as measured and corrected to $D_{20,w}$, are taken to be equal to the value at infinite dilution D_t^0. Findings of a very similar nature for 70S, 50S, and 30S particles have also been obtained by Koppel (1973). Both results indicate compact particles as opposed to an extended flexible conformation.

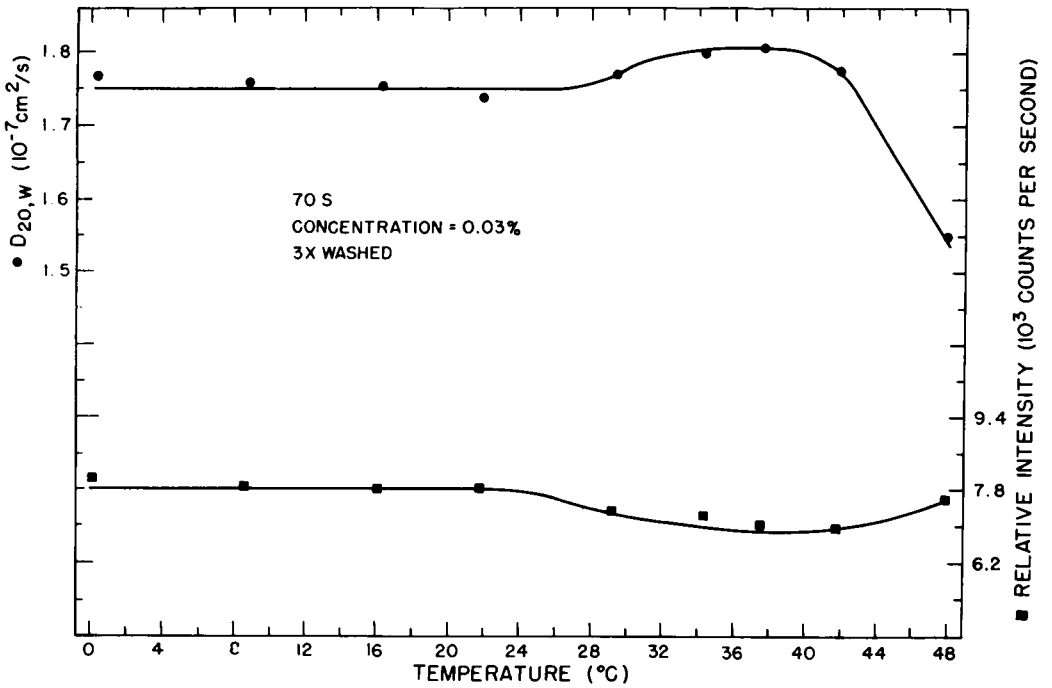

FIGURE 2 $D_{20,w}$ and relative scattering intensity against temperature for ribosomes washed three times.

Fig. 2 is a plot similar to Fig. 1 except the concentration is some three times lower and shows an effect seen only slightly at higher concentrations. From 0°C to around 30°C, there is no dependence of $D_{20,w}$ on temperature. At slightly higher temperatures, $D_{20,w}$ rises gradually with a concomitant decrease in scattered intensity and then finally begins to fall indicating aggregation. This ~2.5% rise in $D_{20,w}$ and 10% decrease in relative intensity could be caused by a small fraction (10–20%) of 70S particles being dissociated into subunits, although it could also be explained by the removal of material from the 70S particle. This latter possibility is not likely, however, as most ribosomal proteins are very small in molecular weight compared with either subunit, and hence it would take a large amount of protein to produce the same effect. A similar dissociation was observed by Tamaoki and Miyazawa (1966) although the lowest temperature they considered was 40°C. It is also interesting to note that in monitoring the aggregation process, $D_{20,w}$ starts to decrease slightly before the relative intensity starts to increase, indicating the ribosomes may be swelling before associating with one another.

Using conditions that minimized or eliminated aggregation, Tal (1969) reported an increase in specific viscosity beginning around 30–40°C which also suggests a conformational change. For particles washed three times, aggregation seems to commence at temperatures just slightly higher than physiological. The actual growth rate of *E. coli* is optimum between 37 and 45°C with only a slight increase in temperature above

45°C resulting in an extremely rapid decline in growth (Stanier et al., 1957). Perhaps one contribution to this decline is aggregation or structural alteration of ribosomes, and the difference between in vivo and in vitro situations is a measure of the unphysiological character of a Tris buffer. It has been shown that monovalent cations such as $Tris^+$ and K^+ interfere with the associating action of Mg^{2+} in the ribosomal system (Zitomer and Flaks, 1972).

It has long been known that Mg^{2+} is extremely important in maintaining the integrity of the 70S particle (Tissiéres et al., 1959). Fig. 3 is the plot of $D_{20,w}$ vs. time as two additions of EDTA are made. The initial solvent contains 10 mM Mg. The first addition makes the solution 3.3 mM in EDTA, the second 6.6 mM. Fig. 3 also includes plots of relative intensity vs. time for periods surrounding the two additions. The separate response of the system to each addition of EDTA as monitored by the relative intensity seems to indicate a two-step process. The first is concluded quite rapidly and the second is approximately exponential-like with a time constant of ~222 s. Zitomer and Flaks (1972), also using a light-scattering technique, indicated that both association and dissociation took place within a second. Our second process is clearly different from this and may be due to the presence of bound messenger RNA, thus creating a more stable complex as is implied by the pressure dissociation studies of Noll et al. (1973). Ribosomes complexed with mRNA have also been shown by Vournakis and Rich (1971) to be substantially more compact than uncomplexed ones, again indicating a tighter structure. It should also be mentioned that the solutions of Zitomer and Flaks were preincubated at 37°C for 30 min, presumably freeing the ribosomes of messenger,

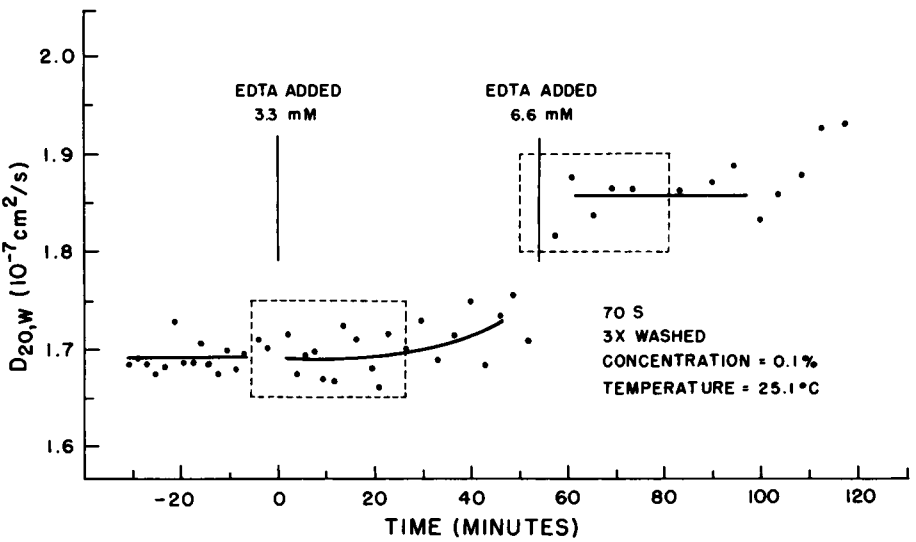

FIGURE 3 A $D_{20,w}$ plotted vs. time as two separate additions of EDTA are made. Initial solvent is buffer A.

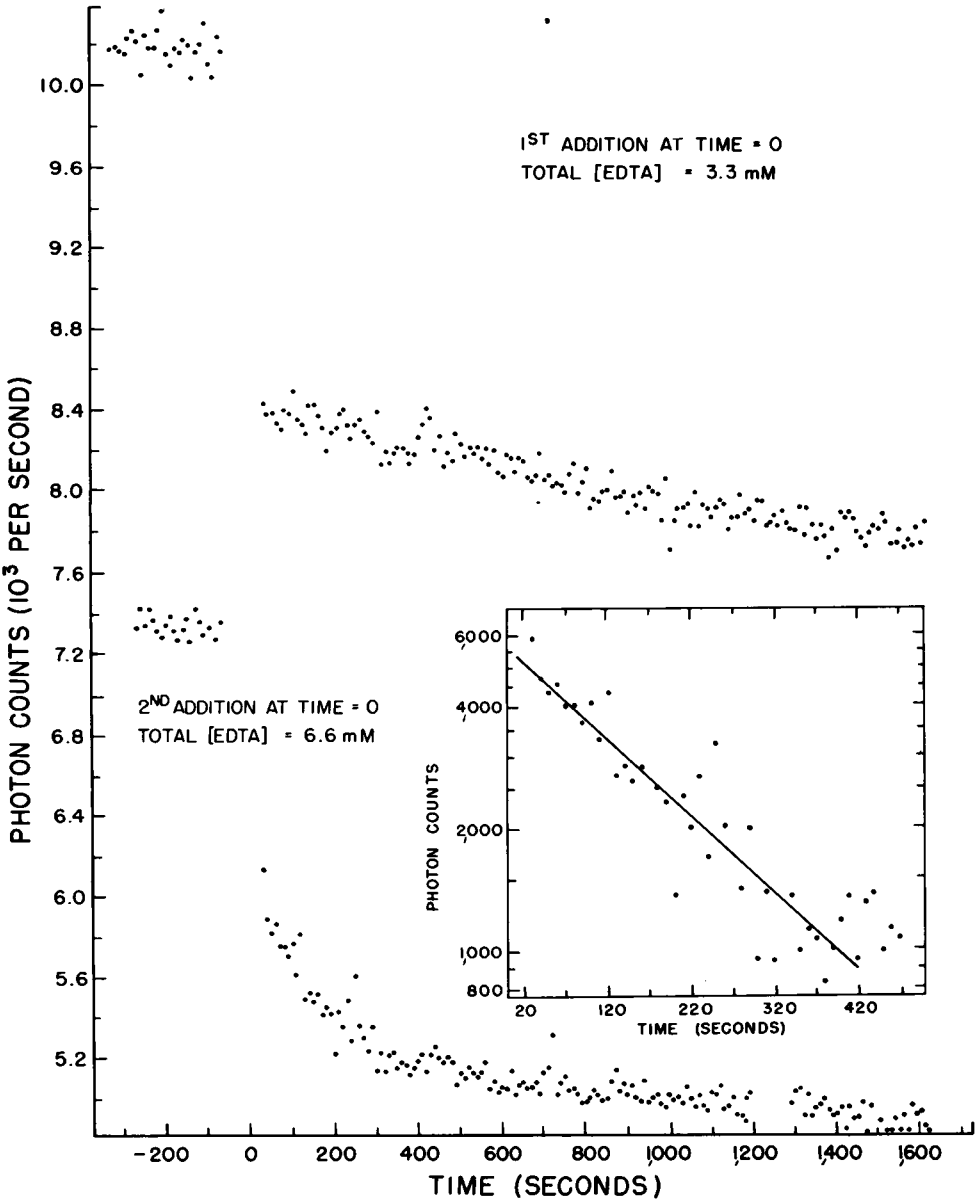

FIGURE 3 B Relative scattering intensity plotted vs. time for the two EDTA additions in Fig. 4 A. The insert is a semi-log plot of the relative scattered intensity vs. time for the second addition of EDTA; the photon counts are for a 5 s period with 24,700 counts subtracted from each point as a base-line correction. In this measurement the computer of average transients was used. The time constant is ~222 s.

while ours were not because of the aggregation problem. Our solutions then must have contained some ribosomes that were complexed with mRNA and some that were not.

The final value of D_t observed in the above experiment is $1.86 \cdot 10^{-7}$ cm^2/s. Assuming complete dissociation to 30S and 50S subunits, and diffusion constants of $D^{50S}_{20,w}$ = $1.9 \cdot 10^{-7}$ cm^2/s (this work) and $D^{30S}_{20,w}$ = $2.9 \cdot 10^{7}$ cm^2/s (Tissiéres et al., 1959), Eq. 3 may be used to predict an effective D_t = $2.10 \cdot 10^{-7}$ cm^2/s for the dissociated solution. Using values of $D^{30S}_{20,w}$ = 2.03 (this study) or $D^{30S}_{20,w}$ = $2.18 \cdot 10^{-7}$ cm^2/s (Koppel 1973) gives effective D_t = 1.92 and $1.97 \cdot 10^{-7}$ cm^2/s, respectively. The fact that a lower D_t is observed than that predicted indicates that one or both subunits have expanded, or some type of association has taken place. Whether this is a natural consequence of dissociation or is due to the chelating action of EDTA is difficult to say, although presumably the solution is still approximately 3 mM in Mg which is normally ample to preserve the integrity of the subunits. Analytical ultracentrifuge examinations of dissociated solutions show two peaks corresponding to 50S and 30S. Using equivalent sphere hydrodynamic models to calculate effective radii from D_t, it is found that the combined volumes of the 50S and 30S subunits is at least 18% greater than that of the 70S particle. Using the dimensions given by Hill et al. in their X-ray scattering studies, the combined volume is some 20% greater than that of 70S. Both of these calculations are for ribosomes that

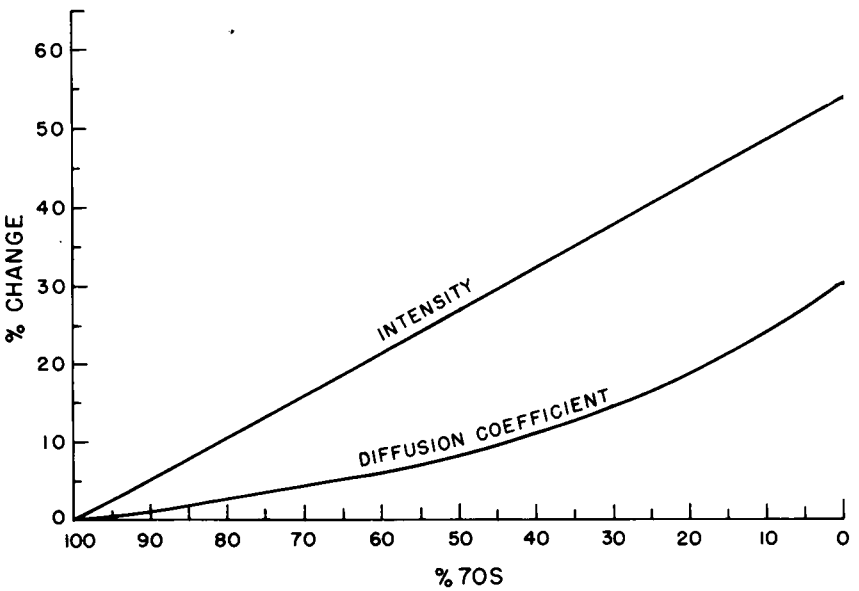

FIGURE 4 Theoretically calculated changes in the relative scattered intensity and diffusion coefficient of a solution containing 70S, 50S, and 30S particles as a function of the percent of 70S particles present. The initial solution was assumed 100% of 70S particles and 0.1% concentration.

have never been exposed to EDTA. It is expected that the subunits will both have increased hydration when dissociated, but it is felt this effect cannot account for such a large change in volume.

The fact that in Fig. 3 the increase of D_t is less pronounced than the decrease of the relative intensity is a property of the experimental technique itself. In a solution of partially dissociated ribosomes, the 70S particles will have greater scattering power than the 50S and 30S subunits due to their size and hence, their contribution to the measured D_t will be greatest. Using 0.1% solution of all 70S particles as reference, a theoretical calculation of the percent change of D_t and relative intensity vs. the concentration of 70S is shown in Fig. 4.

In a separate experiment (not shown) where there was one addition of EDTA to a final concentration of 12 mM, the intensity decrease followed an exponential-like decay and the final $D_{20,w} \cong 1.85 \cdot 10^{-7}$ cm^2/s. In one of the initial experiments of this type with 70S ribosomes that had been isolated via several cycles of differential centrifugation instead of the sucrose gradient technique, a somewhat different effect was observed. After the EDTA was added (12 mM), $D_{20,w}$ was observed at first to increase by 3% accompanied by a 22% decrease in the relative scattered intensity. During the next 90 min, $D_{20,w}$ then decreased 12% and the intensity increased 9% indicating larger particle formation. After this time, both variables remained constant. After many months of storing the ribosomes at liquid nitrogen temperatures, this effect could not be reproduced. It is thought that some specific association was taking place and the effect was a property of the mode of preparation. Otherwise it is unexplained.

Fig. 5 shows an experiment similar to that of Fig. 3. After the solution was made 8.2 mM in EDTA, the scattered intensity decreased in an exponential manner with a time constant of ~95 s. After approximately 1 h, Mg^{2+} was added to increase the Mg^{2+} concentration by 8.2 mM. After this time 84% of the initial intensity was recovered and the diffusion coefficient was some 1.5% lower than the control value. These results indicate that only partial association occurred and even then it is possible that the final conformation was expanded from that of the native 70S. It is also possible that a limited amount of aggregation took place. According to intensity measurements, the association process went to completion much faster than our ability to measure it. When another solution was made 11 mM in EDTA and the wait before the Mg^{2+} addition was 5 min, 95% of the original intensity was recovered upon the addition of Mg^{2+} although the final D_t was some 4% lower than the original control value (not shown).

In the situation where separately prepared 30S and 50S subunits in 1 mM Mg^{2+} buffer A were mixed and the solution suddenly made 13.5 mM in Mg^{2+}, a 10% increase in scattered intensity was immediately observed and then a linear rise to a 23% change after 1 h (not shown). During this time D_t showed a lot of fluctuation and the final value was some 4.4% lower than the initial value. The ultracentrifuge showed three diffuse peaks corresponding to 70S, 50S, and 30S particles, respectively. The long time needed for association is probably a function of the initial state of the subunits. For this experiment, both had been stored at −70°C for about 2 mo and

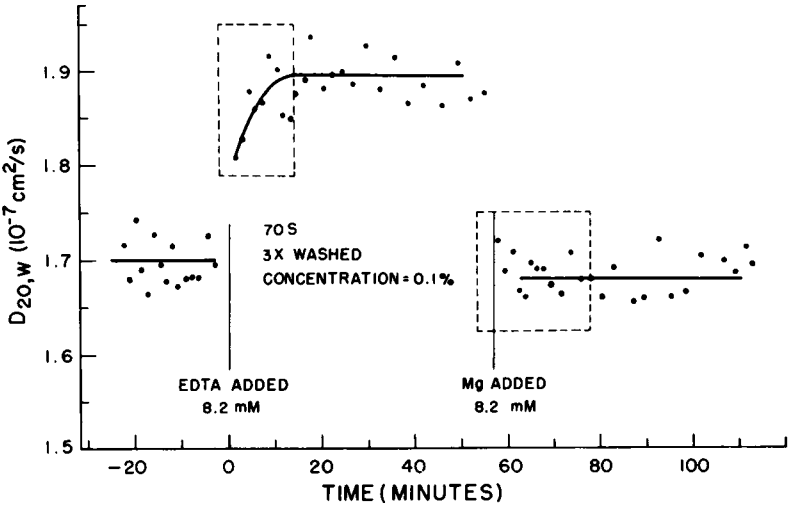

FIGURE 5 A $D_{20,w}$ vs. time for one addition of EDTA and a subsequent addition of Mg^{2+}. Temperature was 25.1°C.

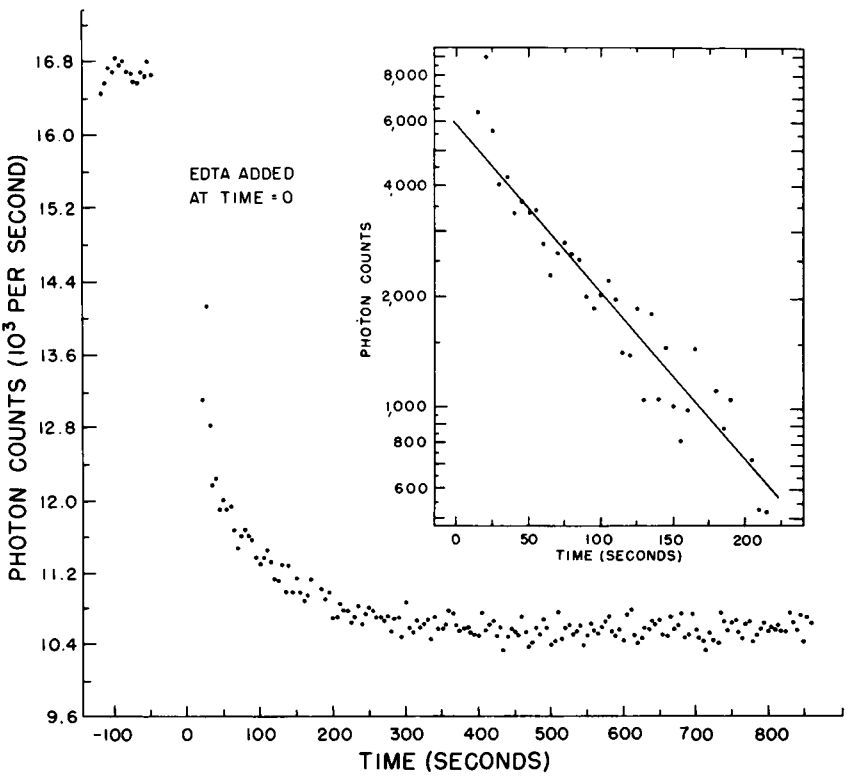

FIGURE 5 B Relative scattering intensity vs. time for the addition of EDTA in Fig. 6 A. Insert is a semi-log plot of this data; photo counts here are for a 2.5 s interval with 26,400 counts being subtracted from each point as a base-line correction. Time constant is ~95 s.

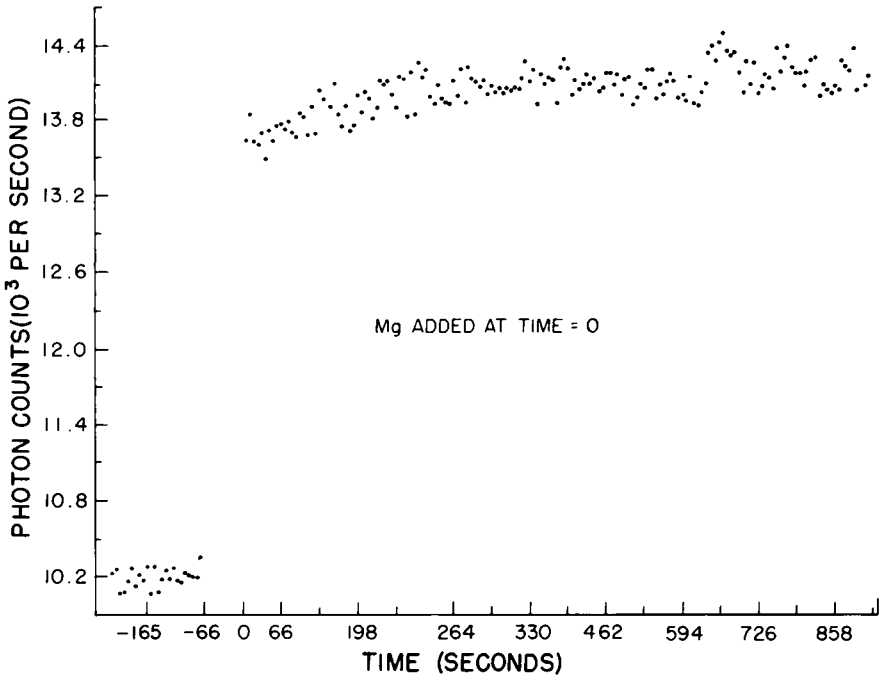

FIGURE 5 C Relative scattering intensity vs. time for the addition of Mg^{2+} in Fig. 6 A. Photon counts are for 3.3-s intervals.

deterioration over this period would certainly account for a slower or incomplete association. Also, the initial value of D_t before Mg^{2+} was added was somewhat lower than expected, thus indicating expanded or aggregated particles. 30S particles have been shown to be especially sensitive to denaturation (Tamaoki and Miyazawa, 1966; Leon and Brock, 1967).

Fig. 6 depicts the situation where *p*-chloromercuribenzoic acid (PCMB) is twice added to a solution of 70S particles. Known to react with S—H groups, PCMB has been shown to cause dissociation of 70S ribosomes (Tamaoki and Miyazawa, 1967). The number of sulfhydryl groups in *E. coli* ribosomes has been measured by Acharya and Moore (1973) to be 38.8±1.0, 22.8±3, and 12.9±0.3 for 70S, 50S, and 30S particles, respectively, and for intact subunits there are 1 and 2.7 reactive SH groups for the 50S and 30S (Slobin, 1971). Although dissociation clearly takes place, it does not seem to be as extensive as in the case of EDTA addition. Also, the effects of the first addition are not instantaneous but appear to progress with time. It has long been thought that the RNAs of the 50S and 30S subunits are involved in 70S formation (Watson, 1964). This work supports the belief that proteins also contribute to this association. In the work of Tamaoki and Miyazawa, sedimentation techniques were used to determine the relative amounts of each component as a function of PCMB concentration and hence, are subject to the criticism of ribosomal dissociation due

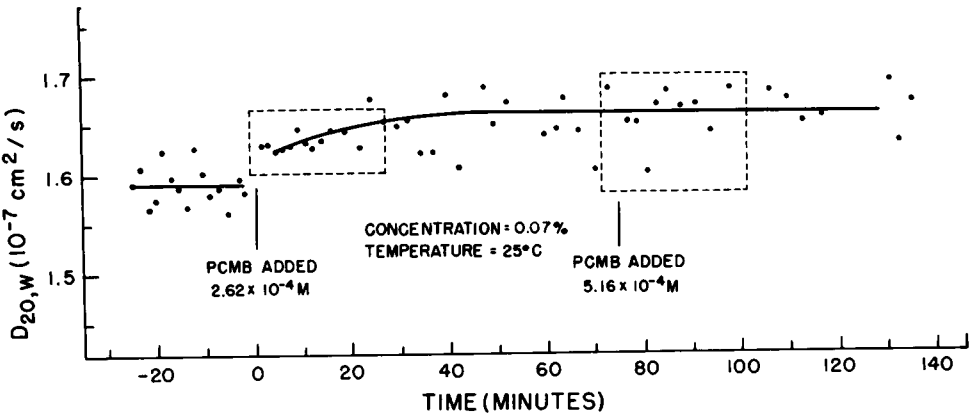

FIGURE 6 A $D_{20,w}$ vs. time as PCMB is added to a solution of 70S particles.

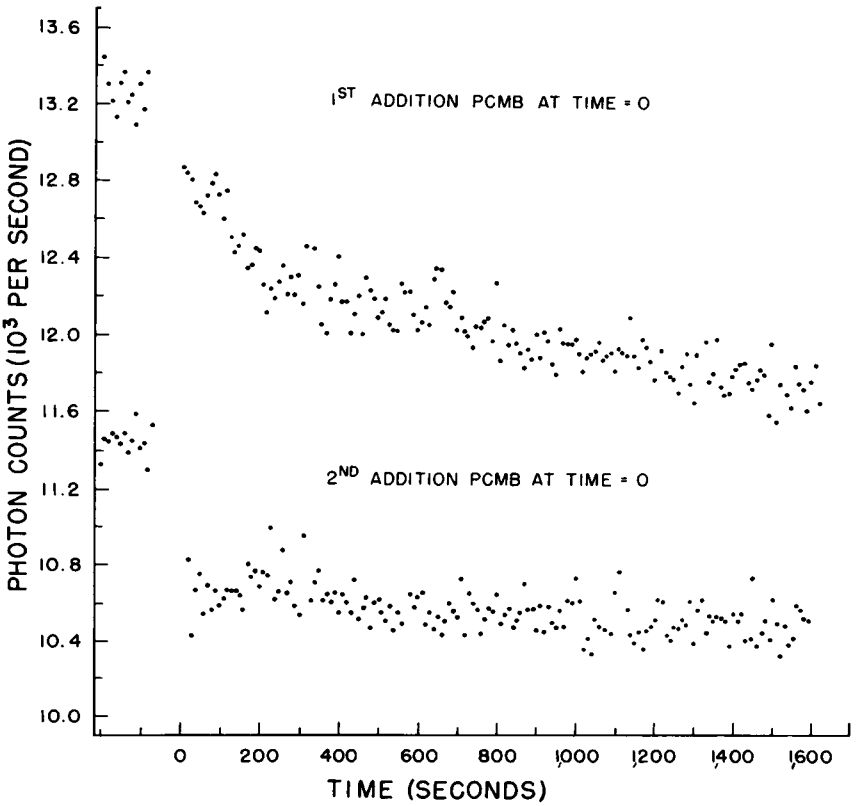

FIGURE 6 B Relative scattered intensity vs. time for the addition of PCMB in Fig. 8 A. Solvent is buffer A without DTT.

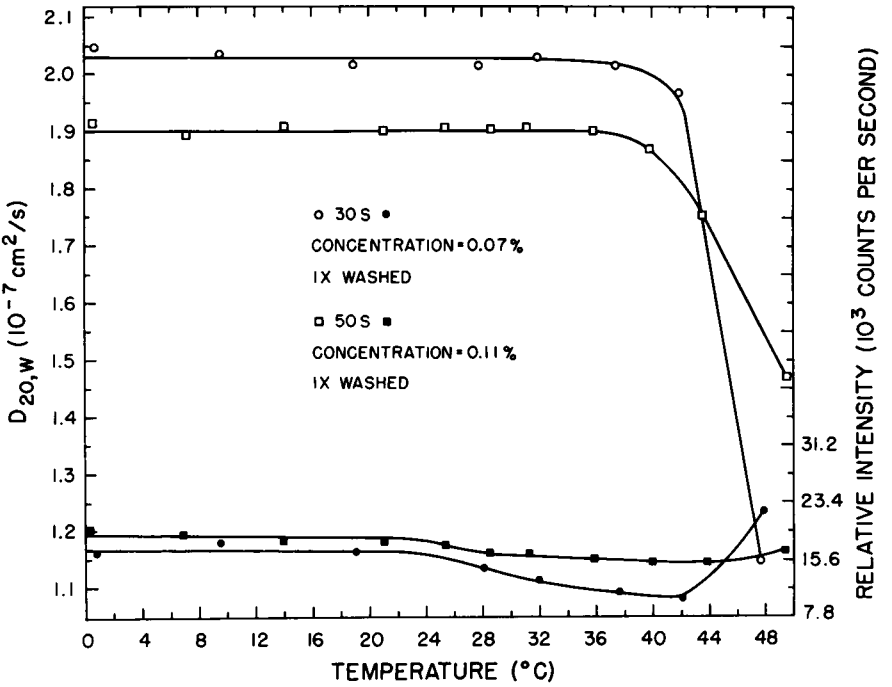

FIGURE 7 $D_{20,w}$ and relative scattering intensity vs. temperature for solutions of 30S and 50S particles, respectively. Mg^{2+} concentration is 1 mM in buffer A. Open characters represent $D_{20,w}$ and closed, intensity.

to increased pressure (Infante and Baierlein, 1971). Our method is entirely free of this drawback.

Fig. 7 is a plot of D_t and also relative scattered intensity for once washed 50S and 30S components vs. temperature. Like the 70S particles, aggregation starts at temperatures slightly higher than physiological and $D_{20,w}$ decreases prior to the increase in the scattered intensity, again indicating that swelling precedes aggregation. Upon addition of EDTA to a solution of 50S subunits to a final concentration of 9.6 mM (initial Mg = 10 mM), there was no significant drop in the relative intensity although D_t decreased slowly some 4% over a period of 4.3 h, indicating a small conformational change but no appreciable loss of material. If the 50S solution was made 13.8 mM in EDTA, D_t immediately dropped approximately 5.3%, remaining there for about 2 h. A slow decrease then started (not shown). The intensity showed only a decrease which could be accounted for by dilution. Over 2 h it stayed approximately constant, although showing greater fluctuation. Again, no loss of material from the 50S particle is implied. These results agree quite well with the sedimentation work of Gesteland (1966) who reduced the Mg concentration by slow dialysis against an EDTA solution. If it is assumed that no material is lost with the EDTA treatment, our particles more closely resemble Gesteland's 36S conformation than his 21S form.

For the 30S particles, the drop in $D_{20,w}$ and rise in relative intensity at higher tem-

peratures are more closely related than those for the 50S subunits and the decrease in $D_{20,w}$ more dramatic even though both experiments were similar with respect to time of heating etc. Measurements at a particular temperature took 10–20 min and temperature changes and equilibration 15–30 min. The similarity in critical aggregating temperatures for the unlike subunits is striking. The subunits appear more heat stable than unwashed intact ribosomes and are very similar to well-washed ribosomes. In the range of 28–42°C, a 3.3% decrease in relative intensity is seen without a noticeable change in $D_{20,w}$. This could be due to material dissociating from the 30S particle as the intensity would be most sensitive to this. If a 30S solution 1 mM in Mg^{2+} is suddenly made 5.2 mM in EDTA (not shown) the relative scattered intensity immediately drops by about 50% and $D_{20,w}$ some 13%. These results indicate the 30S subunit has not only been broken, but each part has subsequently expanded. A sedimentation run of the solution shows one hypersharp peak of ~12.6S. Using the Svedberg equation, a molecular weight of ~400,000 daltons is calculated. This is strictly an approximation as the values of S and D used are not at infinite dilution. However, it does indicate that the 30S subunit has been broken approximately in half. The hypersharp peak is probably an indication of a conformation having a substantial dependence of S upon concentration which would be expected for a flexible noncompact molecule. Gesteland (1966) showed that upon slow dialysis vs. a 1 mM EDTA solution, 30S subunits over the period of 4 h progressed from 30S to more expanded conformations of 26S to 16S with only a small loss in protein content. As seen here, a more extreme treatment produces more drastic changes.

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