PHYSICOCHEMICAL AND LIGHT SCATTERING STUDIES ON RIBOSOME PARTICLES

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ABSTRACT The light scattering technique has been used to measure the molecular weight of *Escherichia coli* ribosomes. The 30S, 50S, and 70S components have been isolated and purified. The refractive index increment dn/dc was found to have the same value, (0.20 ± 0.01) cm³/g, for the three species. The molecular weights are $(1.0 \pm 0.1) \cdot 10^6$, $(1.7 \pm 0.1) \cdot 10^6$, and $(2.9 \pm 0.3) \cdot 10^6$ daltons respectively. Some information about the dimensions in solution (radius of gyration) and the interaction constant (second virial coefficient) have been obtained, and their significance is discussed.

INTRODUCTION

If we consider the present state of knowledge of the essential role played by the ribosome particles in protein synthesis, an investigation into the physicochemical properties of ribosomes in solution would appear to offer great promise.

Although many aspects of this process have been clarified, the mechanism by which the ribosome subunits cooperate in making all the molecular species involved in polypeptide synthesis work together has not yet been understood. A better understanding of the structure of the ribosome is more than likely essential to the interpretation of its function.

Recently, the biochemical approach has led to the determination of a number of important facts, mainly concerning the identification of the various ribosome proteins (Waller and Harris, 1961; Leboy et al., 1964; Gesteland and Staehelin, 1967; Traut et al., 1967; Traub et al., 1967; Fogel and Sypherd, 1968).

The physical structure of ribosomes has not been so extensively studied. Nothing has been done since the first physicochemical investigations were made (Tissières et al., 1959; Kurland, 1960; Hamilton et al., 1962), although more refined techniques are now available.

Other studies have been carried out with the electron microscope (Hall and Slayter, 1959; Huxley and Zubay, 1960; Spirin et al., 1963; Hart, 1965; Bruskov and Kiselev, 1968). However, the possibility of interpretation of electron micrographs of

shadowed or stained ribosomes is not very good, because of both the poor contrast and the complexity of the structure to be determined, the fact also being borne in mind that fixatives such as formaldehyde may give rise to artifacts in the shape observed.

The only data which the electron microscope can give are the over-all dimensions of the ribosome particles, though it must be taken into consideration that under these conditions the ribosomes are in an anhydrous state, and that their true dimensions in the cell may be extremely different, because of the solvation of the particles.

The physiological state of ribosomes is better achieved in solution. In this instance it is possible to obtain information on the ribosome structure, molecular weight, and possibility of bond formation, with various methods.

The molecular weight of ribosomes has mainly been determined (Tissières et al., 1959) on the basis of sedimentation velocity measurements, using the Scheraga-Mandelkern (1953) equation

$$M^{2/3} = s_{w,25} \frac{\eta^{1/3} \eta_0 N}{\beta(1 - V\rho_0)}, \qquad (1)$$

where $s_{w,25}$ is the sedimentation coefficient η_0, η, are the intrinsic viscosities of the solvent and solute respectively, N, Avogadro's number, ρ_0, the density of the solvent, V, the partial specific volume of the solute, and β, an empirical form factor.

An alternative is offered by the Svedberg equation

$$M = s_{w,25} \frac{RT}{D(1 - V\rho_0)}, \qquad (2)$$

where R is the thermodynamic gas constant, T, the absolute temperature, and D, the diffusion coefficient, while the other symbols are the same as for equation 1.

It can be seen that the sedimentation method does not give a direct determination of molecular weight, involving as it does a number of parameters which are scarcely known for ribosomes, as well as other empirical factors.

A better approach may be made by the light scattering technique. Earlier data (Hamilton et al., 1962) gave only a rough measurement of the molecular weight of rat liver ribosomes, probably because of heterogeneity and impurities in the sample.

We shall set out here the results of light scattering measurements on *E. coli* ribosomes, by means of which we were able to obtain the molecular weight with a higher accuracy. Light scattering has the advantage over sedimentation techniques of being an absolute measurement which needs only the parallel measurement of the refractive index increment. Moreover, the radius of gyration and the interaction constant (second virial coefficient) may be obtained also.

Recently (Hill et al., 1969) the molecular weight of *E. coli* ribosomes was also obtained by means of the sedimentation equilibrium in the analytical ultracentrifuge. This is also an absolute method, although not exempt from various difficulties of interpretation.

MATERIALS AND METHODS

Ribosome Extracts

Ribosomes were obtained from *E. coli* strain RNase$^-$ 10$^-$ Gesteland (derived from AB 301, an Hfr Met$^-$ strain) with a method derived from Tissières et al. (1959) as described elsewhere (Danusso and Reale Scafati, 1968).

In this way we obtained a suspension of ribosomes which were mainly in the monomeric 70S state, with some 100S dimers, in Tris-HCl 10^{-2} M $MgCl_2$.

Ultracentrifuge Analysis

The extracted ribosomes, as well as the separate components to be observed in the light scattering apparatus, were checked in an analytical ultracentrifuge Spinco model E (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.), with the band sedimentation velocity

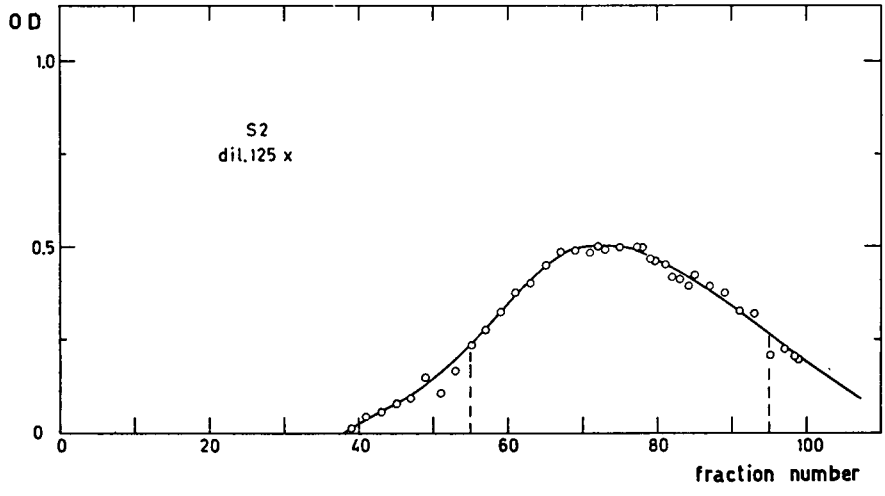

FIGURE 1 *a* Elution profile of chromatographic column.

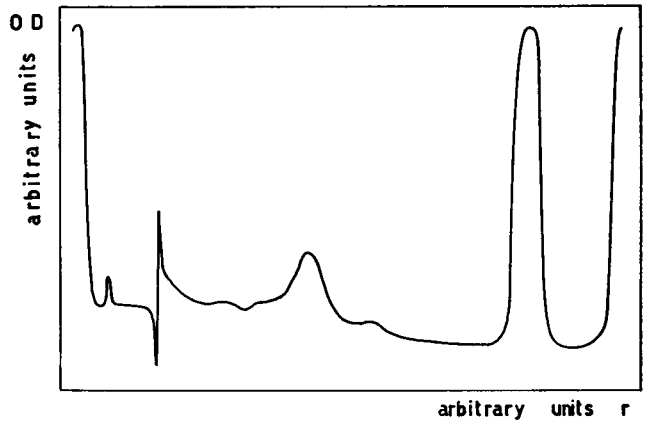

FIGURE 1 *b* Ultracentrifuge analysis of purified 70S ribosomes. Microdensitometry after 30 min of a run at 32,000 rpm.

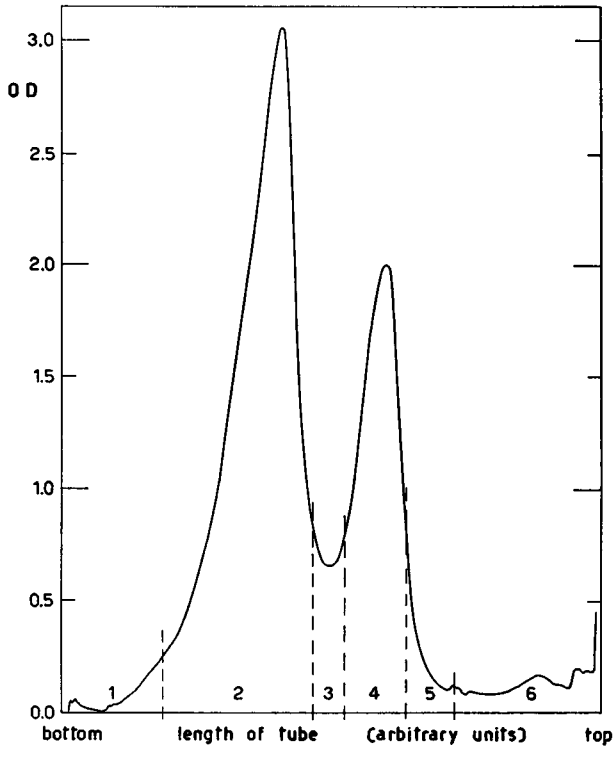

FIGURE 2 Elution profile of the ribosome subunits after a sucrose gradient as registered with a Gilford spectrophotometer.

technique in heavy water, as described elsewhere (Danusso and Reale Scafati, 1967). With the same technique the sedimentation coefficient of each component was determined, and the results were converted to the standard $s_{w,20}$ values.

The band sedimentation technique has the advantage over normal sedimentation methods of requiring only a small quantity of ribosomes, and of being free from Johnston-Ogston effects.

Purification

For the molecular weight to be obtained, it was necessary to separate and purify each ribosome component before introducing it into the light scattering apparatus.

70S After some preliminary attempts, we found that the 100S dimers may be eliminated in the presence of 10^{-2} M Mg^{++}, by being treated with a small quantity of pancreatic RNase which probably destroys the fragment of messenger RNA that keeps the two monomers together. The RNase itself must be eliminated from the sample by purification in a Sepharose column.

Typical experimental conditions were the following: 2 ml of ribosome extract in Tris, at a concentration of up to 50 mg/ml, were treated with 2 μg/ml RNase for 30 min at room temperature, then layered on a 35-cm-high column of Sepharose 4B, equilibrated with the same 10^{-2} M Mg^{++}, 10^{-2} M Tris. The column was eluted for about 3 hr, 100 fractions of 10 drops each being automatically collected with a Beckman model 133 refrigerated collector

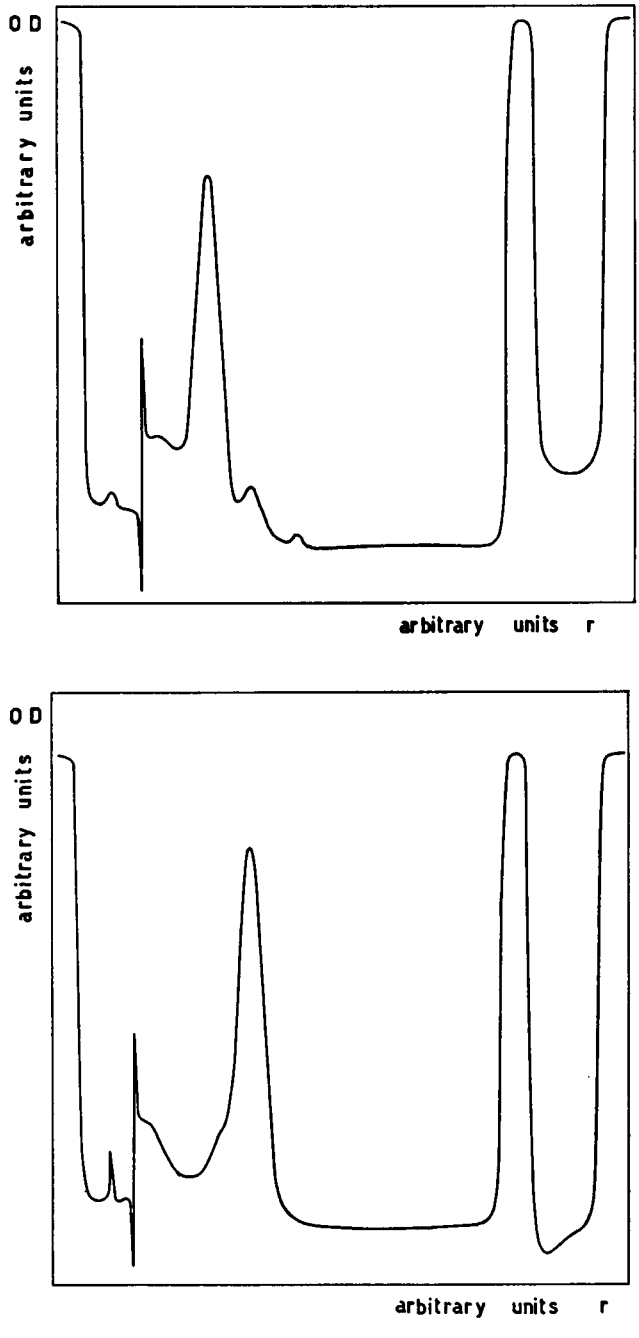

FIGURE 3 Ultracentrifuge analysis of purified 30S and 50S ribosomes. Microdensitometry after 30 min of a run at 32,000 rpm. (*a*) 30S subunit; (*b*) 50S subunit.

(Beckman Instruments, Inc., Palo Alto, Calif.). The eluting sample was passed through the 2 mm flow cell of a Gilford spectrophotometer (Gilford Instrument Labs, Inc., Oberlin, Ohio). Fig. 1 shows the ultracentrifuge analysis of the 70S preparation after passage through the column.

30S and 50S Subunits In order to obtain the 30S and 50S subunits, the ribosome extract was dialyzed against 10^{-2} M Tris, 10^{-4} Mg^{++} buffer. The dissociation was verified in the analytical ultracentrifuge. Then 0.5 ml of sample at a concentration of $\sim$ 15 mg/ml was layered on top of a 0–30% sucrose gradient solution in Tris 10^{-4} M Mg^{++} buffer, in each of the six tubes of an SW 25.3 rotor of the Beckman L2-65 preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). After being spun at a velocity of 25,000 rpm for 13 hr, the tubes were eluted, and drops collected after the solution had been passed through the 2 mm flow cell of the Gilford spectrophotometer. The result is given in Fig. 2, while the separation of the components is shown by the ultracentrifuge analysis (Figs. 3 *a* and *b*). After separation, we had to carry out long and repeated dialysis of the samples against a large volume of Mg^{++} 10^{-4} M Tris 10^{-2} M buffer. The Visking tubes used were treated by the method developed by McBrain and Steven (1956) in order to increase the exchange velocity. Any traces of sucrose had to be very carefully eliminated for the light scattering and refractive index increment measurements, because of the high refractive index increment (0.145% ml/g at 20°C) of sucrose solution as compared to that of ribosomes (about 100 times less). The Mölish test was used to check whether this elimination was complete.

Light Scattering Measurements

The apparatus used in the experiments was a SOFICA model 42.000 (Société Française de Contrôle et d'Analyse, St. Denis, France), with Hg vapor lamp of which the 5460 A line was used, and a 1P21 phototube which rotates to reveal the light scattered at any angle between 30 and 150° from the incident beam.

A number of difficulties arising in other types of apparatus from the need for various corrections (for multiple refractions and reflections of incident light) are removed by a simple device. The cell containing the sample solution to be measured is immersed in a benzene or toluene bath. Because the refractive index of benzene is equal to that of glass, spurious refractions or reflections are minimized, and only the refractive index variation when light passes through the water solution needs to be taken into account.

The optical diagram of the apparatus is given in Fig. 4.

The reading is taken from a galvanometer which shows the current at the phototube produced by the scattered light I_ϑ at the corresponding angle ϑ (with a solid angle resolution of $\pm 1°30'$). This, in turn, may be expressed in terms of Rayleigh's ratio, R_ϑ, by means of a cylindrical glass standard, whose scattering factor is calibrated against pure benzene. Rayleigh's ratio for benzene is $R_B = 16.3 \cdot 10^{-6}$ cm^{-1} (for $t = 25°C$) so that

$$R_\vartheta = \frac{I_\vartheta}{I_B} R_B \frac{(1 + \cos^2 \vartheta)}{\sin (\vartheta/2)} .$$

The factor $(1 + \cos^2\vartheta)/(\sin[\vartheta/2])$ accounts for the two possible polarizations of the incident unipolarized light $(1 + \cos^2\vartheta)$, and for the apparent volume effects (n_0^2/n^2) $(\sin[\vartheta/2])$ due to refractive index differences at the glass-water interface.

The mean dimensions ϕ of the ribosomes ($\sim$ 200 A, determined by electron microscopy)

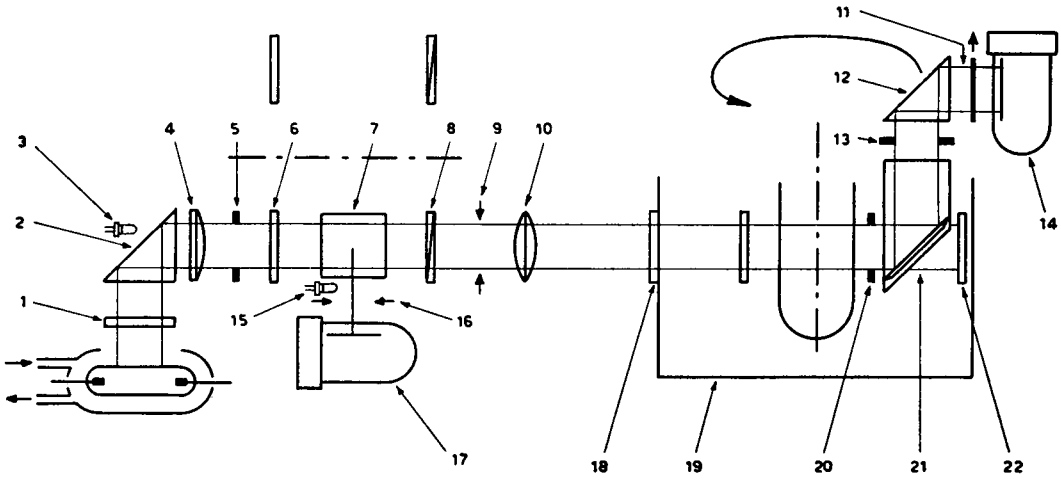

FIGURE 4 Optical diagram of the light scattering apparatus: (1) anticaloric glass window, (2), (12) total reflection prisms, (3), (15) compensation lamps, (4) condenser lens, (5) source slit, (6) filter, (7) scattering glass, (8) polarizer, (9) adjustable slit, (10) principal lens, (11) shutter, (13) exit slit, (14) measuring phototube, (16) iris diaphragm, (17) standard phototube, (18) window glass, (19) toluene bath, (20) entrance slit, (21) air gap total reflection prism, (22) light trap.

compared with the wavelength, λ, of the incident light, 5460 A, are such that $\phi \sim \lambda/25$. The internal interference effects, although small, have then to be taken into consideration.

With the double extrapolation Zimm plot method (Zimm, 1948) it is possible to gain some light scattering information on the dimensions and shape of the ribosome, although this may be more cumbersome than the simple $c \to 0$ extrapolation.

According to Zimm, R measurements must be made at various angles and at a number of values of the concentration, c, of the sample.

By plotting $c/I = c/I_\vartheta \times (\sin[\vartheta/2)/(1 + \cos^2\vartheta)$ vs. $(\sin^2[\vartheta/2]) + Kc$, (where K is only an appropriate numerical factor, in order to obtain an intelligible graph), one gets a double set of curves, which may be extrapolated to $\vartheta \to 0$, $c \to 0$. The common extrapolated value $(c/I)_{\substack{c=0 \\ \vartheta=0}}$ multiplied by I_B/R_B gives C/R_ϑ, and may be introduced into the following expression, from which the molecular weight, M is calculated:

$$\frac{2\pi^2 n_0^2 (dn/dc)^2}{\lambda^4 N} \left(\frac{c}{R_\vartheta}\right)_{\substack{c=0 \\ \vartheta=0}} = \frac{1}{M}; K\left(\frac{c}{R_\vartheta}\right)_{\substack{c=0 \\ \vartheta=0}} = \frac{1}{M}. \tag{3}$$

Equation 3 is true for the extrapolation. Because in general

$$K\frac{c}{R_\vartheta} P(\vartheta) = \frac{1}{M} + 2Bc + \cdots\cdots,$$

where $P(\vartheta)$ is a geometrical form factor, and according to Zimm

$$\lim_{\vartheta \to 0} P^{-1}(\vartheta) = 1 + \frac{16\pi^2 R_g^2 \sin^2(\vartheta/2)}{3\lambda_0^2},$$

where R_g is the radius of gyration, it can be seen that the initial slope of the zero concentration line gives

$$\frac{(\text{Initial slope})_{c=0}}{\text{Intercept}} = \frac{16\pi^2}{3} R_g^2 \left(\frac{n}{\lambda_0}\right)^2,$$

while the initial slope of the zero angle line gives

$$(\text{Initial slope})_{\vartheta=0} = 2B.$$

Refractive Index Increment

In equation 3 the quantity dn/dc appears. Because of the dependence of M on the second power of dn/dc, it is important to obtain this value for the ribosome samples with good accuracy. We have used a differential refractometer in the manner described by Abbe, in which the 5460 A wavelength of a Hg lamp passes through a two-sector thermostatable cell, one sector containing the solvent and the other, the ribosome suspension. The deviation Δd of the image over a scale is proportional to Δn, according to

$$\Delta n = K \Delta d.$$

The calibration constant has been determined and is $K = (0.987 \pm 0.005) \cdot 10^{-3}$.

Preparation of the Solutions

One of the well known difficulties of light scattering measurements is the elimination from solutions of all impurities such as dust, precipitates, and aggregates.

In fact, all these impurities may give spurious light scattering effects, particularly if they are of large dimensions.

Impurities may be removed from the solvent by filtration and the small residual effect may be subtracted from the measurements, but it is more difficult to eliminate the spurious particles which are present in the sample itself. First of all, only those particles whose dimensions differ widely from those of ribosomes can be removed. Secondly, care must also be taken that the purification methods do not damage the ribosomes.

The best method for this purpose is centrifugation of the solutions at appropriate speeds, just before they are poured into the carefully washed and dried light scattering cuvettes. The purity of the solutions may be checked by measurement of the dissymmetry of the scattered light. Large particles, in fact, give a high forward scattering so that the ratio of the intensity scattered at, say, 45° to that at 135°, is higher than unity. As for the dissymmetry of ribosomes, it is in any case small.

After some preliminary attempts, we obtained good results by filtering the solvent buffer with a Jena glass filter model G5f (Jenaer Glass Werke, Schott und Genoden, Jena, Germany), at a very low pressure. A number of 25 ml solutions of the sample at various concentrations were made (with the filtered solvent), and these were in turn centrifuged at 30,000 g for 30 min. Only the upper 20 ml of the supernatant were used in the cells of the light scattering apparatus while 1 ml was used for concentration and dn/dc measurement.

Determination of Concentration

The concentration of the solutions was determined spectrophotometrically, using a Cary model 15 spectrophotometer (Cary Instruments, Monrovia, Calif.).

The extinction coefficient $E_{1\%}^{1\,cm}$ of the ribosomes under our conditions has been deduced from a number of dry weight measurements, as reported in a previous paper (Danusso and Reale Scafati, 1968) and results as

$$E_{1\%}^{1\,cm} = 132 \pm 1.5 \text{ ml/g at a 2600 A wavelength.}$$

On the same ribosome preparation, the dry weight, optical density, RNA, and protein content were measured, the last two by the orcinol and Lowry methods respectively.

The results are as follows:

Dry weight: $(75.9 \pm 0.9)\ 10^{-6}$ g/m for OD 2600 = 1
RNA content: 58.9% ±1%
Protein content: 41.1% ±1%

RESULTS

Refractive Index Increment

At the low concentrations used for the light scattering experiments, *dn/dc* measurements were not easy to make, and the uncertainty of each result was large. This meant that a number of determinations had to be made for each separated component, and the best fitting straight line had to be obtained with the least square method from the experimental Δd and optical density data.

The results are reported in Figs. 5–7.

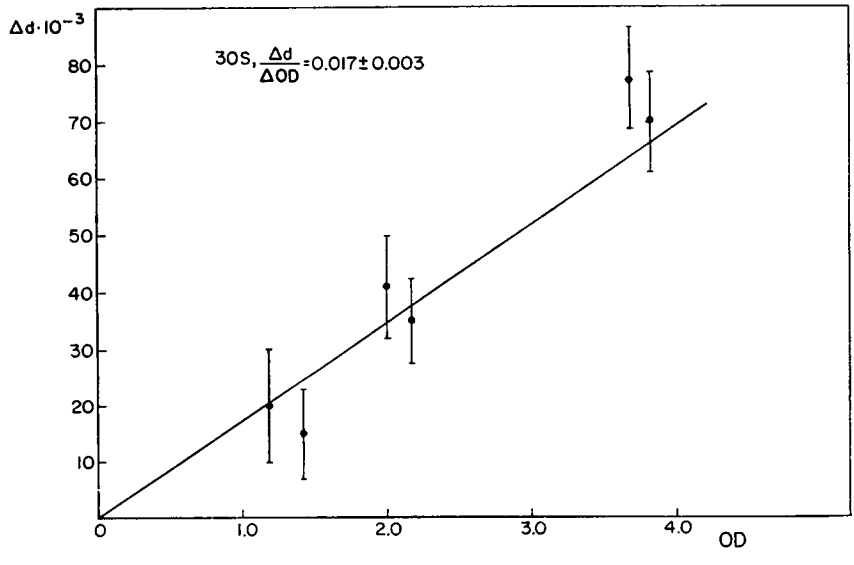

FIGURE 5 Best fit of the experimental $\Delta d/\Delta$ OD results for *dn/dc* determination of 30S subunit.

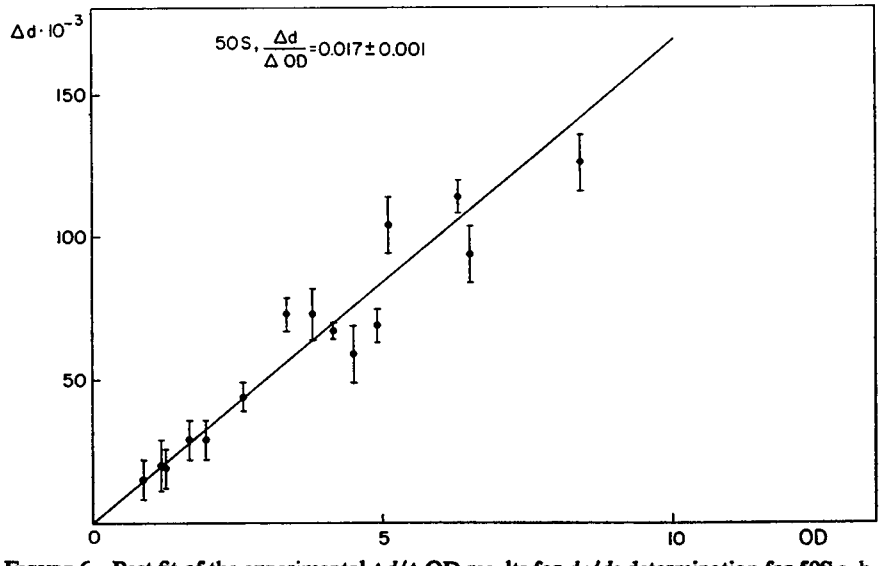

FIGURE 6 Best fit of the experimental $\Delta d/\Delta$ OD results for *dn/dc* determination for 50S subunit.

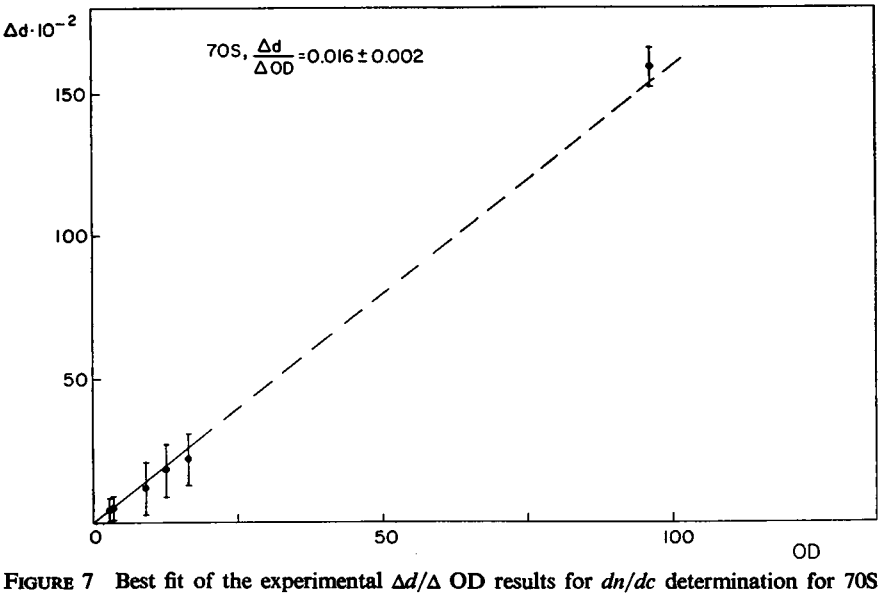

FIGURE 7 Best fit of the experimental $\Delta d/\Delta$ OD results for *dn/dc* determination for 70S ribosome.

It can be seen that the three slopes $\Delta d/\Delta$(OD) are identical within the experimental accuracy, and that the linearity in the explored range is fairly good.

All the experiments were made at 15°C. A common average value of *dn/dc*

$$dn/dc = (0.20 \pm 0.01) \text{ cm}^3/\text{g},$$

was used in all cases.

Light Scattering

As mentioned before (see Materials and Methods), a number of solutions of each sample were poured directly into the light scattering cuvettes from the ultracentrifuge tube after clarification, then stored in a toluene bath at 5°C during each experiment. Each cuvette was then placed in the apparatus for the reading of I_ϑ at various angles. The temperature was kept at 15°C by means of a circulating refrigerator bath. A typical experiment, on five samples of different concentration plus the solvent, took about 3 hr. Generally two or three measurements were made, and the average taken, although there were only slight fluctuations.

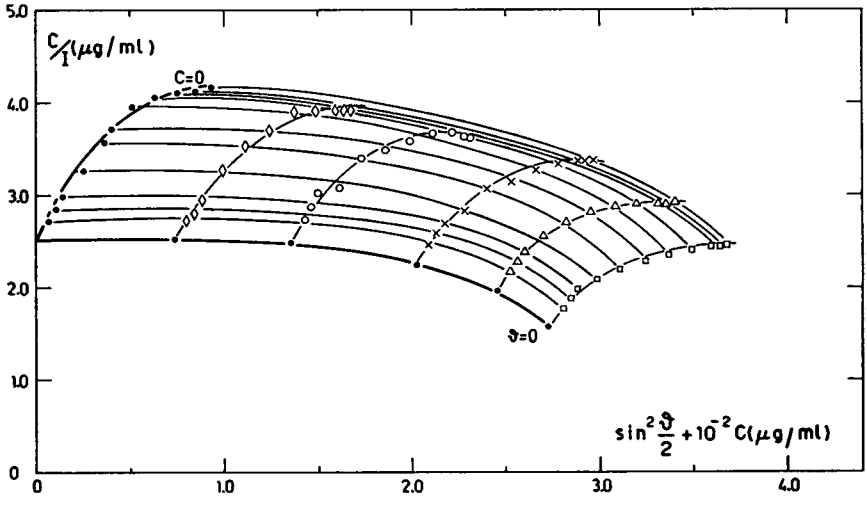

FIGURE 8 Zimm plot of 70S ribosomes. Intercept value $2.55 \cdot 10^{-6}$ g/ml.

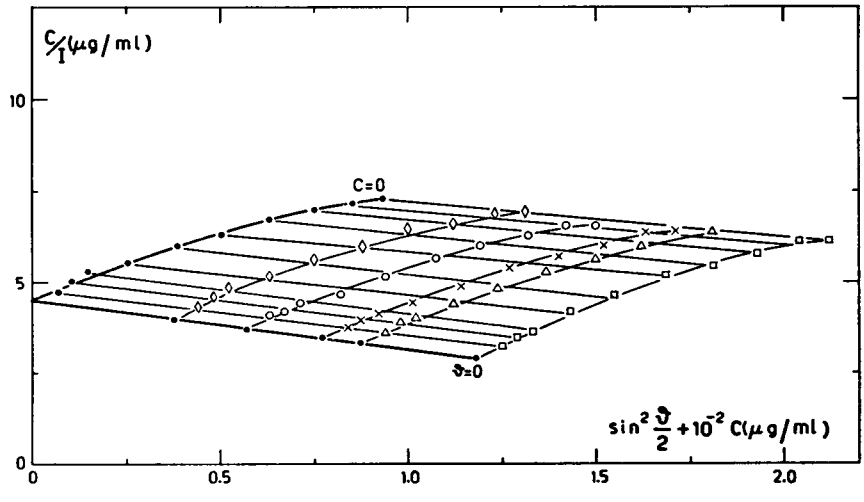

FIGURE 9 Zimm plot of 50S ribosomes. Intercept value $4.65 \cdot 10^{-6}$ g/ml.

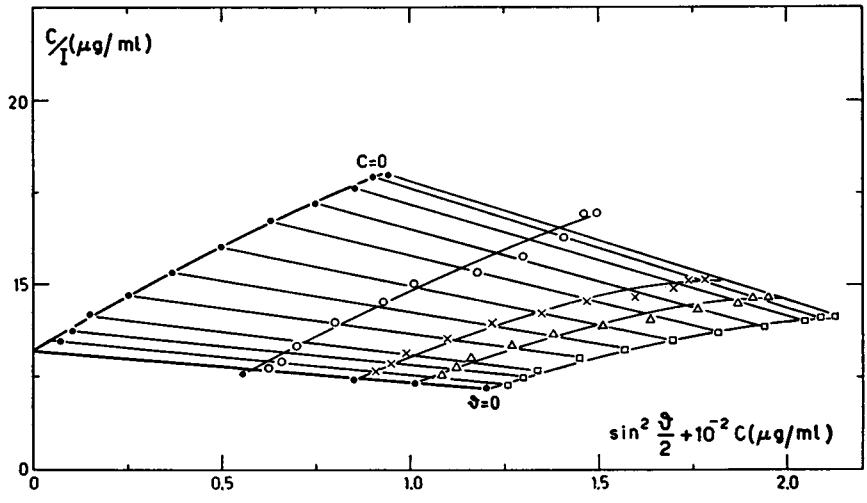

FIGURE 10 Zimm plot of 30S ribosomes. Intercept value $7.05 \cdot 10^{-6}$ g/ml.

TABLE I
30S

Experiment no.	Sedimentation coefficient	Intercept	M	Initial slope $c \to 0$	Slope to intercept ratio $c \to 0$	R_g	D sphere	Initial slope $\vartheta \to 0$	B
	Svedberg						*A*		
G5	28.0	7.95	$0.95 \cdot 10^6$	16.0	2.0	77.0	206	−4.5	$-2.25 \cdot 10^{-2}$
G8	27.4	7.05	$1.08 \cdot 10^6$	10.5	1.49	67.0	178	−4.0	$-2.0 \cdot 10^{-2}$
G11	27.8	6.4	$1.18 \cdot 10^6$	12.0	1.87	74.9	198	−3.0	$-1.5 \cdot 10^{-2}$
G16	26.8	7.20	$1.05 \cdot 10^6$	12.0	1.65	70.6	187	0	0
G18	26.4	8.55	$0.89 \cdot 10^6$	14.0	1.64	70.3	186	+1.0	$0.5 \cdot 10^{-2}$
		Avg M = $(1.0 \pm 0.1) \cdot 10^6$				Avg D_s = 191 ±4 A			

From these results, $I(\vartheta) = (I_\vartheta - I_o) \cdot (\sin[\vartheta/2])/(1 + \cos^2\vartheta)$ was plotted vs. ϑ (and vs. c) for a double interpolation of the experimental points with a continuous curve, correcting visually for statistical fluctuations. It was not considered advantageous to get the best fit in a more refined way, because the form of the $I(\vartheta, c)$ function is not known, while such corrections at this early stage were useful for better drawing the c/I curves in the Zimm plot.

Repeated experiments were carried out for each ribosome component and the best value for the molecular weight was given by the average result.

Figs. 8–10 are typical Zimm plots for 70S, 50S, and 30S respectively.

Tables I–III give all the information that has been derived for each ribosome

TABLE II
50S

Experiment no.	Sedimentation coefficient	Intercept	M	Initial slope $c \to 0$	Slope to intercept ratio $c \to 0$	R_g	D sphere	Initial slope $\vartheta \to 0$	B
	Svedberg						*A*		
G4	49.0	4.50	$1.69 \cdot 10^6$	8.0	1.8	73.7	196	−0.7	$-0.35 \cdot 10^{-2}$
G8	41.0	4.32	$1.76 \cdot 10^6$	6.5	1.49	67.0	178	−1.0	$-0.5 \cdot 10^{-2}$
G10	45.7	4.50	$1.69 \cdot 10^6$	4.5	1.0	54.7	146	−1.0	$-0.5 \cdot 10^{-2}$
G11	48.4	4.20	$1.80 \cdot 10^6$	8.0	1.9	75.4	210	−1.0	$-0.5 \cdot 10^{-2}$
G14	47.0	4.70	$1.61 \cdot 10^6$	4.0	0.85	50.5	134	0	0
G15	52.0	4.65	$1.63 \cdot 10^6$	11.5	2.48	86.4	230	+1.5	$+0.75 \cdot 10^{-2}$
G16	46.0	4.58	$1.65 \cdot 10^6$	9.0	1.98	72.2	206	+1.0	$+0.5 \cdot 10^{-2}$
G17	44.5	4.25	$1.79 \cdot 10^6$	4.0	0.94	53.1	142	+1.0	$+0.5 \cdot 10^{-2}$
			Avg M = $(1.7 \pm 0.1) \cdot 10^6$				Avg D_s = 180 ±11 A		

TABLE III
70S

Experiment no.	Sedimentation coefficient	Intercept	M	Initial slope $c \to 0$	Slope to intercept ratio $c \to 0$	R_g	D sphere	Initial slope $\vartheta \to 0$	B
	Svedberg						*A*		
S1	64.0	2.55	$2.98 \cdot 10^6$	3.5	1.37	64.4	172	0	0
S2	67.5	2.78	$2.72 \cdot 10^6$	11.2	4.03	110.3	283	1.0	$0.5 \cdot 10^{-2}$
S3	65.4	2.45	$3.09 \cdot 10^6$	6.5	2.60	87.9	234	0.5	$2.5 \cdot 10^{-2}$
			Avg M = $(2.9 \pm 0.3) \cdot 10^6$				Avg D_s = 230 ±30 A		

species. In these tables not only the molecular weight, but also the radius of gyration R_g and the second virial coefficient B are given.

DISCUSSION

Some considerations may be made on the results summarized in Tables I–III.

First of all, the value of the extrapolation $(c/I)_{c \to 0}$ can be rather well reproduced from one experiment to the next.

The uncertainty in the molecular weight for 30S ribosomes is of $\sim$ 10% due to greater difficulty in getting enough of this subunit in the purified form, while better results have been obtained for 50S ribosomes.

As for 70S ribosomes, these are also difficult to purify completely, particularly because of the presence of aggregated material due to the higher Mg concentration.

This probably also accounts for the curvature of the $(c/I) - c$ curves at given angles, in the sense that at high concentrations some aggregated material is present and disappears as the concentration is lowered.

The second virial coefficient, given by the initial slope of the $\vartheta = 0$ extrapolated curve, is appreciably zero in our experimental conditions for 70S ribosomes, and presumably also for the two subunits, although some fluctuations have been observed so that we can only say that it is in any case less than $2 \cdot 10^{-2}$ cm³/g in its absolute value.

As for R_g values, the results are fairly good for 50S subunits and somewhat higher with respect to electron microscope results. The limited number of tests gives a rather low accuracy for the 70S ribosome. The results for the 30S subunit, however, are too high, in comparison with both the 50S subunit and the electron microscope observations. The assumed shape was in any case a spherical one, and this hypothesis is acceptable for 50 and 70S, but definitely not for 30S components to which a lenticular shape may better be ascribed.

The ionic strength is rather low under our conditions:

$$\tfrac{1}{2} \sum m_i z_i^2 \simeq 5 \cdot 10^{-2} \text{ for 70S ribosomes}$$
$$\simeq 3 \cdot 10^{-2} \text{ for 50 and 30S ribosomes.}$$

It is possible that the values of B, R_g and M all depend in some related way on the ionic strength.

The hydration of the molecule and its interactions with the solvent are strongly determined by the presence of ions in solutions. Some attempt has been made at finding differences in the Zimm plot features of the ribosomes with the addition of KCl at various concentrations. Work is still in progress in this direction, and further studies are under way on the pH and temperature dependence.

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