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A Contrast Variation Study of *Escherichia coli* Ribosomes Reassembled from Protonated and Deuterated Subunits

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Abstract. Neutron low angle scattering studies on *E. coli* ribosomes reassembled from protonated and deuterated subunits indicate that the association of the two subunits occurs without major distortion of their shape or modification of the distribution of the protein and RNA components.

Key words: Neutron low angle scattering – Contrast variation.

In recent years a variety of methods have been used to study the structure of bacterial ribosomes (for a review see Kurland, 1977). Among these, neutron small angle scattering has proven extremely useful in many applications. The analysis of the scattering functions obtained by the contrast variation method in terms of spherical harmonics has yielded information about the shape of the 50S subunit in solution (Stuhrmann et al., 1977) while label triangulation techniques (Moore et al., 1977; Hoppe et al., 1975) provided direct measurements of protein-protein distances.

However, the unique feature of neutron scattering lies in the fact that it is a straightforward method to probe the distribution of the components – protein and RNA – in the ribosomes. In particular, it has been shown that the 50S subunits display a strong segregation of the proteins and RNA (Stuhrmann et al., 1976) and that their assembly from free components is apparently accompanied by a reduction of the specific volume of the RNA (Crichton et al., 1977) as opposed to the situation in the 30S subunits where the two components are rather homogeneously distributed (Beaudry et al., 1976; Stuhrmann et al., 1978). Further, our previous measurements using contrast variation on native particles (Stuhrmann et al., 1978) suggested that the association of the two subunits in the 70S ribosomes occurs without major distortion of their shape or gross redistribution of the components. To verify this result an alternative approach involving the reassociation of deuterated and native subunits was used.

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Three types of 70S particles were reassembled from deuterated 50S and protonated 30S, from protonated 50S and deuterated 30S and from deuterated 30S and 50S. These 70S ribosomes are subsequently referred to as DH, HD and DD particles respectively.

The results confirm our previous conclusions at least qualitatively. They justify the attempt to identify parts of the 70S ribosomes with the isolated subunits both in electron microscopy and in neutron small angle scattering models. They also exemplify once more the advantage of using the contrast variation method with selectively deuterated biological material.

Materials and Methods

Selectively deuterated 30S and 50S subunits with a degree of deuterium substitution of approximately 90% in the protein and 45% in the RNA as well as native subunits were isolated from *E. coli* (MRE 600) as described previously (Stuhrmann et al., 1978; Moore et al., 1975).

Reassociation of the three types of 70S particles[1] (DD, HD and DH), was performed starting from protonated or deuterated subunits as required. In each case 300 A_{260} units and 520 A_{260} units of the appropriate 30S and 50S preparations respectively were clarified by low speed centrifugation, dialysed against TMA-1 buffer at 4° C (10 mM tris HCl pH 7.4, 10 mM Mg acetate, 30 mM NH_4Cl, 6 mM 2-mercaptoethanol) and preincubated separately for 30 min at 37° C in the same buffer. The different 30S and 50S preparations were then mixed to obtain DD, HD and DH ribosomes respectively and incubated at 37° C for 1 h in a dialysis bag immersed in TMA-1 buffer. The slight excess of 30S particles in the reassociation mixtures was maintained to facilitate the separation of subunits from the reassociated 70S and reduce the contamination by 50S subunits. After incubation the reaction mixture was cooled to 0° C and clarified by low speed centrifugation.

Under these conditions, 95–97% of the 50S starting material was incorporated into 70S particles. The reassociated 70S ribosomes were separated from remaining subunits by centrifugation on a preparative sucrose gradient in a Beckman SW27 rotor. A gradient consisting of 4 ml dilute reaction mixture (150–170 A_{260} units/ml) and 4 ml 7.5% sucrose, both in TMA-1 buffer, was loaded on 28 ml of 10–40% sucrose gradients in the same buffer.

After centrifugation for 18 h at 18000 rpm and 4° C the gradients were fractionated into approximately 40 fractions on an ISCO Gradient Analyser. Aliquots of the fractions were diluted to measure absorption at 260 nm (A_{260}). The fractions containing 70S particles were pooled, brought to 20 mM magnesium acetate and precipitated with one volume of cold (−20° C) ethanol. After 2 h precipitation at −40° C, the precipitate was centrifuged, ethanol was evaporated under vacuum (20 min at 0° C) and the pellets redissolved in and dialysed against scattering buffer 1 (tris HCl 10 mM, pH 7.4, magnesium acetate 10 mM, KCl 100 mM, β-mercaptoethanol 6 mM).

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[1] The S values of the deuterated particles are of course different but for convenience they are referred to by the S values for native particles

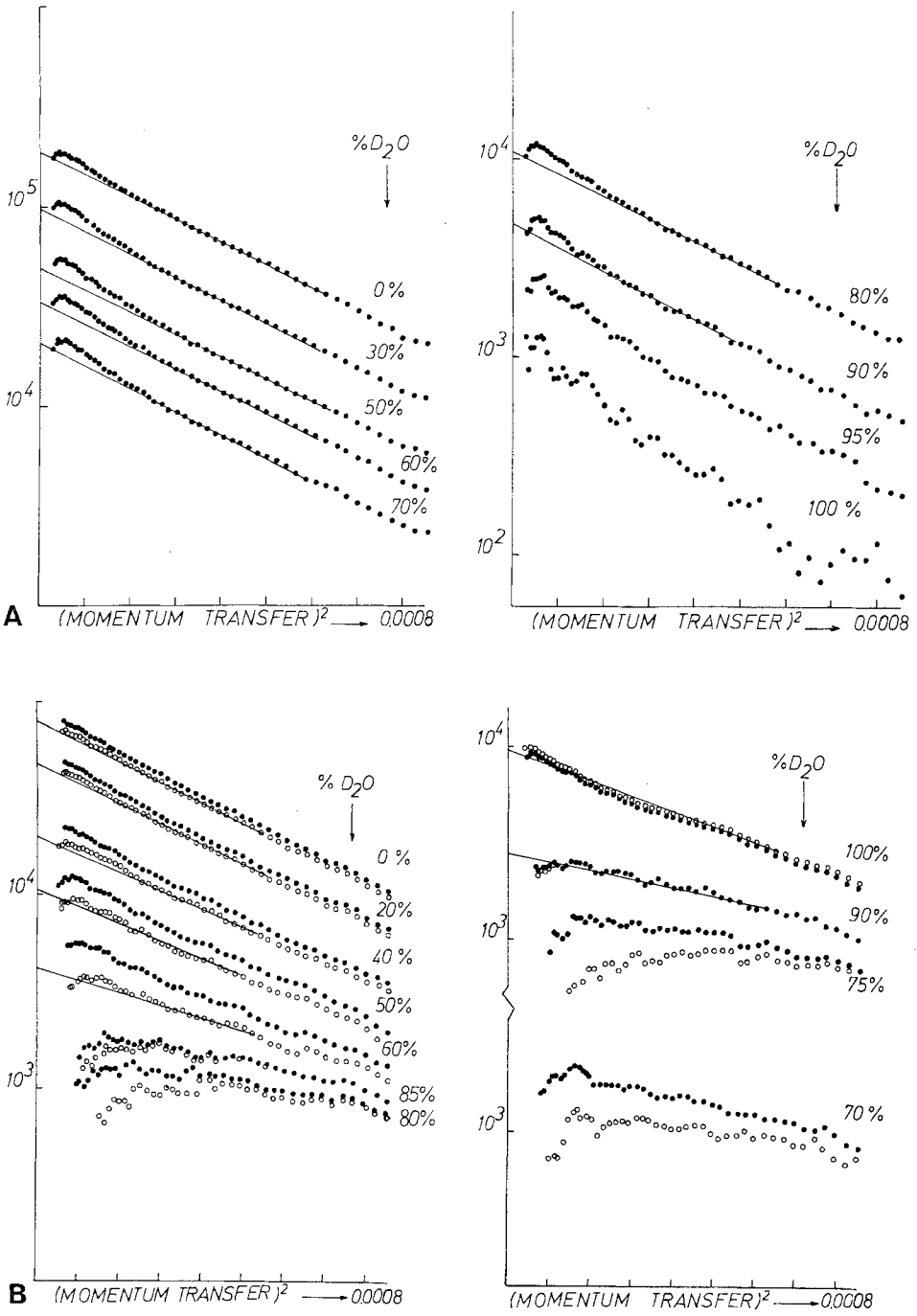

Fig. 1. Guinier plots for the DD (**A**) and HD (**B**) particles. The full circles for the HD ribosomes correspond to the scattering intensity before correction for the contamination by DD particles. The open circles are the curves after correction. The straight lines indicate the regions used for the determination of the radius of gyration

The amount of dimers and free 30S and 50S subunits contaminating the different samples was determined by sucrose gradient centrifugation and resulted in values which were not significantly different but if anything lower than those obtained previously for 70S preparations (3% 100S, 5% 50S and 2.5% 30S) (Stuhrmann et al., 1978).

The solutions corresponding to the different contrasts were prepared by mixing appropriate amounts of two batches which had been extensively dialysed against buffer in H_2O or in D_2O.

Tests for functional activity were carried out as described previously using the method of Fahnestock et al. (1974). All types of particles were found to have similar activities.

Neutron scattering experiments were done at the high flux beam reactor of the Institut Max von Laue–Paul Langevin using the small angle scattering device D11 (Ibel, 1976).

The experimental conditions were the same as reported previously (Stuhrmann et al., 1978) the scattering curves being recorded in a range of momentum transfer ($\kappa = 4\pi \sin \Theta/\lambda$) extending from 4.0 10^{-3} Å^{-1} to 0.5 Å^{-1}. All samples had a concentration of 10.0 $\pm$ 0.5 mg/ml as determined from their absorption at 260 nm. The scattering curves shown in Figure 1 were obtained by subtraction of the background due to the buffer. No corrections were made for the small amount of aggregates or free subunits. The HD and DH samples were not entirely homogeneous since the deuterated subunit preparations contained approximately 15% of the other subunit as indicated by sucrose gradient centrifugation. Since an excess of 30S subunits was used during the reassociation, this resulted in the presence of 7 and 21% DD particles in the HD and DH samples respectively.

A correction was made for the scattering curves of the HD particles to eliminate the contribution of the contaminating DD particles as shown in Figure 1. The corrected curves ($I(\kappa)_{\text{corr.}}$) correspond to the difference:

$$I(\varkappa)_{\text{corr.}} = \frac{I(\varkappa)_{\text{experimental}} - I_{\text{DD}}(\varkappa) \cdot 0.07\ \text{c}}{0.93\ \text{c}}, \tag{1}$$

where $I_{\text{DD}}(\kappa)$ is the intensity which would be scattered by a 1% (w/v) solution of DD particles in the same H_2O/D_2O mixture. This can be readily calculated from the experimental curves of the DD sample. c is the total concentration as measured spectrophotometrically. The calculated contribution of the contaminating particles to the forward scatter as a function of the solvent composition is further illustrated in Figure 2. It should be noted that the points falling in the region where the correction is very important (65–85% D_2O) are not used in the interpretation.

The extrapolated zero angle intensities and radii of gyration were evaluated graphically from plots of the logarithm of the scattering intensity versus the square of momentum transfer (Guinier plots). Counting errors were not propagated in this work. In any case, the qualitative conclusions which are drawn are not affected by variation of the actual numerical values over a wide range. Further, although systematic differences between samples prepared in different ways, e.g. native 70S particles and ribosomes obtained by reassociation of subunits are to be expected, the differences between the present values and those reported previously cannot be considered to be significant.

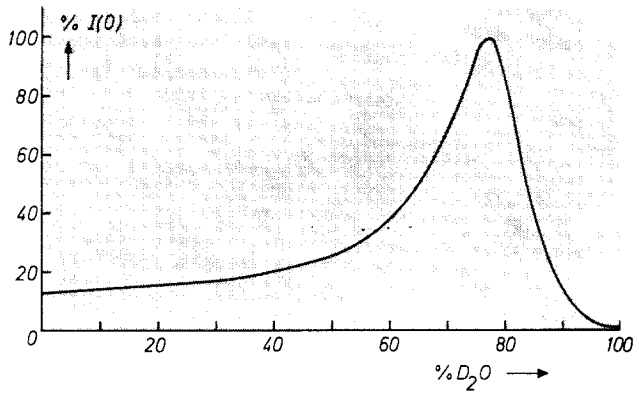

Fig. 2. Percentage of the extrapolated forward scattering [% $I(0)$] due to the contaminating DD particles in the HD sample versus the scattering density of the solvent

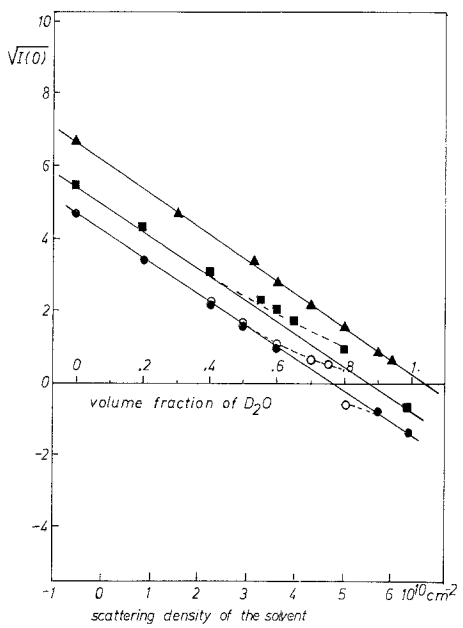

Fig. 3. The square root of the extrapolated forward scatter versus the scattering density of the solvent for the DD (▲), HD (●) and DH (■) particles. The deviations from linearity in the latter two cases are due to the contamination by DD particles. The open circles (○) correspond to the values found for the HD particles after correction for this contamination

Results and Discussion

For a homogeneous sample consisting of identical particles with equal scattering density a plot of the square root of the extrapolated zero angle scattering versus the scattering density of the solvent results in a straight line which intercepts the abscissa when the average scattering density of the solute (ϱ_p) equals that of the solvent. This is for instance the case for the DD ribosomes (Fig. 3), the average scattering density being 6.63 10^{10} cm^{-2}. For a mixture of isomorphous particles with different

average scattering densities the square root of the forward scatter reaches a non-zero minimum when the scattering density of the solvent equals the average scattering density taken over all particles. This phenomenon occurs for the HD and DH samples as seen from the uncorrected scattering curves in Figure 1. The value of $\sqrt{I(0)}$ at the minimum is related to the root mean square deviation of the distribution of scattering densities in the particles (Stuhrmann and Duee, 1975). In the present case there are only two types of particles: HD and DD or DH and DD respectively. Hence $\sqrt{I(0)}$ at the minimum is easily related to the degree of contamination x:

$$\text{Min } \{\sqrt{I(0)}\} \simeq 0.6\ 10^{10}\ \text{cm}^{-2} = [x(6.63 - \varrho_{\min})^2 + (1-x)(4.70 - \varrho_{\min})^2]^{1/2}\ 10^{10}\ \text{cm}^{-2}\,. \tag{2}$$

With $\varrho_{\min}$ 5.2 10^{10} cm this leads to a value of x of approximately 0.05 in agreement with the results of the chemical analysis. The average scattering density of the DD ribosomes is known experimentally and that of the HD and DH particles can easily be calculated to be 4.7 10^{10} cm^{-2} and 5.48 10^{10} cm^{-2} respectively from the scattering densities of the subunits (3.52 and 6.63 10^{10} cm^{-2}) and their volume fractions. This is also the value found after correction for the DD particles as indicated in Figure 2. The plot for the DH sample is given here only as an illustration. It was judged that the contamination by DD ribosomes was too high to allow any further interpretation especially since one could only have used data at lower contrasts than that of the matching point. The slopes of the lines for the three types of ribosomes are identical within experimental error as should be the case for particles having the same effective volume. The value of the molecular weight which can be calculated from the magnitude of the forward scatter obtained from the data in Figure 1 for the DD ribosomes is 2.4 10^6 according to Equation 1 if a concentration of 0.0087 g/ml is used to account for the contamination by dimers and free subunits.

$$M = \frac{\mathrm{I}(0)}{I_{\mathrm{H_2O}}} \cdot \frac{N}{4\pi D} \cdot \frac{\left[\exp\left\{\dfrac{D \cdot N \cdot \sigma_{\mathrm{H_2O}}}{M_{\mathrm{H_2O}}}\right\} - 1\right]}{(\varrho_p - \varrho_{\mathrm{H_2O}})^2\, \bar{\varrho}_c^2\, \bar{\nu} \cdot c}\,. \tag{3}$$

$\dfrac{I(0)}{I_{\mathrm{H_2O}}} = 51.9$

$\sigma_{\mathrm{H_2O}}$ = total cross section of water (226 10^{-24} cm^2)

N = 6.02 10^{23}

$\bar{\nu}$ = 0.59 cm^3/g specific volume of the solute

c = 0.0087 g/cm^3 concentration of solute

D = thickness of the sample: 1 mm

ϱ_p = scattering density of the solute at zero contrast (6.63 10^{10} cm^{-2})

$\varrho_{\mathrm{H_2O}}$ = scattering density of water (−0.56 10^{10} cm^{-2})

$\bar{\varrho}_c^2$ = loss factor due to H/D exchange (0.85)

This value is in reasonable agreement with that reported earlier by Hill et al. (1969) 2.65 ± 0.2 10^6 and corresponds exactly to the sum of the molecular weights generally accepted for the free subunits (1.5 and 0.9 10^6).

To interpret the scattering data for a system consisting of two interacting subunits it is useful to represent the excess scattering density of the system $\varrho(\vec{r})$ as the sum of the contributions of the two subunits.

For the 70S ribosomes this yields using the usual notation of the contrast variation method with appropriate subscripts to designate the terms corresponding to each subunit:

$$\varrho_{70S}(\vec{r}) = (\varrho_{50S} - \varrho)\,\varrho_{c,50S}(\vec{r}) + (\varrho_{30S} - \varrho)\,\varrho_{c,30S}(\vec{r}) + \varrho_{s,50S}(\vec{r}) + \varrho_{s,50S}(\vec{r}) \equiv \bar{\varrho}_{50S}\,\varrho_{c,50S}(\vec{r}) + \bar{\varrho}_{30S}\,\varrho_{c,30S}(\vec{r}) + \varrho_{s,50S}(\vec{r}) + \varrho_{s,30S}(\vec{r})\,. \quad (4)$$

$\varrho_c(\vec{r})$ and $\varrho_s(\vec{r})$ are the shape and the internal structure function as usually defined (Stuhrmann and Kirste, 1965; Ibel and Stuhrmann, 1975)

The contrasts at which the two subunits are seen ($\bar{\varrho}_{50S}$ and $\bar{\varrho}_{30S}$ respectively) are the differences between the average scattering densities of the subunits in the bound state at their matchpoint and that of the solvent. This structure gives rise to a scattering intensity which is given in Equation 5.

$$I(\varkappa) = \bar{\varrho}^2_{50S}\,I_{c,50S}(\varkappa) + \bar{\varrho}^2_{30S}\,I_{c,30S}(\varkappa) + 2\,\bar{\varrho}_{50S}\,\bar{\varrho}_{30S}\,I_{c,30S,\,50S}(\varkappa) + \bar{\varrho}_{50S}\,I_{c,50S,\,s,30S}(\varkappa) + \varrho_{50S}\,I_{cs,50S}(\varkappa) + \bar{\varrho}_{30S}\,I_{cs,30S}(\varkappa) + \bar{\varrho}_{30S}\,I_{c,30S,\,s,50S}(\varkappa) + I_{s,30S}(\varkappa) + I_{s,50S}(\varkappa) + 2\,I_{s,50S,\,30S}(\varkappa) \quad (5)$$

$$I_{c,50S}(\varkappa) = A_{c,50S}(\varkappa)\cdot A^*_{c,50S}(\varkappa)$$

$$I_{c,50S,\,s,30S}(\varkappa) = A_{c,50S}(\varkappa)\cdot A^*_{s,30S}(\varkappa)$$

$$I_{cs,50S}(\varkappa) = A_{c,50S}(\varkappa)\cdot A^*_{s,50S}(\varkappa)$$

$$I_{s,50S}(\varkappa) = A_{s,50S}(\varkappa)\cdot A^*_{s,50S}(\varkappa)\,.$$

All other symbols are evident. A_c and A_s are the amplitudes corresponding to $\varrho_c(\vec{r})$ and $\varrho_s(\vec{r})$.

If one assumes that the 70S particles are formed by simple superposition of the two subunits it becomes possible in principle to separate the terms due to the interaction of the subunits. This requires however a very accurate knowledge of the basic scattering functions for the various particles.

In the case of the HD particles two situations are of special interest. When $\bar{\varrho}_{30S}$ is zero, Equation 5 reduces to

$$I(\varkappa) = \bar{\varrho}^2_{50S}\,I_{c,50S}(\varkappa) + \bar{\varrho}_{50S}\,I_{cs,50S}(\varkappa) + I_{s,50S}(\varkappa) + [\bar{\varrho}_{50S}\,I_{c,50S\,s,30S}(\varkappa) + I_{s,30S}(\varkappa) + 2\,I_{s,30S,\,50S}(\varkappa)]\,. \quad (6)$$

The terms in the square brackets all depend on the contribution to the scattering due to the internal structure of the 30S subunit. Unless a very drastic rearrangement of the protein and RNA occurs in this subunit upon complexation these terms can be

neglected in first approximation especially in the very low angle region. This is justified because this subunit is nearly homogeneous and the contrast at which the 50S moiety is seen is relatively high under those conditions. Thus the very low angle region of the scattering curve corresponding to this situation, and which can easily be obtained by extrapolation should give information about the 50S subunit in the 70S particle.

When $\bar{\varrho}_{50S}$ is zero the terms involving the internal structure of the large subunit cannot be so easily neglected a priori. Fortunately at this particular contrast the small subunit is also seen at a sufficiently high contrast to dominate the very low angle region of the scattering curve.

The most straightforward way to obtain this type of information is to study the variation of the radius of gyration with the contrast $\bar{\varrho}$ (i.e. the difference between the average scattering density of the solute at $\bar{\varrho} = 0$ and that of the solvent).

This quantity varies with the contrast as described by Equation 7 (Ibel and Stuhrmann, 1975)

$$R^2 = R_c^2 + \frac{\alpha}{\bar{\varrho}} - \frac{\beta}{\bar{\varrho}^2}. \tag{7}$$

α is the second moment of the scattering density fluctuation or internal structure $\varrho_s(\vec{r})$. The parameter β is zero when the centre of the excess scattering density corresponds to that of the shape function $\varrho_c(\vec{r})$ defining the particle at all contrasts. If the position of the centre of the excess scattering density of the solute, $\varrho(\vec{r})$, changes with the contrast, this results in a curvature of the R^2 vs $1/\bar{\varrho}$ plot.

The measurement of β however is complicated by the fact that being a second order effect it requires accurate data at low contrast. This is especially true in most biological systems where the scattering densities of the components are not very different. These low contrast data are more difficult to obtain because the intensity is weak. Further these scattering curves are also most affected by the presence of impurities in the sample (Fig. 2).

Graphs of R^2 versus $1/\bar{\varrho}$ are shown in Figure 4 for the DD and HD particles. The values of R_c, the radius of gyration at infinite contrast, are 85 $\pm$ 2 Å, somewhat larger than found with the preparations of native 70S particles, which had not been obtained by a reconstitution process. However, as the latter preparations had a slightly higher contamination in free 50S and 30S subunits the difference with the previously reported value of 81 $\pm$ 2 Å cannot be considered to be significant. The value of α, the slope of the plot in Figure 4, is positive for both types of ribosomes ($\alpha_{DD} = 1.1\ 10^{-3}$, $\alpha_{HD} = 0.5\ 10^{-3}$) as expected for a solute where the higher scattering density material is located on average further from the centre of the particle. This is also in agreement with the negative α found in the native particles where the scattering density of the protein is lower than that of the RNA.

The separation between the centres of the two subunits (Δ) can be determined from the very strong curvature of the graph for the HD particles ($\beta = 4.2\ 10^7\ \text{cm}^{-2}$) using Equation 8.

$$\Delta = \sqrt{\frac{\beta}{\bar{\varrho}_{50S}^2}} + \sqrt{\frac{\beta}{\bar{\varrho}_{30S}^2}}, \tag{8}$$

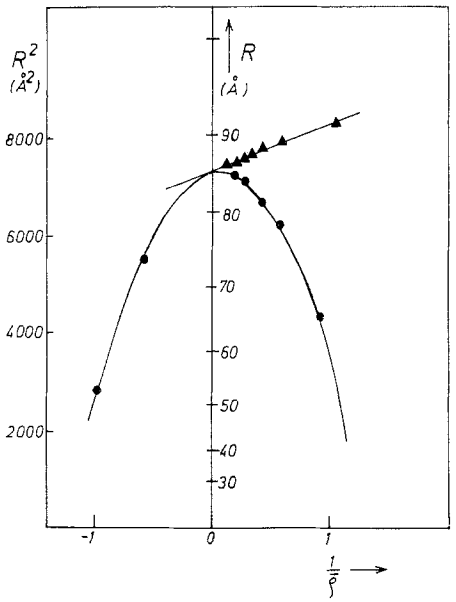

Fig. 4. The square of the radius of gyration plotted against the reciprocal of the contrast for the DD (▲) and HD (●) particles

where $\bar{\varrho}_{50S}$ and $\bar{\varrho}_{30S}$ are the contrasts corresponding to the matchpoints of the subunits in the 70S particles.

As shown in the appendix, this formula is very general and in the particular case of a two-phase system, i.e. a system consisting of two parts having well defined scattering densities the result is equivalent to that given by the Parallel Axis Theorem.

Two conditions should be satisfied to use the formula. First, the volume of the subunits must remain unchanged upon complexation so that the average scattering densities in the 70S ribosomes equal that of the free subunits. Since the volume of the 70S particle is equal to the sum of the volumes of its subunits this is not a severe restriction.

Further, the centre of the scattering density of the complexed subunits must be independent of the contrast (i.e. $\beta_{30S} = \beta_{50S} = 0$). This is not certain but since it has been shown previously that the value of α for the 70S ribosomes can be calculated directly from those of the free subunits it seems unlikely that large rearrangements of RNA and protein occur (Stuhrmann et al., 1978). Under these assumptions a value of 88 ± 15 Å is found for Δ.

Δ can also be calculated from the radii of gyration at infinite contrast and the volume fractions of the free subunits using the Parallel Axis Theorem. This requires the additional assumption that the radii of gyration at infinite contrast do not change when the subunits interact.

With the present value of R_c one finds that $\Delta = 93 \pm 15$ Å. Previously a value of 76 ± 15 Å had been found (Stuhrmann et al., 1978).

The apparent radius of gyration of the solute when the 30S subunit is on average matched ($\varrho_{solv.} = 6.63\ 10^{10}$ cm^{-2}) is approximately 78 Å which can be compared with the 79 Å which one would expect for the free 50S subunit at this contrast.

Similarly the radius of gyration in a solvent containing 59% D_2O is 68.5 Å as expected for the free 30S subunit. The limits of Δ set an upper limit of 5 Å and 10 Å to the possible change of the radius of gyration of the 50S and 30S subunit respectively.

However, if such large changes occurred the relationship between the α-values of the different native particles (Stuhrmann et al., 1978) would not hold.

Another way of illustrating the consistency of the data is to calculate the distance between the centres of the protein distributions of the two subunits in the 70S particle. Using the radii of gyration for the native particles in 68% D_2O and applying the Parallel Axis Theorem value of $\Delta = 88 \pm 10$ Å is found. The distance between the centres of the RNA distributions given by the radii of gyration in 42% D_2O is 81 $\pm$ 10 Å.

Even with the presently available data it seems thus extremely unlikely that changes in the radii of gyration of the subunits of more than 5% would occur upon association.

Bacterial ribosomes are thus most likely formed by the simple superposition of the two subunits without any detectable change in their shape or in the distribution of the protein and RNA components at least at this low resolution.

As already mentioned, the differences between the absolute values of the present and previous results cannot be considered to be significant. The variations observed give an idea of the reproducibility of the results on samples prepared in a different way or differing in their homogeneity and show once again that the standard deviations resulting from the propagation of counting statistics are generally underestimated. Further, they confirm the qualitative model for the structure of ribosomes and also exemplify the fact that useful additional information can be obtained by combining the various approaches to neutron scattering, i.e. solvent exchange methods and specific deuteration.

Appendix

In the case of a system consisting of two components of well-defined scattering density (ϱ_1 and ϱ_2) it follows from the definition of β (Ibel and Stuhrmann, 1975) that:

$$\beta V^2 = [(\varrho_1 - \varrho)V_1\Delta_1 + (\varrho_2 - \varrho)V_2\Delta_2]^2 , \tag{1}$$

where V = total volume of the particle, V_1 = volume of phase 1, V_2 = volume of phase 2, ϱ = average scattering density of the solute: $\varrho V = \varrho_1 V_1 + \varrho_2 V_2$.

$|\Delta_1|$ and $|\Delta_2|$ are the distances between the centres of the distribution of component 1 and 2 respectively and the centre of the particle defined by Equation (2)

$$V_1\Delta_1 + V_2\Delta_2 = 0 . \tag{2}$$

Making use of this relationship it is straightforward to show that:

$$\beta V^2 = (\varrho_1 V_1\Delta_1 + \varrho_2 V_2\Delta_2)^2 . \tag{3}$$

Developing this equation and using Equation (2) again one finds:

$$|\Delta_1| = \frac{V}{V_1}\left[\frac{\beta}{(\varrho_2 - \varrho_1)^2}\right]^{1/2} \quad \text{and} \quad |\Delta_2| = \frac{V}{V_2}\left[\frac{\beta}{(\varrho_2 - \varrho_1)^2}\right]^{1/2} . \tag{4}$$

Since $\Delta = |\Delta_1| + |\Delta_2|$ and $\frac{V}{V_1} + \frac{V}{V_2} = \frac{V^2}{V_1 V_2}$,

$$\Delta = \frac{V^2}{V_1 V_2}\left[\frac{\beta}{(\varrho_2 - \varrho_1)^2}\right]^{1/2}. \tag{5}$$

This can easily be transformed into Equation (8).

When the scattering density of the solvent equals that of the first component the contrast is given by:

$$\varrho - \varrho_{\text{solvent}} = \varrho - \varrho_1 = \frac{(\varrho_2 - \varrho_1)V_2}{V} = \bar{\varrho}_1 . \tag{6}$$

$$\text{Thus} \quad |\Delta_2| = \left[\frac{\beta}{\bar{\varrho}_1^2}\right]^{1/2} \quad \text{and similarly} \quad |\Delta_1| = \left[\frac{\beta}{\bar{\varrho}_2^2}\right]^{1/2}. \tag{7}$$

Hence, it follows that

$$\Delta = \sqrt{\frac{\beta}{\bar{\varrho}_1^2}} + \sqrt{\frac{\beta}{\bar{\varrho}_2^2}}. \tag{8}$$

The Parallel Axis Theorem states that the distance (Δ) between the centre of mass of the two components having radii of gyration R_1 and R_2 and scattering mass fractions f_1 and f_2 respectively and R_T the radius of gyration of the whole system are related by:

$$\Delta = \left[\frac{R_T^2 - f_1 R_1^2 - f_2 R_2^2}{f_1 f_2}\right]^{1/2}. \tag{9}$$

Both Equation (8) and (9) require the knowledge of the exact scattering densities of the components in situ.

However, it can be shown that for any system of two subunits the distance (Δ_0) between the centres of the two subunits at the zero contrast of the whole system is given by:

$$\Delta_0 = \frac{V^2}{V_1 V_2}\left[\frac{\beta}{(\varrho_2 - \varrho_1)^2}\right]^{1/2} \tag{10}$$

regardless of the internal structure of each of the subunits considered separately.

ϱ_1 and ϱ_2 are the average scattering densities of the subunits. Moreover, if $\beta_1 = \beta_2 = 0$ for the subunits in the complex the distance between their centres is independent of the contrast. In particular this is also the distance at infinite contrast (Δ_∞) i.e. the distance between the centres of the shape functions which describe the subunits in the complex.

In the general case ($\beta_1 \neq \beta_2 \neq 0$) one can show that

$$|\Delta_0 - \Delta_\infty| \leq \left[\frac{\beta_1}{(\varrho_1 - \varrho)^2}\right]^{1/2} + \left[\frac{\beta_2}{(\varrho_2 - \varrho)^2}\right]^{1/2}. \tag{11}$$

Thus, if a large β value exists, or can be produced, in a complex system it is better to estimate the distance between the centres of the components using this parameter rather than the Parallel Axis Theorem.

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