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Biological Macromolecules in Solvents of Variable Density: Characterization by Sedimentation Equilibrium, Densimetry, and X-ray Forward Scattering and an Application to the 50S Ribosomal Subunit from *Escherichia coli*†

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ABSTRACT: Solvents of variable density are commonly used for structural analysis of biological macromolecules. Solvent density can be altered by either the use of water isotope mixtures or addition of small solute molecules to the solution. Under such conditions the composition of the macromolecules may vary because of preferential interactions with the solvent components. Among the techniques which may be used to study these interactions, it is well-known that three approaches, sedimentation equilibrium, densimetry, and X-ray forward scattering, provide essentially similar information. In the thermodynamic approach, the data obtained by any of these techniques are usually interpreted in terms of macroscopic quantities, density increment or electron density increment, and then the particle is described by three physical parameters, molecular weight, partial specific volume, and one phenomenological interaction parameter ξ which is a function of solvent composition. Going further requires the introduction of a model. In the following, we consider *globular* macromolecules: we show that whenever linear variation of the density increment or electron density increment is observed as a function of solvent density, a more detailed description of the macromolecule is obtained without introducing a model in terms of molecular weight, partial specific volume, and two constant parameters β_1 and β_3 (grams of water and of small solute molecules per gram of macromolecule). In other words, it is possible in that particular case to define an equivalent macromolecule which is invariant with respect to solvent composition. The use of this "particle approach" is presented in the context of more detailed structural analysis with solution scattering techniques. In addition, the respective advantages of using sedimentation equilibrium, densimetry, and X-ray forward scattering, either alone or coupled, to determine molecular weight, partial specific volume, and interaction parameters are discussed, and new possibilities are proposed. As an illustration, we describe the combination of sedimentation equilibrium (SE) and solution X-ray scattering (SXRS) to determine a number of physical parameters relevant to the 50S subunit from *Escherichia coli* ribosomes. Molecular weight and partial specific volume were determined from SE experiments in water isotope mixtures and from coupling of SE and SXRS experiments in water. Interaction parameters were determined independently from SE and SXRS experiments in solutions containing various amounts of sucrose. The results obtained demonstrate that the composition of the 50S subunit is invariant over the range of sucrose concentration studied (0–50%) and that only water is associated to the subunit. This amount of water is calculated to be 0.41 ± 0.06 g/g from SE data and 0.37 ± 0.02 g/g from SXRS data.

In recent years, the use of solvent scattering density variation in scattering experiments has proved to be a powerful tool for structural analysis of biological systems in solution. The variation of scattering density is achieved either by addition of low molecular weight electron-dense compounds to the solution (X-ray) or by alteration of the H_2O/D_2O ratio (neutron). The information that may be obtained under these conditions is illustrated in a large number of publications [see the principles of the approach in Stuhrmann & Kirste (1965) and recent reviews by Jacrot (1976) and Luzzati & Tardieu (1980)].

However, one is well aware that the composition of the particle under study may vary according to solvent composition because of H/D exchange (SNS)[1] or because of preferential interactions between the particle and the diffusible components of the solvent (SXRS). In SNS, it is generally assumed that H/D exchange can be corrected for on the basis of a linear variation of the exchange with D_2O content (Jacrot, 1976). On the contrary, whenever contrast variation is used in SXRS, there is a general concern about the fate of the particle in the rather unusual environment of a solution several molar in salt or containing a high concentration of sucrose.

Working with SXRS, we have looked for the best way to analyze the composition of the particle under study and in particular to characterize the interactions between the diffusible components of the solvent and the macromolecule. Several physical approaches can be considered for this purpose. We selected three which essentially measure the same class of physical parameters: densimetry, sedimentation equilibrium, and X-ray forward scattering.

This paper is intended as an attempt to put together the various approaches which allow us to describe the particle at different levels of complexity. We begin with the description of the particle developed by Eisenberg (Casassa & Eisenberg, 1964; Cohen & Eisenberg, 1968; Eisenberg, 1976), which makes use of an additional parameter, (besides molecular weight and partial specific volume) the "interaction parameter ξ". ξ takes into account possible preferential interactions between the particle and all the diffusible components of the solvent and has the advantage of being measurable from either

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[1] Abbreviations used: SE, sedimentation equilibrium; SNS, solution neutron scattering; SXRS, solution X-ray scattering; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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SE, densimetry, or SXRS. More elaborate descriptions of the particle require the introduction of a model. We show here that if the interaction parameter displays a certain type of variation with increasing solvent density, a more detailed description of the particle is obtained without the introduction of any model: for the measurements under consideration the particle behaves as if constant amounts of diffusible components were bound to it whatever the solvent composition. In other words, it is possible to define an equivalent particle which is invariant with respect to the solvent. Furthermore, it is sometimes possible to show that the particle behaves as if only water were associated to it. In the second section we review the various practical possibilities of the three approaches, either alone or coupled, in the determination of the physical parameters which are of interest to us, that is, molecular weight, partial specific volume, and the various interaction parameters. However, the main limitation of the approaches discussed here is that they are only sensitive to excesses of density with respect to the solvent. Thus they do not provide a means of detecting solvent permeability, i.e., the composition of the particle under study and the equivalent particle defined may differ by a certain amount of solvent which would never be detected. Without entering into detailed structural analysis, we indicate that further information including the question of solvent permeability may, in favorable cases, be provided by the analysis of the global properties of the SXRS intensity curves when these data are available.

Combination of densimetry measurements and SXRS has already been used in our laboratory (Aggerbeck et al., 1978). As an illustration, we report here on the application of the coupling of SE and SXRS to a system whose structural analysis is presently under progress: the 50S subunit from *Escherichia coli* ribosomes. The experiments presented here were undertaken to determine molecular weight and partial specific volume and to document the behavior of the 50S ribosomal subunit in the solutions containing variable amounts of sucrose which were used for the SXRS contrast variation experiments.

Definition of Experimental Conditions

In the following, the solute particles under consideration are *globular particles*, i.e., particles in which none of the dimensions is 1 order of magnitude larger than the other dimensions. In addition, *these particles do not display polyelectrolyte behavior in* solution. The solute particles will be referred to in the following as component 2.

The particles are studied either in water or water isotope mixtures, D_2O, $H_2{}^{18}O$, and $D_2{}^{18}O$ (two-component systems assuming H/D exchange correction), or in aqueous solutions containing, in addition, small solute molecules like salt, sucrose, and glycerol (three-component systems). The small molecules used to increase the solvent density, irrespective of charges, are component 3, water being component 1. However, biological systems are usually studied in aqueous buffers of low ionic strength (typical range 0.01–0.1). Consequently, in such cases, we shall operationally define component 1 as the solvent in the absence of component 3.[2] Thus, in the following, solvent means component 1 plus possibly component 3, and solution means the solvent plus the solute particles. The components of the solvent are sometimes referred to as the diffusible components.

Table I: Parameters Used

M_j, m_j, μ_j	molecular weight (g/mol) and number of electrons of component *j* (e) and their ratio $\mu_j = m_j/M_j$
μ_s	ratio of the number of electrons/molecular weight of the solvent
ρ_0, ρ_{e0}	density (g/cm^3) and the electron density (e/cm^3) of the solvent, $\rho_0 = \rho_{e0}/\mu_s N$
ρ, ρ_e	density (g/cm^3) and electron density (e/cm^3) of the solution
V, n	volume of the sample (cm^3) and number of solute particles in this volume
N	Avogadro's number
c_j, c_{ej}, c_{vj}, c_{evj}	concentration in g/g, e/e, g/cm^3, and e/cm^3 of component *j*
ν_j, Ψ_j	partial specific volume (cm^3/g) and electron partial specific volume (cm^3/e) of component *j*, $\Psi_j = \nu_j/\mu_j N$

The experiments are always supposed to be performed at *constant chemical potentials* of the diffusible components, even when not explicitly stated; the equations derived in the following are only valid under these conditions. Operationally, this is obtained by equilibrating the solutions against the solvent by exhaustive dialysis. In practice, such an equilibration is only critical for densimetry experiments.

The interpretation of the experiments is done by assuming that the solutions fulfill a number of conditions, in particular, the following. (1) The particles are identical. (2) The solutions are ideal, i.e., the distribution of the particles is random, without correlation in position or orientation. Operationally, this condition is attained by extrapolation of the parameters obtained to vanishing concentration of component 2. (3) The solutions are incompressible. (4) There is no redistribution of the diffusible components in the centrifugal field in the absence of component 2. (5) Outside the regions perturbed by the solute molecules, the solvent may be treated as a medium of uniform density, and the fluctuations introduced by the addition of small solute molecules can be discarded.

These conditions are in general well fulfilled by solutions of biological particles. For an extensive discussion of some of these points, see Eisenberg (1976), Tanford (1961), and Luzzati & Tardieu (1980). In addition, for the sake of simplicity, we suppose in the following that the partial specific volumes of the various components remain constant.

Notation

Various notations can be found in the literature, which are difficult to reconcile. Even if we try to make use of the most commonly adopted definitions, we are faced with the problem that some parameters like ρ have a different meaning in thermodynamics and in scattering techniques. We have adopted the compromises shown in Table I. The intensity at the origin normalized to one electron of solute, $i_n(0,\rho_{e0})/c_{e2}$, can experimentally be obtained by extrapolation of the Guinier plot to $s = 0$ (Guinier & Fournet, 1955).

Theory

Characterization of Biological Macromolecules in Solvents of Variable Density by SE, Densimetry, and SXRS. (*I*) *Description of the Composition of the Particle.* (*1*) *Review of Basic Equations of SE, Densimetry, and SXRS.* In the following, we first are obliged, for the sake of clarity, to recall the basic equations of SE, densimetry, and SXRS. The derivation of these equations may be found elsewhere (Luzzati, 1960; Tanford, 1961; Casassa & Eisenberg, 1964; Pessen et al., 1973; Eisenberg, 1976; Lee et al., 1980). The nomen-

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[2] A practical difficulty may appear in this definition in the case where the same salt is used in the buffered solution and as the density-varying agent; this was the case, for example, when NaBr was used to study low-density lipoproteins (Luzzati et al., 1979). In this case, the buffer will still be considered as water, and variation in density will be calculated by taking into account the differences in salt concentration.

clature for SXRS was chosen in order to make the similarities of the equations more apparent than with the classical thermodynamic notation.

SE and densimetry measure properties of macroscopic systems which are conveniently expressed in terms of the density increment $\partial\rho/\partial c_{v2}$ of the solution with respect to the solvent, introduced by the presence of component 2. The corresponding X-ray data, i.e., those relevant to the thermodynamic properties of the system, are the intensity at the origin. When normalized to one electron of solute, it can be expressed as a function of the electron density increment. The following set of relationships is obtained:

$$\rho - \rho_0/c_{v2} = \partial\rho/\partial c_{v2} \qquad (1)$$

$$(2RT/\omega^2)(\mathrm{d}\ \ln c_2/\mathrm{d}r^2) = M_2\partial\rho/\partial c_{v2} \qquad (2)$$

$$i_n(0,\rho_{e0})/c_{e2} = m_2(\partial\rho_e/\partial c_{ev2})^2 \qquad (3)$$

These density increments may be expressed in different ways from the molecular properties of the solute. We find it convenient to introduce the expression of the concentrations

$$c_{v2} = nM_2/VN \quad c_{ev2} = nm_2/V \qquad (4)$$

and call ΔM the excess of mass associated with 1 mol of solute and Δm the excess of electrons associated with one solute particle with respect to the solvent:

$$\Delta M = NV(\rho - \rho_0)/n \qquad \Delta m = V(\rho_e - \rho_{e0})/n \qquad (5)$$

Eq 1–3 become

$$\rho - \rho_0/c_{v2} = \Delta M/M_2 \qquad (6)$$

$$(2RT/\omega^2)(\mathrm{d}\ \ln c_2/\mathrm{d}r^2) = \Delta M \qquad (7)$$

$$i_n(0,\rho_{e0})/c_{e2} = \Delta m(\Delta m/m_2) \qquad (8)$$

Equations 6–8 mean, in other words, that densimetry measures the excess of mass associated with 1 g of particle with respect to the solvent, SE the excess of mass associated with 1 mol, and SXRS the product of the corresponding quantities associated with one particle measured in electrons.

(*2*) *Molecular Weight and Partial Specific Volume.* In two-component systems, the excess of mass associated with the particle is the one associated with the dry particle ΔM_2, and it may be expressed in a simple way as a function of molecular parameters: molecular weight and partial specific volume of the particle. Equations 1–3 become, as $\Delta M = \Delta M_2 = M_2 - M_2 v_2 \rho_0$

$$(\rho - \rho_0)/c_{v2} = 1 - v_2\rho_0 \qquad (9)$$

$$(2RT/\omega^2)(\mathrm{d}\ \ln c_2/\mathrm{d}r^2) = M_2(1 - v_2\rho_0) \qquad (10)$$

$$i_n(0,\rho_{e0})/c_{e2} = m_2(1 - \Psi_2\rho_{e0})^2 \qquad (11)$$

These equations are the usual basis of molecular weight and partial specific volume determinations which will be discussed in section II.

(*3*) *Interaction Parameters.* In *three-component systems*, since preferential associations between the particle and the diffusible components of the solvent may occur, the excess of mass is composed of two parts, ΔM_2 which corresponds to the excess of mass associated with the dry particle and another contribution which measures the preferential associations. Eisenberg (Casassa & Eisenberg, 1964; Eisenberg, 1976) has introduced a convenient formulation and defines interaction parameters ξ_j and ξ_{ej} which include contributions from all the diffusible components, expressed in terms of component *j*. For instance, ξ_1 measures the amount of water which would have to be bound to the particle to give rise to the measured excess of mass:

$$(\rho - \rho_0)/c_{v2} = 1 - v_2\rho_0 + \xi_1(1 - v_1\rho_0) \qquad (12)$$

$$(2RT/\omega^2)(\mathrm{d}\ \ln c_2/\mathrm{d}r^2) = M_2[1 - v_2\rho_0 + \xi_1(1 - v_1\rho_0)] \qquad (13)$$

$$i_n(0,\rho_{e0})/c_{e2} = m_2[1 - \Psi_2\rho_{e0} + \xi_{e1}(1 - \Psi_1\rho_{e0})]^2 \qquad (14)$$

In the same way, it is possible to define an interaction parameter ξ_3, since $\xi_1(1 - v_1\rho_0)$ may also be written $-\xi_3(1 - v_3\rho_0)$ in eq 12–14. The ξ_j or ξ_{ej} values are unambiguously defined and may be measured by SE, densimetry, or SXRS experiments as a function of solvent density. The ξ_j and ξ_{ej} are functions of ρ_0 and ρ_{e0}, but for a given density ρ_0 and its corresponding electron density ρ_{e0}, we have the relationship $\xi_{e1} = (\mu_1/\mu_2)\xi_1$ or $\xi_{e3} = (\mu_3/\mu_2)\xi_3$. Let us finally recall that, as constantly emphasized by Eisenberg, the operational parameters ξ_j cannot be interpreted in terms of molecular mechanisms, without introduction of a model.

At that stage, in order to go further, thermodynamicists generally introduce a model in terms of solvation or component 3 binding. We wish to show here that a more detailed description of the particle may be obtained, without the introduction of any model, if constraints are added on the variation of the interactions parameters as a function of solvent density.

(*4*) *Equivalent Particle.* In the following, we consider the case where *linear variation* of density increments or electron density increments are observed in the presence of component 3 as a function of ρ_0 or ρ_{e0}. This case is of special interest for us, since it is the only one that has been encountered until now in scattering experiments (Tardieu et al., 1976; Sardet et al., 1976; Sperling, 1977; Aggerbeck et al., 1978; Luzzati et al., 1979; this work, see below).

For the sake of simplicity, we develop the consequence of a linear variation only for density increments. The equations for electron density increments are similar. The linear variation of density increments (or excess of mass) can be expressed by

$$\partial\rho/\partial c_{v_2} = A - C\rho_0 \qquad (15)$$

where *A* and *C* are numerical constants whose values are the *y* intercept and slope of the straight line. As emphasized by Eisenberg (1976), a linear variation of $\partial\rho/\delta c_{v_2}$ vs. ρ_0 does not imply the invariance of ξ_1. In fact, the consequence for the ξ_1 value is a homographic type of variation with ρ_0

$$\xi_1 = [(A - 1) - (C - v_2)\rho_0]/(1 - v_1\rho_0) \qquad (16)$$

However, it is now possible to write the density increment as a function of two parameters β_1 and β_3 which are *constant whatever the solvent composition*:

$$\partial\rho/\partial c_{v2} = 1 - v_2\rho_0 + \beta_1(1 - v_1\rho_0) + \beta_3(1 - v_3\rho_0) \qquad (17)$$

Indeed, by equating eq 16 and 17 and if we separate the terms into the following system of equations

$$1 + \beta_1 + \beta_3 = A \qquad (18)$$

$$v_2 + \beta_1 v_1 + \beta_3 v_3 = C \qquad (19)$$

it is clear that we can solve the system for β_1 and β_3:

$$\beta_1 = [(A - 1)v_3 - (C - v_2)]/(v_3 - v_1) \qquad (20)$$

$$\beta_3 = [(C - v_2) - (A - 1)v_1]/(v_3 - v_1) \qquad (21)$$

β_1 and β_3 are operational quantities, whose values can be unambiguously determined from a series of experiments performed at various densities. We have thus obtained a more detailed description of the particle in terms of two β's instead of one ξ. We wish to emphasize that this more detailed description is obtained without the introduction of any model. This description does not require an intermediate determination of the ξ_j parameters;[3] it directly results from the additional constraint of linearity. In physical terms it means that the particle behaves as if constant amounts β_1 (g/g) of component 1 and β_3 (g/g) of component 3 were associated to it whatever the solvent composition. In other words, we have defined an equivalent particle composed of the dry particle itself associated with β_1 (g/g) of component 1 and β_3 (g/g) of component 3.

Since the same demonstration could be achieved from linear variation of electron density increments, we have at the same time shown that a linear variation of $\partial\rho/\partial c_{v_2}$ vs. ρ_0 implies a linear variation of $\partial\rho_e/\partial c_{ev2}$ vs. ρ_{e0} and vice versa. In conclusion, each of the three techniques under consideration provides us with a description of the particle in terms of a "minimal equivalent particle" comprising β_1 (g/g) of water and β_3 (g/g) of component 3.

Since these three techniques measure excesses of mass with respect to the solvent, the difference between the equivalent particle and the particle under study becomes clear: as far as composition is concerned, they can only differ by a certain amount of solvent, which means, in other terms, that the particle under study may well be partially permeant to solvent. In the same way, the equivalent particle we define and the models proposed by the thermodynamicists may well differ by a certain amount of solvent.

This equivalent particle occupies an invariant volume V^*

$$V^* = M_2(v_2 + \beta_1 v_1 + \beta_3 v_3)/N \qquad (22)$$

and the mean density and electron density within this volume are

$$\rho^* = (1 + \beta_1 + \beta_3)/(v_2 + \beta_1 v_1 + \beta_3 v_3) \qquad (23)$$

$$\rho_e^* = (1 + \mu_1\beta_1 + \mu_3\beta_3)/(\Psi_2 + \Psi_1\beta_1 + \Psi_3\beta_3) \qquad (24)$$

From these relationships, other expressions of density and electron density increments may be derived which are commonly used, especially in scattering experiments, to determine the position at which the density increment vanishes:

$$\partial\rho/\partial c_{v2} = V^*N(\rho^* - \rho_0)/M_2 \qquad (25)$$

$$\partial\rho_e/\partial c_{ev2} = V^*(\rho_e^* - \rho_{e0})/m_2 \qquad (26)$$

Yet, it has to be noted that the treatment we present does not at all require us to achieve "matching" of the particle.

Of special interest is the case where the experimental point recorded in the absence of component 3 lies on the straight line. The coordinates of this point are $1/v_1$ and $M_2(1 - v_2)$. In such a case we have the relationship

$$A - 1 = (C - v_2)/v_1 \qquad (27)$$

then, eq 20 and 21 simplify to

$$\beta_1 = A - 1 \qquad (28)$$

$$\beta_3 = 0 \qquad (29)$$

and it comes from eq 16 and 20

$$\beta_1 = \xi_1 = \text{constant} \qquad (30)$$

Equations 28–30 lead to the conclusion that in the particular case where the point in water lies on the straight line, the particle behaves as if only a constant amount of water is associated to it. Since the three techniques considered are equivalent in this respect, the demonstration that only water is associated to the equivalent particle may be achieved by any of them.

(*II*) *Experimental Determination of Physical Parameters Relevant to the Composition of the Particle.* The parameters to be determined are the molecular weight M_2, the partial specific volume v_2, the interaction parameters ξ_j or ξ_{ej}, and in favorable cases the parameters β_j which define the equivalent particle. Among SE, densimetry, and SXRS, only SE is able to provide us with all these parameters by itself. Furthermore, whenever densimetry or SXRS is involved, it requires an independent determination of the absolute concentration of the solute. In the following, after briefly recalling the procedures widely used to determine these parameters, we propose new possibilities, based on combination of SE, densimetry, and SXRS.

(*1*) *M_2 and v_2 Determinations.* These determinations are most easily performed in water whenever it is possible. Usually, the partial specific volume is measured directly by densimetry (Kratky et al., 1973), and then SE or XSRS provides the molecular weight (eq 10 and 11) (Luzzati, 1960; Tanford, 1961; Schachman & Edelstein, 1973).

In SE and densimetry experiments, water isotopes $H_2{}^{18}O$, D_2O, and $D_2{}^{18}O$ may be used to change the solvent density. It has been shown that such an approach can be applied to determine M_2 and v_2 by SE alone (Edelstein & Schachman, 1967, 1973). Measurements performed as a function of ρ_0 give a straight line (eq 10), whose ordinate at the origin and slope are equal to M_2 and M_2v_2, respectively.

Another possibility, not used to our knowledge until now, is the coupled use of SE and SXRS. Combination of eq 10 and 11 leads to the elimination of M_2:

$$(2RT/\omega^2)[\mathrm{d}\ln c_2/\mathrm{d}r^2]/[i_n(0,\rho_{e0})/c_{e2}] = (1/\mu_2)(1 - v_2\rho_0)/(1 - \Psi_2\rho_{e0})^2 \qquad (31)$$

This equation is then solved for v_2. Equation 31 has two solutions, one of which has no physical meaning. In general, the choice is unambiguous, since the range of v_2 is known and the two solutions are quite different. If both values happen to be very close, a second SE experiment at a different density will provide the answer. Equation 31 may also degenerate into a first degree equation if the ratio of density to electron density is the same for both solvent and solute particles, that is, if $\mu_2 = \mu_s$.

We mentioned at the beginning of this section that SXRS and densimetry require knowledge of the concentration while SE does not. An attractive possibility is the triple use of SE, densimetry, and SXRS. The ratio of SXRS and densimetry measurements is independent of absolute concentration determination:

$$[(\rho - \rho_0)/c_{v2}]/[i_n(0,\rho_{e0})/c_{e2}] = (1/m_2)(1 - v_2\rho_0)/(1 - \Psi_2\rho_{e0})^2 \qquad (32)$$

Thus eq 32 and 10 may then be solved for M_2 and v_2 and the concentration determined by using either eq 11 or 9.

If the density of the particle under study is close to 1, measurements in H_2O may be difficult or even impossible. In fact, this difficulty is only serious for SXRS measurements, since SE and densimetry experiments can usually then be performed in water isotopes. However, even if it is not possible to obtain the M_2 value from measurements in water, it can also be determined by making use of multicomponent systems. Indeed, if SE and densimetry are performed at the same density ρ_0, M_2 is immediately derived from eq 1 and 2. Also, in principle, electron-density increments could be determined from density increments measured by densimetry and then m_2 determined from eq 3. However, in practice, if v_2 is not known, the calculation of electron density increments from density increments or vice versa is often not possible; such a calculation

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[3] Of course the ξ_j may be written as a function of β_1 and β_3 (eq 15 and 20):

$$\xi_1 = \beta_1 + \beta_3(1 - v_3\rho_0)/(1 - v_1\rho_0)$$

requires the knowledge of the chemical composition of both solution and solvent, which are not in general known with sufficient accuracy. Other possibilities to determine M_2 and v_2 together with other parameters are mentioned below.

(*2*) *Interaction Parameters.* Once M_2 and v_2 are known, the ξ_j or ξ_{ej} values at any density ρ_0 are immediately derived (eq 12–14) from any measurements by SE, densimetry, or SXRS. In the case where only M_2 is known (see above) the coupling of SE or densimetry with SXRS may be of particular interest. If SXRS and SE or SXRS and densimetry are performed at one electron density ρ_{e0} and the corresponding density ρ_0, eq 12 and 14 or 13 and 14 can in principle be solved for v_2 and ξ_1. To our knowledge, such an approach was never tried, and we do not know whether the accuracy of the experimental data would be sufficient for the calculations. Although we assume here that we are working with systems whose partial specific volumes can be considered as constant, we think it worth mentioning that such experiments could in principle be used to follow possible changes of partial specific volumes as a function of solvent density.

(*3*) *Equivalent Particle.* Whenever linear variations of density or electron-density increments are observed, an equivalent particle can be defined.[4] The parameters which describe this equivalent particle, β_1 and β_3 (or β_{e1} and β_{e3}) are immediately derived from SE, densimetry, or SXRS experiments by using eq 20 and 21. When the point recorded in the absence of component 3 lies on the straight line recorded in the presence of component 3, it is immediately clear that β_3 is equal to 0. We mention once more that if the point in H_2O cannot be measured, the difficulty may be overcome by performing SE or densimetry experiments with water isotopes.

If M_2 is not known, it is clear from the form of eq 25–26 that the molecular weight may be obtained for example from the ratio of the slopes of the straight lines obtained by SE and densimetry or by SXRS and densimetry (Aggerbeck et al., 1978). The partial specific volume v_2 is then directly derived from any experiment performed in water. Obviously, the coupling of SXRS, densimetry, and SE offers many possibilities, and trying to review all of them would be tedious.

(*III*) *Additional Information from Overall Properties of SXRS Curves.* We have demonstrated previously that if density or electron-density increments vary linearly as a function of ρ_0 or ρ_{e0}, it is possible to define an equivalent particle, whose composition is identical with the one of the particle under study, but for a possible solvent permeability. We have reached here the limits of thermodynamics. Without entering into detailed structural analysis, we want to mention here that information on this last point may be obtained from some of the overall properties of the X-ray scattering curves and, in particular, from the value of the autocorrelation function at the origin.

The autocorrelation function at the origin measures the average of the *square* of the electron-density contrast associated with the particle which depends on the solvent permeability of the particle. However, the value of the autocorrelation function at the origin not only depends upon the *composition* of the particle but also upon the *distribution* of the electron density associated to the particle. Discussing the conditions which allow us to separate the respective contributions of distribution and composition would lead us too far. But that the value of the autocorrelation function at the origin is relevant to a more elaborate description of the composition of the particle can be illustrated by two examples.

In the first example, we consider the case of a particle which is studied in water. We suppose that the particle is such and its hydration distributed in such a way that the electron density associated with the particle can be considered as constant. In this case, the ratio of the intensity and autocorrelation function at the origin measures the hydrated volume. This property has often been used to determine the hydration of the particles under study (Kratky & Pilz, 1978) when M_2 and v_2 are known. It is of interest to note that this is a typical case where thermodynamic measurements cannot provide the answer since, in water, hydration is equivalent to solvent permeability.

In the second one, we assume that the particle under study is impermeant to solvent and the distribution of electron density associated with the particle is invariant with solvent composition. In such a case, it has been demonstrated (Aggerbeck et al., 1978) that the variation of the autocorrelation function at the origin follows a straight line as a function of $\Delta\rho_{e0}^2$. The slope of this line only depends upon the composition of the particle and is equal to the invariant volume associated with the equivalent particle divided by m_2. In that respect it is of interest to note that such measurements allow us to demonstrate, for example, that the hydration is the same in the presence and in the absence of component 3, while thermodynamic measurements do not detect the hydration in water.

Experimental Results

Characterization of the 50S Ribosomal Subunit from E. coli by Combination of Sedimentation Equilibrium and X-ray Forward Scattering. (*A*) *Experimental Procedures.* (*1*) *Materials.* $H_2{}^{18}O$ and $D_2{}^{18}O$ (99% pure) and D_2O (99.8% pure) were obtained from the C.E.A. (Saclay, France). RNase-free sucrose was purchased from Merck.

(*2*) *Preparation.* 50S ribosomal subunits were obtained from *E. coli* strain MRE600 according to the procedure of Dondon et al. (1974). Briefly, ribosomes were washed in a high salt solution, 1 M NH_4Cl and then centrifuged in a 5 mM Mg^{2+} buffer with a Beckman Ti 15 zonal rotor at 22 000 rpm during 15 h. This purification yields an homogeneous population of 70S A ribosomes (Wishnia et al., 1975; Debey et al., 1975), also called tight couples (Hapke & Noll, 1976). The 50S ribosomal subunits were prepared by dissociation of the purified 70S ribosomes and separated by zonal centrifugation in a 10 mM Mg^{2+} and 400 mM NaCl buffer. Purified subunits were stored at −80 °C in the following buffer (buffer B1): 10 mM Tris-HCl, pH 7.5, 8 mM $Mg(CH_3CO_2)_2$, 100 mM NH_4Cl, and 1 mM DTT. All the sedimentation and scattering experiments were performed in buffer B1; for the sedimentation experiments, only 0.01 mM DTT was included in the buffer. Concentrations were determined by UV absorption on a Cary 118 c spectrophotometer by using the value $E^{1\%,1\mathrm{cm}} = 145$ at 260 nm for the extinction coefficient (Hill et al., 1969).

(*3*) *SE Experiments.* A Beckman Model E ultracentrifuge equipped with photoelectric scanner was used. Generally, nine samples were run simultaneously in three double-sector Yphantis cells. Normal double-sector cells were also used on occasion. Rotors ANG, AND, or ANF (designed for very low speed ultracentrifugation) were chosen according to the speed of the centrifugation or the number of samples. The density increments $M_2(\partial\rho/\partial c_{v2})$ were obtained from the slope of the equilibrium plot of ln c_2 vs. r^2 (where c_2 is the light absorption at 265 or 280 nm and r the radial distance). SE measurements were routinely made at different concentrations and rotor speeds with very good agreement. The sample concentration was chosen to give an absorbance at the observation wave-

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[4] In practice, the variation of density increments may be linear only over a limited range of densities. If it is so, an equivalent particle may always be defined in that range.

Table II: Physical Parameters Relevant to the 50S Ribosomal Subunit[a]

techniques used	M_2	v_2 (cm^3/g)	ρ^* (e/Å^3)	β_1 (g of H_2O/g of RNP)	V_1 (Å^3)	reference
picnometry		0.592 ± 0.006[b]				Hill et al., 1969
SE in water	(1.55 ± 0.05) × 10^6[b]					Hill et al., 1969
SE in water isotopes	(1.54 ± 0.12) × 10^6	0.600 ± 0.036				this work
SE and SXRS in water	(1.56 ± 0.05) × 10^6	0.597 ± 0.008				this work
SXRS in sucrose			0.463	0.33[e]	2.55 × 10^6	Tolbert, 1971[c]
SXRS in sucrose			0.455 ± 0.007	0.37 ± 0.02	2.5 × 10^6	this work[d]
SE in sucrose				0.41 ± 0.06		this work

[a] The errors correspond to the standard deviations from the mean. [b] Values at $t = 4$ °C. [c] Values at $t = 3$ °C. [d] Values at $t = 10$ °C. [e] The value given by Tolbert is 0.404 g of H_2O/g of ribosome. In fact, using all of his data, we calculate 0.33 g of H_2O/g of ribosome, which is slightly less than our value and compatible with the difference in both values given for ρ^*.

length in the range 0.1–0.4 OD unit. The temperature of the experiment was controlled by a RTIC unit and maintained at 10 °C.

The samples of 50S ribosomal subunits were prepared from a stock solution in buffer B1 and diluted ~20-fold in one part of buffer B11 (buffer B1, 11-fold concentrated) and ten parts of the appropriate mixture of H_2O and $H_2{}^{18}O$, D_2O, or $D_2{}^{18}O$, respectively. Solutions in sucrose were obtained in a similar way by dilution of an aliquot of subunit stock solution in the appropriate mixture of B1 and BS66 (buffer B1 containing 66% sucrose, w/w). All solutions were prepared by mixing adequate volumes and checked by weight measurements. When D_2O and $D_2{}^{18}O$ were used, H/D exchange was accounted for by using the values for exchangeable hydrogen atoms, 16 exchangeable H/1000 daltons of protein and 9 exchangeable H/1000 daltons of RNA (Jacrot, 1976), which leads to $M_{D_2O} = 1.011 M_{H_2O}$, where M_{D_2O} is the molecular weight in 100% D_2O; in x% D_2O, $M_x = (1 + 0.011x)M_{H_2O}$.

All the samples in water isotope mixtures or in low sucrose concentration were found to give linear equilibrium plots after 24 and 36 h of centrifugation. For the higher sucrose concentrations (20% and higher) 48–72 h were necessary to reach equilibrium. On occasion, the lower portion of the cell, near the meniscus ($\ln c_2 < -3$), was variable and discarded. The plots of $\ln c_2$ vs. r^2 always gave straight lines. The parameters of the linear regression were computed by using a programmable Hewlett-Packard calculator with digitizer and plotter. In order to ascertain whether the regression lines obtained from equilibrium plots were representative of all the particles in solution, we calculated recovery yield from the ratio of the integrated optical density at equilibrium to the initial value before centrifugation (le Maire et al., 1978).

(*4*) *SXRS Experiments.* The X-ray source was an Elliott rotating copper anode generator, with microfocus setting. The linear beam was reflected and focused by a gold-coated bent glass mirror and limited by appropriate slits. The detector was a linear position-sensitive counter (Gabriel & Dupont, 1972). The experimental procedures are described elsewhere (Tardieu et al., 1976). As the scattered intensity was compared with the energy of the incident beam, the experimental curves were normalized and put on an absolute scale (Sardet et al., 1976; Luzzati, 1960). Collimation corrections were done according to Lake's (Lake, 1967) procedure. The scattering angle s is defined by $s = 2 \sin \theta/\lambda$ (λ = 1.54 Å), $i(s)$ is the distribution of scattered intensity for point collimation, and $i_n(s)$ the normalized intensity; the intensity at the origin was determined by extrapolation of the Guinier plots.

50S ribosomal subunit solutions were prepared by dilution of a concentrated stock solution with buffer B1 or with the appropriate mixture of B1 and BS66. Solutions were made 1 mM in DTT immediately prior to experiments. Concentrations were in the range 4–15 mg/mL. All the experiments were performed at constant temperature: 10 ± 0.2 °C. The electron density expressed in e/Å^3 of a solvent containing x% (w/w) sucrose was calculated by using the formula $\rho_{e0} = 0.3351 - 1.409 \times 10^{-4}\,\rho_0$, where ρ_0 is the density of the solvent. The ratio μ_2 = number of electrons/molecular weight was determined by using the following data: RNA 23S composition, 25% A, 22% C, 32% G, and 21% U (C. Branlant, personal communication); RNA 5S sequence from Brownlee et al. (1968); protein fraction, 30%. The ratio for protein was taken to be 11.870, a standard value, true within <1% for most proteins.

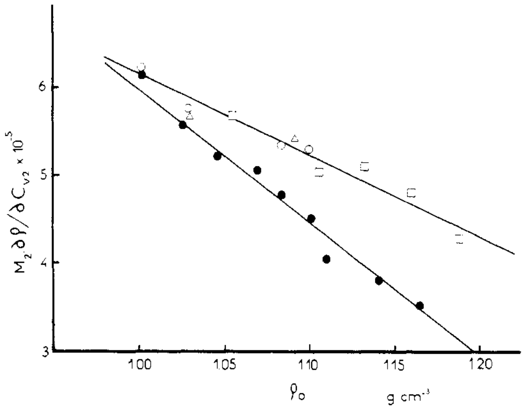

FIGURE 1: SE of the 50S ribosomal subunit. In water isotope mixtures: empty circle, $H_2O/H_2{}^{18}O$; empty triangle, H_2O/D_2O; empty square, $H_2O/D_2{}^{18}O$. Filled circles correspond to SE experiments performed in sucrose solutions. All the experiments were performed at 10 °C. The experiments in water mixtures were run at 3620 and 4485 rmp; experiments in sucrose were performed at 4020 rpm. The correlation coefficients of the regression are as follows: water, r^2 = 0.972; sucrose, r^2 = 0.986.

(*B*) *M_2 and v_2 Determination.* (*1*) *SE Experiments in Water Isotopes.* Experiments were performed in different mixtures of $H_2O/H_2{}^{18}O$, H_2O/D_2O, and $H_2O/D_2{}^{18}O$. Densities ranged from 1.001 to 1.185 g/cm^3. The results, corrected for H/D exchange when necessary, are represented on Figure 1. A linear regression analysis leads to the values of v_2 and M_2 given in Table II.

(*2*) *SE and SXRS in Water.* Several SE or SXRS experiments have been performed in water. We have taken the average value of each parameter and used the nomograph as shown in Figure 2. M_2 and v_2 values are given in Table II. It can be seen that the agreement between the two determinations is excellent. The value for the molecular weight is in very good agreement with the previously published value by Hill, while the value for v_2 is slightly higher (Hill et al., 1969).

The relatively low accuracy of the first method (SE experiments in water isotopes), which has already been discussed (Edelstein & Schachman, 1973), is confirmed by the experiments reported here: the precision on the 50S subunit v_2 value

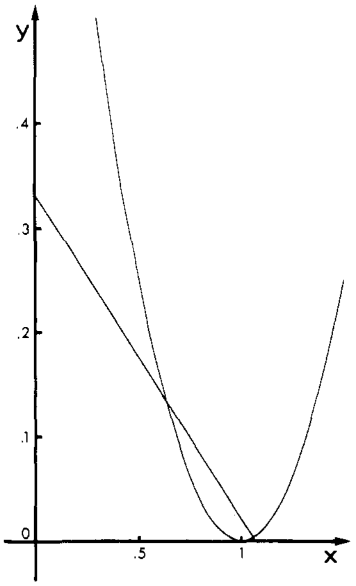

FIGURE 2: Coupling of SE and SXRS in the absence of component 3. A graphic method for determination of partial specific volume is presented here. We have the following equations (see eq 10 and 11)

$$i_n(0,\rho_{e0})/c_{e2}\mu_2 = \mathrm{SXRS} = M_2(1 - v_2\rho_{e0}/N\mu_2)^2 \quad \text{(F1)}$$

$$(2RT/\omega^2)\ (\mathrm{d}\ \ln c_2/\mathrm{d}r^2) = \mathrm{SE} = M_2(1 - v_2\rho_{e0}/N\mu_s) \quad \text{(F2)}$$

where μ_s is the ratio number of electrons/molecular weight for the solvent, $\mu_s = \rho_{e0}/N\rho_0$. Let us write $\lambda = \mu_s/\mu_2$ and $x = v_2\rho_{e0}/N\mu_2$. The elimination of M_2 in eq F1 and F2 leads to

$$(1 - x)^2 = (\mathrm{SXRS}/\lambda\mathrm{SE})(\lambda - x) \quad \text{(F3)}$$

This equation can be solved in x, that is, in v_2. In the figure, a parabola $y = (1 - x)^2$ is drawn in the expected range of x. The experimental SXRS and SE data are represented by a straight line according to $y = (\mathrm{SXRS}/\lambda\mathrm{SE})(\lambda - x)$. The two intercepts of the straight line with the parabola are the solutions in x, thus in v_2. One value of v_2 has no physical meaning. In the case presented here, the choice is unambiguous since one solution corresponds to $v_2 > 1$ which obviously has to be discarded. SXRX = 209 100 ± 4200 is the average of seven determinations. SE = 629 900 ± 12 700 is the average of five determinations. $\lambda = 1.061$, thus $x = 0.634$ and $v_2 = 0.597 \pm 0.008$.

in our hands is only ±6%, to be compared with the calculated ±1% for the same parameter determined by picnometry (Hill et al., 1969). In this respect, combination of SE and SXRS is far more attractive: the precision of ±1.4% on the partial specific volume of the 50S ribosomal subunit compares well with the above-mentioned ±1% from Hill et al. (1969). The errors on the molecular weight are also similar, ±3% in both determinations. However, one has to keep in mind that SXRS measurements require the monodispersity in size of the populations of particles under investigation.

(*C*) *Interaction Parameters Determination.* (*1*) *SE in Sucrose.* Experiments were performed at different sucrose concentrations up to 37% (w/w). Results are shown on Figure 1. All the data can be fitted by a straight line, and it should be stressed that the point obtained in water lies on the sucrose straight line within experimental uncertainty. This leads to the values of $\beta_3 = 0$ and of $\beta_1 = 0.41 \pm 0.06$ g of water/g of subunit for the hydration of the particle.

(*2*) *SXRS in Sucrose.* A total of 15 experiments have been recorded at different sucrose concentrations ranging from 6% to 52% (w/w). We are here only concerned with the variation of the intensity at the origin as a function of the electron density of the solvent. The plot $[i_n(0,\rho_{e0})/c_{e2}]^{1/2}$ vs. ρ_{e0} can be seen in Figure 3. A linear regression gives the straight line drawn in the same figure and leads to the value of β_1 given in Table II. This value of 0.37 ± 0.02 g/g is in fair agreement with the first determination provided by SE. Furthermore, the point in water lies on the straight line. The β_1 value obtained here is slightly higher than that measured by Tolbert (1971) on the basis of four experiments at different sucrose concentrations.

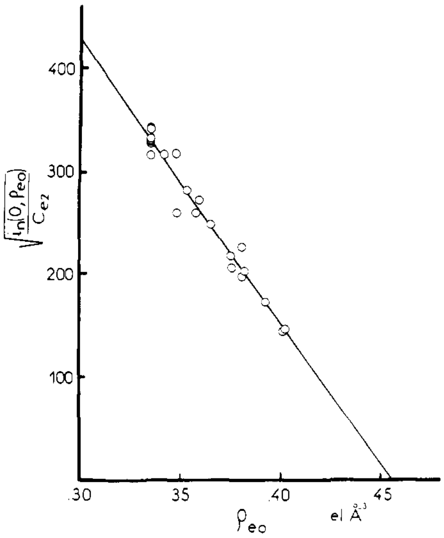

FIGURE 3: SXRS in sucrose; plot of $[i_n(0,\rho_{e0})/c_{e2}]^{1/2}$ vs. ρ_{e0}. The correlation coefficient of the regression is $r^2 = 0.969$.

We have seen that the plots obtained from either SE or SXRS are linear and that the points recorded in water lie on the respective straight lines. It should be emphasized that the point in water has been recorded many times and that, for instance, four independent SXRS determinations vary within <1%. The conclusion that $\beta_3 = 0$ or that only water is associated to the 50S subunit is thus well established. The values obtained for β_1 from either SE or SXRS are in fair agreement, but it is clear from the calculated standard deviations that the SXRS determination is far more accurate and is to be preferred.

Discussion

We have discussed in this paper the question of the composition of the particle which is studied in SXRS experiments performed in solvents of variable density. We first of all wish to explain the choice of what is called by Eisenberg "the particle approach" (Eisenberg, 1981) used here. As far as thermodynamics is concerned, treatment based on fluctuation analysis advocated by Eisenberg may have to be preferred for its generality and elegant formulation. However, the ultimate goal of our work is the elucidation of the structure of the particle under investigation. Far from restricting ourselves to the study of forward scattering, we make use of the widest possible range of scattering angles and try to retrieve as much structural information as possible. Clearly, the "particle concept" is basic to our work, hence, our choice of the particle approach at the expense of generality and elegance as witnessed by the list of experimental conditions laboriously drawn up at the beginning of this paper. Secondly, it has to be emphasized here that the knowledge of the composition of the particle is not at all required, for most of the treatment of the X-ray data. However, when it comes to an interpretation in terms of a structural model of the macromolecule, its chemical composition becomes of foremost importance.

We have presented here a rigorous analysis of the behavior of a macromolecule in solvents of variable density. Starting from the description of the particle in terms of the interaction parameters ξ_j introduced by Eisenberg (1976), we propose, in the case where $[i_n(0,\rho_{e0})/c_{e2}]^{1/2}$ or SE or densimetry values vary linearly as a function of solvent density, to make use of another description of the particle which does not require the introduction of a model; we define an equivalent particle composed of the dry particle to which constant amounts β_1 (g/g) of water and β_3 (g/g) of component 3 are associated. β_1 and β_3 are operational quantities, whose values can be unambiguously determined from experiment. Indeed, we think this description particularly convenient in that case, as the equivalent particle is invariant with respect to the variation of solvent density while the interaction parameters are not. Furthermore, in the case where the point recorded in water lies on the straight line, the description we propose makes immediately clear that we are dealing with hydrated particles. In fact, it has been common practice for us in recent years to say that we were studying hydrated particles and to show that all the experimental data were in agreement with this hypothesis (Sardet et al., 1976; Aggerbeck et al., 1978; Sperling, 1977). All the data recorded in solvents of variable density yield linear plots where the point recorded in water lies on the straight line. As shown in this paper, these data are sufficient to demonstrate that, except for solvent permeability, no explanation, other than hydration, was consistent.

Some points have been briefly evoked which probably could be pursued in the future: we mentioned in the last section that some properties of the SXRS intensity curves may be helpful in answering the question of solvent permeability; neutrons might also be of help but have not been considered here since, in general, solvent density variation is not achieved with small solute molecules. Also we considered only three components, but the treatment could be extended to four.

A last point needs some comments. We assumed in the beginning that the partial specific volumes are constant. In fact, they may vary. However, they are not expected to vary by more than a few percent. The effects on density increment variation will thus be 1 order of magnitude lower than those due to hydration, for example and are not expected, at the present stage, to be detected by the approaches presented here. Thus, we have neglected them.

Finally, we have emphasized in this paper the similarities between SXRS intensities at the origin, SE, and densimetry. We have shown that their combinations, as illustrated by the SE and SXRS experiments presented above, can offer useful ways to determine some physical parameters of a particle. We wish to point out now their differences. SE and densimetry measure excess of mass, while SXRS measures excess of electrons. Such a difference could in principle be helpful in the case of nonlinear variations to describe the interactions between the particle and the solvent. More important are the practical differences between the three approaches, which involve completely different experimental procedures. Thus, their coupled use provides us with the best way to check that all the measurements made are correct.

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