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Neutron scattering measurements of separation and shape of proteins in 30S ribosomal subunit of *Escherichia coli*: S2–S5, S5–S8, S3–S7

(ribosome structure/pair separation distances)

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ABSTRACT **Neutron scattering measurements done on *E. coli* 30S ribosomal subunit specimens in which specific pairs of proteins were deuterated have enabled us to estimate the distances between the labeled proteins. The distances between centers of gravity of three protein pairs have been determined: S2–S5 (105 Å), S3–S7 (115 Å), and S5–S8 (35 Å). A method for extracting shape information about these proteins from the neutron scattering profiles is demonstrated. The method shows that S5 and S8 are compact and S2 is extended.**

Three years ago we proposed that neutron scattering could be exploited as a means of mapping the locations of the proteins of large multicomponent assemblies (1). In the following communication we report the first successful application of the method to pairwise measurements of the distances separating the centers of proteins in the 30S ribosomal subunit of *Escherichia coli*. Additionally, a method of data analysis is applied which allows us to make some deductions about the shapes of the proteins whose separations are measured. Proper experimental controls are crucial in the initial presentation of results which follow from a novel experimental approach. Accordingly, data are shown indicating that our results are unlikely to derive from systematic error in the experiment or from an inadequate biochemical preparation.

The central idea of the method is that the scattering curve given by two objects separated by a fixed distance, when averaged over all orientations with respect to the incident radiation, includes a rippling interference contribution. This communication contains information about the distance between the centers of scattering mass of the two objects, and can therefore be used as a measure of their separation (1–4). Neutron scattering is a suitable means of detecting such interference contributions since the scattering density of the parts of a structure can be adjusted by specific deuteration. In the case of the small ribosomal subunit, a pair of proteins deuterated in most nonexchangeable hydrogen locations is placed in the otherwise undeuterated structure by reconstitution (5), creating a large difference in scattering density between the deuterated proteins and the other ribosome components. The scattering density of the surrounding buffer is then adjusted by addition of D_2O so that it approximately equals that of the ribosome as a whole, resulting in a situation in which the contribution of the bulk of the ribosome to the low angle neutron scattering is suppressed relative to that of the pair of deuterated proteins (1). In early experiments the principles of such contrast manipulations were shown to produce the expected effects on the scattering curves of ribosomal subunits (6, 7). We now show that the contribution of the interference cross term from a pair of deuterated proteins to the scattering curve can be measured and consequently that the determination of pair separation distances is a practical reality.

A method for the assessment of the contribution of the shapes of the two proteins of a pair to the interference cross term is suggested which is based on the length distribution profile of all cross-correlation vectors between the two proteins (a radial Patterson function). Thus, it now appears that a three-dimensional map in which the locations and general shapes of proteins in the 30S ribosome are shown can result from continued exploitation of the methods we have established. A more detailed treatment will appear elsewhere (Proceedings of the 1975 Brookhaven Symposium on Biology).

MATERIALS AND METHODS

Cells and Ribosomes. Deuterated ribosomal proteins were prepared from *E. coli* MRE600 grown on protonated glucose in minimal medium dissolved in 100% D_2O (7, 9). Protonated ribosomal proteins were prepared from the same strain grown in H_2O medium following Held *et al.* (10). The protonated components used, unpurified, for the bulk of each reconstitution were prepared from *E. coli* Q13. The method used for isolating 30S ribosomal subunits has been described elsewhere (11).

Ribosomal Proteins and RNA. 30S ribosomal proteins were purified from 30S subunits by chromatography on carboxymethyl cellulose columns in urea at pH 5.6, followed by chromatography on Sephadex G-100 (11). Schwartz/Mann enzyme grade urea was used throughout. 16S RNA was made by phenol extraction of Q13 subunits (12).

Reconstitutions. Q13 30S protein was fractionated on carboxymethyl cellulose and the fractions corresponding to the proteins to be manipulated in a given experiment set aside. The remaining protein was pooled and concentrated by ultrafiltration. The pooled material was then split into four equal portions and the missing components added back in purified form, deuterated or protonated, as required. These mixtures were then used for reconstitution following Traub *et al.* (12). A preparation containing 400 A_{260nm} of RNA was used in each of the four reconstitutions needed for a distance experiment. The molar ratio of RNA to protein was 1:2 in the reconstitution mixtures; the concentration of RNA was 10 A_{260nm}/ml. Mixtures were incubated with stirring for 150 min at 40°C, the reconstituted material recovered by centrifugation and purified on sucrose gradient to remove extraneous protein. About 50% of the input optical density was recovered as purified, reconstituted subunits.

Assessment of Reconstitutions. The protein mixtures in-

volved in each reconstitution were examined by two-dimensional gel electrophoresis as was the protein composition of the reconstituted products following the scattering experiments (13). Sedimentation profiles were determined before and after each experiment. The incorporation activity of the samples was checked by assaying their activity in a poly(U)-directed phenylalanine incorporating system (11).

Preparation of Scattering Samples. Each interprotein distance finding experiment requires measurements on four samples: A sample in which both proteins whose relative locations are in question are deuterated, the two possible singly deuterated samples, and a sample in which all components are protonated. The measurement is most efficiently done using two samples which are (*a*) mixtures of equal amounts of the double and unsubstituted preparations and (*b*) equal amounts of the two singly substituted preparations. The intersubunit distance information in the scattering data is contained in the difference in scatter between mixtures (*a*) and (*b*) (3, 4, 8). Mixtures (*a*) and (*b*) were formed from reconstituted subunit preparations, appropriately substituted with deuterated proteins, following careful determination of concentrations by measurement of optical density at 260 nm. The mixtures were then dialyzed against three changes of 0.5 mM magnesium acetate, 50 mM KCl, 6 mM 2-mercaptoethanol, 10 mM Tris·HCl (pH 7.5) in 56.8% D_2O (vol/vol) over a period of 36 hr (7). In this buffer, protonated 30S subunits are contrast matched, i.e., they have the same average scattering density as the buffer, so that their scattering at zero angle vanishes. The dialyzed preparations were then centrifuged into thin-walled aluminum sample chambers which had cylindrical cavities just large enough to accommodate the 30S material in each sample when pelleted. Adaptors were made to hold the sample chambers in a swinging bucket rotor so that a flat, compact pellet would be deposited in each. After centrifugation, each sample chamber was fitted with a thin quartz lid and sealed in a holder which fit the scattering apparatus. After the scattering data were collected, the amount of ribosomes in each chamber was determined by resuspending its contents in buffer, and measuring the optical density.

Arrangement of the Scattering Experiment. Neutrons of $\lambda = 2.37$ Å were selected by reflection from a pyrolytic graphite monochrometer from the D_2O moderated reactor spectrum of the Brookhaven High Flux Beam Reactor. The neutrons had a chromatic band width of $\Delta\lambda/\lambda = 0.02$, full width at half maximum. Following the monochromator, the neutrons pass through a graphite filter set to remove neutrons at $\lambda/2$, a monitor of the direct beam, and a 142 cm collimator defining a cylindrical beam of 6.4 mm cross-section and 0.26° divergence. The beam then passes through the sample and is stopped by a beamstop in front of a two-dimensional position-sensitive detector (14) which detects neutrons scattered from the sample. Samples were mounted in an automatic sample changer which permits sequential measurement of up to eight specimens in repeatable cycles, maintaining temperature control and preventing condensation of water from the surrounding air. The flux impinging on the specimen was 2.4×10^7 neutrons cm^{-2} min^{-1}. Specimen to detector distances of 1–2 m were used in different experiments. Background due to gas scatter was reduced by flushing the area in front of the detector with ^4He for a distance of about 60 cm. Data from the counter face were radially integrated to give the average scatter of each specimen per unit area as a function of scattering angle. It is upon these profiles that further analysis was done.

RESULTS

The objective of our experimental measurements is to obtain the interference cross term for a pair of ribosomal proteins in isolation from other contributions to the scattering profile. In order to obtain as large an interference signal as possible, the deuterated proteins in each specimen should be present at as near stoichiometric levels as possible. However, provided the level of incorporation of deuterated proteins into reconstituted particles is the same from sample to sample, the only penalty paid for failure to obtain a high level is loss of signal strength. Of the five proteins used in deuterated form in this work, three are required to obtain properly sedimenting particles: S5, S7, and S8 (22). The fact that the reconstituted particles formed in their presence sedimented at 30 S argues for their uptake in stoichiometric amounts. The two other components used were S2 and S3 which do not influence sedimentation. S3's incorporation into reconstituted particles was shown by two-dimensional gel electrophoresis which demonstrated its presence in normal amount, as well as confirming the presence of S5, S7, and S8. S2, the only component considered normally recovered in less than molar amount in native subunits, was present in greater than normal amounts in reconstituted particles, as judged by stain intensity on gels. The fact that the particles formed on reconstitution were active in protein synthesis supports the view that satisfactory incorporation of deuterated proteins into reconstituted particles was obtained.

The experimental strategy adopted for measuring the interference signal involves the collection of scattering data from: (*a*) a mixture of equal amounts of the double and unsubstituted ribosomes, (*b*) a mixture of equal amounts of the two singly substituted preparations, (*c*) the final 58.6% D_2O dialysis buffer in which samples *a* and *b* are suspended, and (*d*) the counter background with the beam blocked at the sample position. If the concentrations of the ribosomes in samples (*a*) and (*b*) are identical, then their difference gives the interference cross term directly; if they are not, there will be contributions due to the beam independent background and the buffer scatter. In practice, the concentrations were held to within 10% of each other so that corrections for the beam independent background and the buffer scatter were small, permitting a reduction in the counting time required for these samples (see Fig. 2 legend).

In order to convince ourselves that the cross terms we obtained were reasonably attributable to the presence of the deuterated protein, we compared sample S3–S7 (*b*), the singly substituted ribosome mixture, with a preparation of reconstituted ribosomes having no deuterated proteins. The result of this experiment is shown in Fig. 1. The expedient of contrast matching the bulk of the ribosome has reduced the scatter from the particles so that the single deuterated protein accounts for 30% of the observed intensity in the low angle region. The difference between the scattering curves gives a reasonably straight line in a Guinier plot (15), showing that it has the correct shape for the contributions of single proteins to the scattering curve. Additionally, the plot can be used to obtain the scatter at zero angle (per unit monitor time), for which we calculate a value of 6.8 counts/mm^2 based on complete substitution of the ribosomes with labeled protein. The observed value is 5.4 counts/mm^2, which is in satisfactory agreement with expectation, considering that precise values for the molecular weights of S3 and S7 are not known. (The forward scatter is proportional to molecular weight squared.) These observations show that the contributions of single, deuterated proteins to the scattering curve

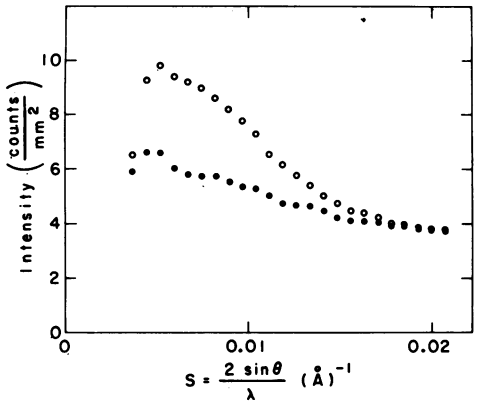

FIG. 1. Scattered intensity as a function of scattering angle for fully protonated 30S subunits and protonated subunits containing either S3 or S7 in deuterated form. About 300 A_{260nm} of fully protonated 30S subunits and 300 A_{260nm} of an equimolar mixture of protonated 30S in which either S3 or S7 were deuterated were prepared by reconstitution (see *Materials and Methods*). The profiles shown are the scattering given by each of the two samples as a function of scattering angle in 56.8% D_2O buffer (see *Materials and Methods*). In both profiles the contribution due to buffer scattering has been subtracted out. The profiles are scaled to allow for the small differences in ribosome amount in the two samples. The vertical scale is neutron events detected per mm^2 of detector surface per approximately 40 min of running time. The results of several cycles of data collection have been averaged. The radial profiles shown are obtained by averaging the two dimensional array of sensitive elements represented by the counter surface circularly about the direct neutron beam at appropriate intervals of radius. The sample to detector distance was 1.94 m. The profile of the fully protonated 30S subunits is given by the solid circles (●); the S3–S7 substituted mixture gave the profile represented by the open circles (○).

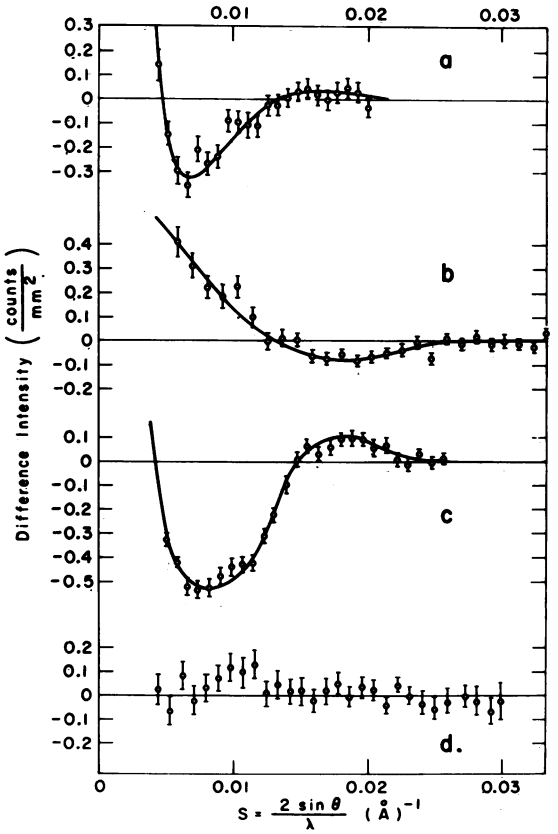

FIG. 2. Protein–protein interference signals. Experimental data were collected in the manner described in the text and combined to isolate the interference cross term using the relationship

$$I_{cross} = \left[\frac{Ia - Id}{Ba} - k_1 \frac{Ic - Id}{Bc}\right] - k_3\left[\frac{Ib - Ic}{Bb} - k_2 \frac{Ic - Id}{Bc}\right]$$

where *Ia*, *Ib*, *Ic*, and *Id* are the curves for the samples consisting of the double + unsubstituted mixture, the single + single substituted mixture, the 56.8% deuterated buffer, and the blocked beam background. k_1 = 1 − (volume of ribosomes in sample *a*), k_2 = 1 − (volume of ribosomes in sample *b*), and k_3 = the ratio of the quantity of ribosomes in *a* to that in *b*. Ba, Bb, and Bc are the undeviated beam intensities transmitted by the specimens scaled to Bc = 1. The cross terms isolated from this expression are presented for four cases: (*a*) S2–S5, (*b*) S5–S8, (*c*) S3–S7, and (*d*) a comparison of unsubstituted reconstituted subunits (see *text*). The error bars shown are the standard errors at each point in the difference curves due to counting statistics. The differences are given in units of neutron events per mm^2 of detector surface per roughly 40 min of data collection as a function of scattering angle. The zero levels for each profile can be calculated to within ±0.2 units on the basis of the available data on the amount of ribosomes in each sample and volume of the sample chambers. More precise values are set by assuming that the difference at high scattering angle is zero, as the theory of this measurement unambiguously predicts. The smooth curves given in profiles a, b, and c were fit by eye. The profiles are scaled to constant ribosome amount for comparison (i.e., 250 A_{260nm}/sample). Profile a was collected at a sample to detector distance of 1.8 m; profiles b, c, and d were collected at 1.25 m, 1.85 m, and 1.6 m, respectively.

are readily observable, and that the magnitude of the contribution of the scatter of a single protein is sufficient to allow a reasonably precise measurement of the interference cross term. A further control experiment was conducted in which four separate reconstitutions of unsubstituted ribosomes were done and the products combined pairwise according to our protocol for a pair separation measurement. When measured and compared, these samples should, in principle, give no cross term signal, and within the error of the experiment none was observed (Fig. 2d). These control experiments help to support the contention that whatever ripple is seen in the difference curve of substituted samples in fact represents the predicted interference cross terms of the protein pairs.

Data from samples (*a*) → (*d*) were combined to give the interference cross terms for a series of pairs of ribosomal proteins consisting of S2–S5, S5–S8, and S3–S7. The curves are shown in Fig. 2. In each case a strongly damped, rippling function is seen which is of the general form expected for an interference cross term (1). The curve for S2–S5 has its first zero at about $(210\ \text{Å})^{-1}$ (Fig. 2a) implying a separation of centers of mass of the two proteins of about 105 Å. The remainder of the curve is more strongly damped than expected for the case of two spheres and shows other distortions characteristic of shape effects (see below). The curve for S5–S8 is even more strongly damped and indicates from its first zero at $(70\ \text{Å})^{-1}$ a separation of centers of mass of about 35 Å (Fig. 2b). In this case the curve has the form expected for two compact proteins, but is damped by form factors since the separation is small. The third case (Fig. 2c), that of S3–S7, shows a strong ripple which, for instrumental reasons, was not measured at the point of the first zero. Thus, the separation measurement must rest on an extension of the curve up to zero, and it is consequently less precise. We estimate the zero to be at $(230\ \text{Å})^{-1}$, implying a separation of centers of about 115 Å. This measurement is a repeat of our first measurement of S3–S7, made a year ago with a less satisfactory preparation, and the distances obtained are

comparable (115 Å versus 106 Å). (The errors in these estimates are discussed below.)

In our original proposal (1) we noted that model calculations had shown that the effects of the shapes of the two proteins being studied were minimal in the region of the first zero, so that its position gives a good measurement of the separation of the protein centers of mass. These calculations were based on models consisting of two arbitrary ellipsoids of revolution having axial ratios ranging from 1 to 5. The ellipsoids were positioned at a variety of relative orientations and center to center separations, and the interference term calculated. Only in cases of extreme axial ratio and small separations were significant (>5%) errors in the position of the first zero observed, and these curves were identifiable in terms of other characteristics of their shape. Beyond the first zero, the curve is progressively more influenced by shape factors, and consequently contains shape information. We suggest that a useful way to represent this information is to do a radial Patterson inversion of the profile (16), which yields the length distribution of all vectors relating the two proteins of the pair.

Application of radial distribution function analysis to the data for S5–S8 and S2–S5 gives the curves shown in Fig. 3. The curve for the S5–S8 pair shows a strong correlation peak which extends from about zero to about 60 Å. S5 and S8, as spheres, would have diameters of 34–35 Å. The solid line in Fig. 3 is the vector length distribution for spheres of that diameter in contact. The close similarity of that theoretical profile and the S5–S8 data leads to the inference that S5 and S8 must be compact.

In the case of S2–S5, the correlation peak is quite broad, extending from roughly 40 to 160 Å. Since the data for S5–S8 suggest a compact shape for S5, S2 must be a substantially extended protein. Application of this method to our data for S3–S7 cannot be relied upon since the data set is so truncated. We expect to repeat this measurement under conditions in which the value at smaller reciprocal spacing can be confidently obtained; however, the indication from the present data is that the correlation peak is a broad one. Given enough such data, it may be possible to construct a three-dimensional map of the 30S subunit giving the location of the center of mass and an approximate shape for each protein.

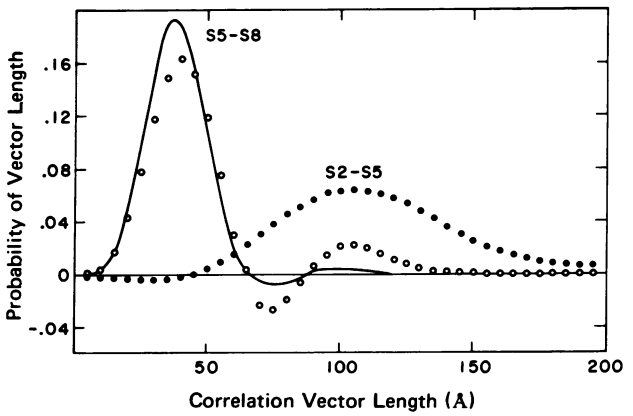

FIG. 3. Length distributions of interprotein vectors. Difference profiles for S2–S5 and S5–S8 (Fig. 2a and b) were used to calculate the distribution of lengths of vectors between the proteins of each pair. To reduce the impact of statistical noise on the calculated profiles, we first smoothed both profiles by a variance weighted least squares, multiple regression fitting to Hermite functions. The smoothed data were then subjected to a radial Patterson inversion to give the probability, $U(R)$, of the interprotein vectors being of length R:

$$U(R) = R\int_0^\infty sD(s)\,\sin(2\pi sR)\,ds$$

where $D(s)$ is the difference profile (16). The resulting probability profiles are plotted, scaled to have an integrated area of 1.0. The errors in the original difference profiles were propagated through the smoothing and inversion processes. The S5–S8 profile has standard errors of 0.01 to 0.015 across the main peak (peak probability = 0.15). The errors build to a maximum of 0.02 in the region of the negative dip at about 70 Å and then die out. The negative and positive features in the profile from 70 Å out are hardly different from 0 by 1 standard deviation. The standard error of the S2–S5 profile is about 0.01 all the way across the peak (maximum probability = 0.065). The solid profile is the vector length distribution for a pair of spheres 34.5 Å in diameter, in contact. A protein–protein interference signal was computed for spheres of that size in contact at intervals in reciprocal space similar to that of the actual S5–S8 data and then inverted as described above.

DISCUSSION

In our initial paper on the use of neutron small angle scattering for quaternary structure determination (1), it was apparent that the small size of the average ribosomal protein relative to the whole ribosome meant that the signal to noise ratio in such an experiment would be small and that the practical feasibility of the technique could not be taken for granted. Thus, the primary purpose of the experiments presented above was to prove the feasibility of the method.

The experimental proofs we offer for the validity of the technique are fundamentally three in number. First, given current neutron fluxes and counting techniques, the contribution of a single, heavily deuterated protein of 25,000 daltons to the scattering of an otherwise protonated ribosomal subunit (approximately 900,000 daltons) is easily detected. It is of the proper magnitude and has a reasonable shape, (Fig. 1). Second, when samples are prepared in a manner identical to that used for interference measurements but with isotopic substitution omitted, no interference signal is seen (Fig. 2d). Third, when the full experiment is done including isotopic substitution, an interference contribution is seen which has an appropriate shape and magnitude (Fig. 2a–c). The exact shape of the interference profile depends on which protein pairs are substituted and is reasonably reproducible from preparation to preparation. Thus, there is a good reason to believe that the detection of the interference signals required by our technique has been demonstrated.

Error in the distance measurements obtained so far came from three sources: (*1*) counting statistics, (*2*) shape effects, and (*3*) biochemical variability. Some multiple regression curve fitting has been done on the three difference profiles shown (23). The uncertainties in the position of the first zero in these fits were 2–3%, which is a measure of the impact of counting statistics on the distance estimates. Model calculations of interference profiles have shown that the error expected from shape effects is of the order of 5%, giving a net uncertainty of about 7% in the distances quoted, independent of biochemical problems. The impact of biochemical variations on the distances found is much harder to estimate. The only distance measurement duplicated so far, that of S3–S7, was reproduced to within 9%. Given the fact that we have measured S5–S8 and S2–S5 only once, we feel that the probable error in the distance estimates obtained is 10–15%. We place similar estimate on the error in the S3–S7 distance feeling that the fact it has been repeated compensates for the uncertainty in extrapolating the data shown in Fig. 2.

In general, our data reinforce the conclusions about protein distribution in the 30S subunit drawn by others on the basis of chemical analysis. For example, proteins S5 and S8 are about 35 Å apart by the neutron technique. Proteins of their molecular weights would have diameters of 36 Å and 33 Å if they were spheres. Thus, the neutron data establish them as nearest neighbors, a result strongly supported by chemical data showing this pair of proteins is readily cross-linked (17, 18). It is interesting that the recent protein distribution models of Morgan and Brimacombe (19) and Traut *et al.* (20), place S3 and S7, as well as S2 and S5 well apart in the 30S subunit, consistent with our data.

An important point to make about interference data is that it not only provides the distance between the centers of gravity of protein pairs, but it also gives the distribution of intersubunit vector lengths. Such data are valuable indication of the shape of proteins within the subunit. For example, the narrow distribution of lengths seen in the S5–S8 pair argues for a relatively compact shape for both proteins *in situ*. The broad length distribution seen in the S2–S5 pair therefore, suggests an elongated shape for S2. The preliminary S3–S7 length distribution profile is also broad, which may imply an elongated shape for one or both members of this pair as well. Recent electron microscope studies on antibody stained 30S subunits have shown that protein S4 also has an extended configuration (21). Thus, our results strengthen the hypothesis that extreme axial ratios may be a common feature among ribosomal proteins.

We feel that these data have established the validity of neutron scattering as an approach to the quaternary structure of large biological assemblies, as originally proposed (1). The results obtained, while only the first step, imply that it should be possible ultimately to build a model of the organization of the ribosome at a level of confidence and resolution not presently offered by any other method.

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