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SUCROSE DENSITY GRADIENT SEDIMENTATION OF E. COLI RIBOSOMES

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The behavior of E. coli ribosomes during sedimentation on sucrose gradients is predicted under a variety of conditions by computer simulations. Since numerous recent kinetic studies indicate equilibration in times short compared to the time of sedimentation, these simulations assume that the system attains local reaction equilibrium at every point in the gradient at all times. For any type of homogeneous equilibrating ribosome population, governed by a single formation constant at one atmosphere pressure for 70S couples, no more than two clearly defined zones will be resolved, although the presence of large dissociating effects due to pressure gradients in high speed experiments will spread the subunit zone. Normally the pattern will consist of a 30S zone and a so-called "70S" zone, which is in reality a mixture of 70S couples and 30S and 50S subunits in local equilibrium. The greater the dissociation into subunits, the more the "70S" zone will be slowed below the nominal rate of 70 Svedberg units. If ribosomes have been collected from the "70S" zone in several successive cycles of purification, the repeated deletion of resolved 30S subunits can result in a preparation with so large a molar excess of 50S subunits that the ensuing sucrose density gradient sedimentation pattern may exhibit a "70S" zone followed by zone of 50S subunits, *instead of* a zone of 30S subunits. Our most important conclusion is that whenever a well-resolved 50S zone is present in a sucrose density gradient sedimentation experiment on E. coli ribosomes, in addition to a 30S and a "70S" zone, under conditions where ribosomes and subunits should be in reversible equilibrium, the preparation must be microheterogeneous, containing a mixture of "tight" and "loose" couples. Moreover in such cases the content of large subunits in the 50S zone must be derived entirely from "loose" couples whereas the 30S zone must contain small subunits derived from both "tight" and "loose" couples. Sedimentation patterns predicted for various mixtures of "tight" and "loose" couples display all the major characteristics of published experimental patterns for E. coli ribosomes, including the partial or complete resolution into three zones, depending on rotor velocity and level of Mg^{2+}.

1. Introduction

The dissociation behavior of ribosomes during high speed ultracentrifugation was successfully explained as a pressure-induced phenomenon by Infante and Baierlein [1] for the case of sea urchin ribosomes. These authors were able to fit observed experimental behavior during zonal ultracentrifugation by means of computer-simulated predictions, assuming a volume of formation of 500 ml per mole of ribosomes. Both experiments and predictions demonstrated that for sea urchin ribosomes no more than two zones were produced: as dissociation takes place, the leading zone, which includes ribosomes and subunits in equilibrium, slows down; in extreme cases it may be converted into *a* zone containing only the large subunit. The trailing zone is composed of the small subunit, at least as long as the subunits in the original ribosomes are equimolar overall. The complications in behavior caused by the advance of the ribosomes into regions of higher pressure, where their dissociation is enhanced, are in qualitative accord with previous studies [2–4] of the effect of pressure on the sedimentation of chemically interacting macromolecular systems. Solvent overlays served unequivocally to prove the pressure effect [1].

In fact, zonal sedimentation behavior of ribosomes at lower ultracentrifuge speeds are also in accord with computer simulations completed a decade earlier, on countercurrent distribution analogs for transport processes [5–7], excluding pressure effects. According to those simulations for a bimolecular complex [6], translated into terms descriptive of ribosomes from E. coli, one expects a leading zone of "70S" ribosomes, followed by a trailing zone of 30S subunits, with essentially no 50S subunit zone. This is illustrated

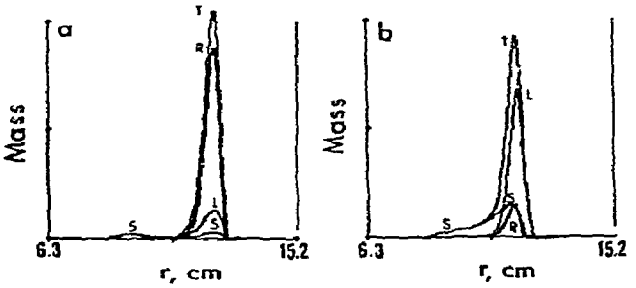

Fig. 1. Simulated sucrose density gradient sedimentation patterns of homogeneous E. coli ribosomes, assuming an overall dissociation constant at 0.9×10^{-9} moles/liter. The rotor velocity is 41 000 RPM. In (a) the volume of formation is assumed to be zero and in (b) the volume of formation is taken as 142 ml/mole (see text). Total (T) and individual mass concentrations are indicated for 30S (S), 50S (L) and 70S (R) species in the spinning rotor (see text). A loading zone of 0.4 ml containing 12.4 A_{260}/ml of ribosomes was assumed, corresponding to a total of 5 A_{260} units (1% solution corresponds to 145 A_{260} units/ml).

in fig. 1a by a simulation of band sedimentation [2] in which the volume of reaction is chosen to be zero. The faster-moving "70S" zone only travels as its nominal rate of 70 Svedbergs if virtually no dissociation takes place. When reversible dissociation occurs, 70S ribosomes are re-equilibrating with 50S and 30S subunits at all times, and therefore cannot outrun a zonal region which must contain an equilibrium mixture of 30S, 50S and 70S species. The consequence is that the more the 70S species dissociates, the slower the "70S" zone must travel, an effect which had been described in complete detail in predicting the zonal transport of reversibly reacting systems of the type $A + B \rightleftharpoons C$ [6,8]. Without recourse to these earlier predictions, this phenomenon was rediscovered by careful direct experiments on E. coli ribosomes [9].

When there is appreciable dissociation caused by pressures, there must be a time-dependent and rotor-speed-dependent sedimentation [1] for the fast zone, effects already discussed [2] in the case of a pressure-sensitive monomer-dimer interacting system. Even when the effect of pressure is large enough to cause complete dissociation of ribosomes into small and large subunits, only two major zones should be apparent. While this was completely supported by findings in the sea urchin ribosome experiments and their simulation [1], yet, in the case of "vacant couple" E. coli ribosomes, usually sucrose density gradient sedimentation shows resolution into three rather well-defined zones, a "70S" zone traveling slower than 70 Svedbergs, a 50S zone and a 30S zone [9–13]. These studies have also shown that, other things being equal, "vacant couples" tend to produce a leading zone which moves more slowly than 70 Svedbergs, whereas the presence of additional species which increase the formation constant of ribosomes tends to make the sedimentation rate of the leading zone approach 70 Svedbergs. This is consistent with predictions [6,8] made in a series of simulations for the system $A + B \rightleftharpoons C$, in which the formation constant of the complex, C, was progressively increased.

One further important characteristic is that there is a strong dependence on Mg^{2+} concentration of the formation constant of ribosomes from their subunits [12,14,15]. When advantage was taken of this dependence in zonal sedimentation experiments [10–12], it was found possible to distinguish between "tight" couples on the one hand, and "loose" couples on the other hand. By sedimentation at some intermediate level of Mg^{2+} concentration, approximately 4–5 mM, it was possible to retain "tight" couples as primarily 70S species, while "loose" couples would be predominantly dissociated into subunits [10–12]. An example of the equilibrium light-scattering behavior of the vacant "tight" couple ribosomes during titration with Mg^{2+} has been given recently [16]. From this, one would expect to see only a small amount of subunits originating from "tight" couples at Mg^{2+} concentrations above 4 mM at low rotor speeds.

2. Generation of 50S zones

Isolation of the "70S" zone from a gradient at low rotor speeds, or in the absence of pressure effects, fig. 1a, tends to produce a ribosome preparation which, although originally equimolar in small and large subunit, must become deficient overall in 30S subunit, through deletion of some resolved 30S species. Eventually, many successive repurifications by this technique could result in a preparation whose zonal sedimentation should [6] show a "70S" zone and a

trailing 50S zone, rather than a trailing 30S zone. Such patterns have actually been reported [10]. On the other hand, there is no mechanism by which a rapidly re-equilibrating ribosome-subunit system would be expected to show three well-defined zones. Consequently, we are led to ask for an alternative explanation for a partially or completely resolved 50S zone *in addition to* a 30S and "70S" zone. Our assertion is that this can result only from microheterogeneity: some of the ribosomal material is in the form of strongly coupled subunits, and some is in the form of only weakly interacting 30S and 50S subunits. This is, in fact, not a radical idea, since the presence of "loose" couples in a ribosomal preparation which consists mostly of "tight" couples constitutes the kind of microheterogeneous system inferred [10–12]. It is noted that the resolution of a 50S moving boundary [17] in analytical ultracentrifugation of ribosomes was interpreted as corresponding to a non-equilibrating system, since it was not in accord with theory of Gilbert and Jenkins [18] for the sedimentation of rapidly re-equilibrating bimolecular complexes. In that study [17] boundary resolution was ascribed to failure to re-equilibrate due entirely to a deficiency in ionic strength or content of Mg^{2+}, and not to any microheterogeneity. Many of the characteristics of zonal separations of E. coli ribosomes have also been attributed [9] to failure to equilibrate chemically at a rate faster than the rate of separation. Since kinetic data are now available for both tight [16] and loose [19,20] couples, and since criteria have been developed [21] for the possible effects of slow kinetics on the enhancement of separation, we can now infer that the reasons given [9,17] would be inadequate to explain the observed behavior, if the sedimentation experiments were being made on homogeneous populations of E. coli ribosomes. Loose couples, in fact, equilibrate very rapidly with subunits and even tight couples equilibrate in times short compared to the time of sedimentation at Mg^{2+} levels greater than 2 mM [16].

3. Simulations

To assess the possible effects of microheterogeneity on the observations in sucrose density gradient sedimentation, we have performed simulations on various

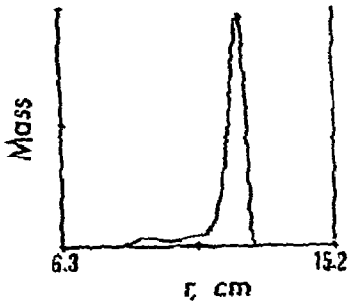

Fig. 2. Simulated sucrose density gradient sedimentation pattern for a mixture of 95% tight couples and 5% loose couples at 22 000 RPM. Dissociation constants of tight couples and loose couples are taken as 0.9×10^{-9} moles/liter and 0.67×10^{-6} moles/liter, respectively. The volume of formation is taken as 142 ml/mole for both tight and loose couples. Loading as in fig. 1. Vertical coordinate represents total mass.

assumed mixtures of loose and tight couples, with the following choice of parameters: the overall dissociation constant for tight couples was taken as 0.9×10^{-9} [16] and that for loose couples was taken from the companion paper [19] as 6.7×10^{-7} moles/liter. The volume of formation of 70S E. coli ribosomes from subunits was taken as 142 ml/mole [22]. The simulations, chosen to represent sedimentation in a buffer containing 5 mM Mg^{2+} in Beckman SW 41 Ti swinging bucket rotors, were performed by using the countercurrent distribution analog of band sedimentation previously described [2] for a pressure-sensitive system of the type $A + B \rightleftharpoons C$. In fig. 1b is

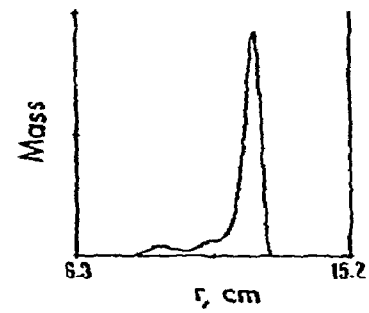

Fig. 3. Simulated sucrose density gradient sedimentation pattern for a mixture of 90% tight couples and 10% loose couples at 22 000 RPM. All other parameters as in fig. 2. Loading as in fig. 1. Vertical coordinate represents total mass.

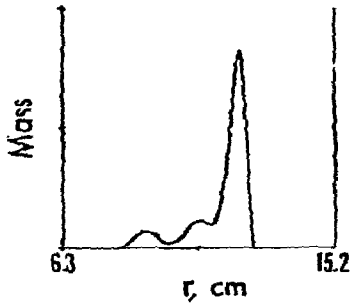

Fig. 4. Simulated sucrose density gradient pattern for a mixture of 80% tight couples and 20% loose couples at 22 000 RPM. All other parameters as in fig. 2. Loading as in fig. 1. Vertical coordinate represents total mass.

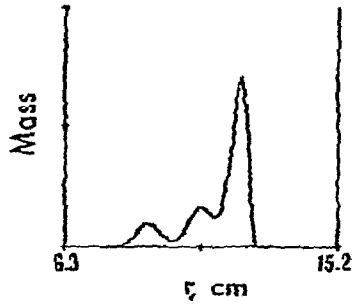

Fig. 5. Simulated sucrose density gradient pattern for a mixture of 70% tight couples and 30% loose couples at 22 000 RPM. All other parameters as in fig. 2. Loading as in fig. 1. Vertical coordinate represents total mass.

shown for comparison the predicted sedimentation patterns for pure tight couples at 41 000 RPM, to illustrate the effect of pressure-induced dissociation. It is seen that in addition to enhancing dissociation, the higher rotor speed also spreads the "30S" zone over a large region. From figs. 1a and 1b, it is seen that *for a homogeneous population of ribosomes, with or without pressure effects, all of the 50S species travels with the leading zone.* Fig. 2 represents the zonal sedimentation pattern representing distribution of total mass, predicted for a system containing 95% tight couples at 22 000 RPM. Also at 22 000 RPM, fig. 3 represents a system with 90% tight couples and 10% loose couples, fig. 4 is for a system containing 80% tight couples, and fig. 5 is for a system containing 70% tight-couples. It is noted that as the 50S zone develops, for increasing percentages of loose couples, the 30S zone, which represents subunits derived from both loose and tight couples, is also accentuated. It should be emphasized again here that these simulations do not in any way allow of any failure to re-equilibrate: at every stage of the separation, complete local chemical equilibration is established, as an essential requirement of the simulation. In fig. 6 is shown a composite diagram for the distribution of mass, which is resolved into contributions from tight couples and contributions from loose couples, for the same conditions as in fig. 5. From fig. 6 it is seen how the contribution to the composite pattern from the virtually totally dissociated loose couples gives rise to a resolved 50S subunit zone which would have been completely absent if only tight couples contributed to the pattern.

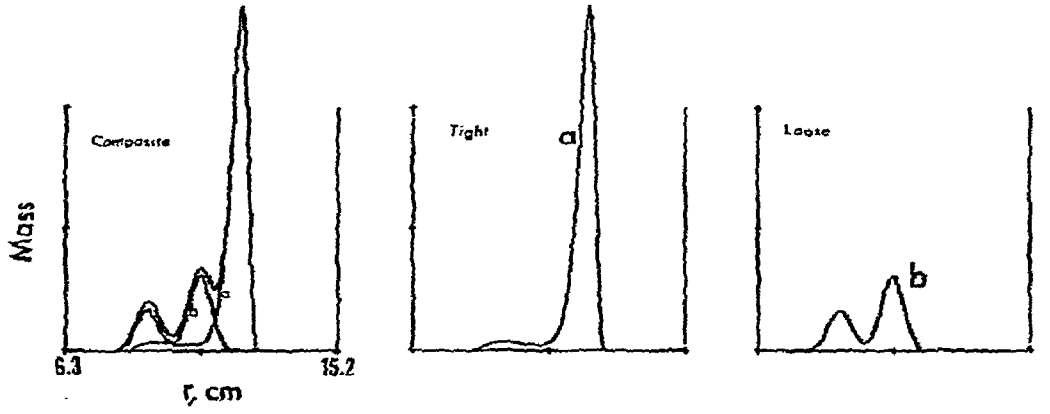

Fig. 6. Contributions to total mass as shown in fig. 5 (30% loose couples), due to tight couples (a) and loose couples (b). Rotor speed is 22 000 RPM.

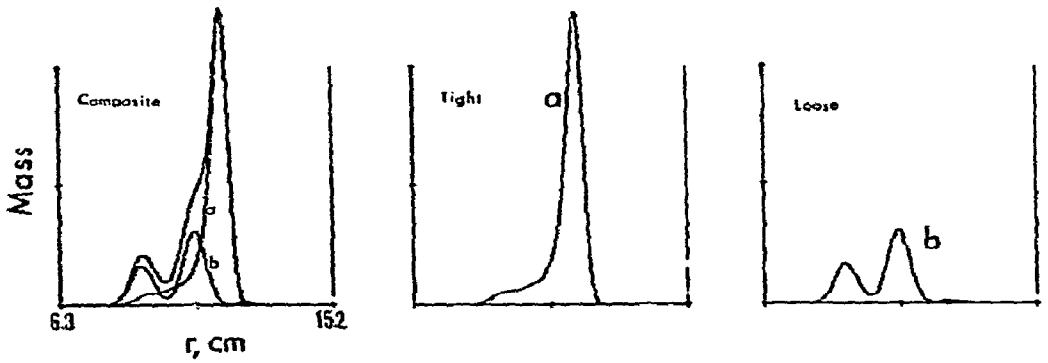

Fig. 7. Contributions to total mass from tight and loose couples for same system as in figs. 5 and 6 (30% loose couples), when the rotor speed is 41 000 RPM, illustrating decrease in resolution caused by pressure-induced dissociation of tight couples (see text).

Again, neither homogeneous tight couples nor homogeneous loose couples alone would give rise to three zones. It is noted from fig. 6 that the 30S zone contains a mixture derived from both tight and loose couples, making it much more difficult to separate the two types of 30S subunits, if such actually exist [11,20,23], than to separate a distinct species of 50S subunit.

In fig. 7 is shown for comparison the same system containing 70% "tight" couples, but the prediction is for sedimentation at 41 000 RPM. It is seen that the excellent resolution of a sharp zone in the 50S region at the lower speed now becomes much poorer, because part of the 30S "tail" due to pressure induced dissociation of tight couples at the higher speed overlaps the otherwise well-resolved 50S zone derived from loose couples. Among other things, this comparison suggests that analyses as well as separations will be more effective when performed at lower rotor speeds.

It should be noted that figs. 1–7 display not only the distribution of total mass, but the redistribution of individual species in the supporting density gradient in the revolving rotor. However, because of the dissociating effect of the pressure gradient, a further re-equilibration will take place among the various species when the pressure is relaxed to one atmosphere [1]. Since conventional methods of analysis require removal of the gradient from the rotor prior to taking local samples which can be used to determine the species present, the inferences which can be drawn from figs. 2–7 as to the species present at each part of the diagram will not correspond wherever 30S and 50S components coexist, to such an experimental species analysis [1,9]. Consequently, since there is no redistribution of total mass during the re-equilibration at one atmosphere, figs. 2–7 should be interpreted as giving the correct distribution of total mass and figs. 6 and 7 should be interpreted as giving the correct assessment of the contributions of loose couples and tight couples to the distribution of total mass.

In the companion paper [19] are described two series of relaxation kinetics experiments performed on "Type I" material isolated as the 70S zone from 26 000 RPM sedimentation in an SW 27 rotor on a 15% to 25% sucrose gradient in 10 mM Mg^{2+}, 10 mM Tris-HCl, 60 mM KCl, pH 7.75. The patterns for material isolated as 70S tight couples and rerun under these conditions should be expected to appear essentially as shown by the tight couple component in figs. 5 and 6. Nevertheless, extensive kinetic work at 10 mM Mg^{2+}, where isolated 70S tight couples would be expected to show no appreciable dissociation [16], has shown large reaction amplitudes [19]. This has led to the conclusion that loose couples have been regenerated rather quickly from the isolated 70S tight couples. In their study of sea urchin ribosomes which led to the discovery of the dissociation effect of pressure, Infante and Krauss [24] found that exposure to 0.5 M KCl led to some irreversible dissociation. In such cases, they reported three-zone patterns. An experimental study to determine the conditions under which this transformation takes place in the case of E. coli ribosomes is now under way [25].

4. Conclusions

Whenever a well-resolved 50S zone is present in a low speed sucrose density gradient sedimentation experiment on E. coli ribosomes, in addition to a 30S and a "70S" zone, under conditions where ribosomes and subunits should be in reversible equilibrium [15,16,19,20], the preparation must be a microheterogeneous mixture containing both tight and loose couples. The intermediate zone will represent some or all of the 50S species derived from loose couples and none of the 50S species derived from tight couples, in essential agreement with recent experiments of Hapke and Noll [23], whereas the 30S zone contains a mixture of the small subunits originating from both tight and loose couples. Loose couples and their subunits, it is noted, at least those derived from tight couples, instead of being irreversibly damaged in the sense of the subunit interaction, react more rapidly than tight couples [19,20]. Conversely, in any preparation of ribosomes containing a homogeneous population (i.e. either all tight or all loose couples) under rapidly re-equilibrating conditions [16,19,20], there will be only two zones in sucrose density gradient sedimentation experiments. For equimolar subunits overall, the slower zone in such a homogeneous preparation will always be composed of 30S subunits, and the faster zone will always contain *all* of the 50S subunit in the sample, whether the 70S species predominates, or is even virtually all dissociated, in the sedimentation experiment. This critique applies to ribosomes free of initiaion factors, tRNA or other non-ribosomal species.

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References

[1] A.A. Infante and R. Baierlein, Proc. Nat. Acad. Sci. U.S. 68 (1971) 1780.
[2] G. Kegeles, L.M. Rhodes and J.L. Bethune, Proc. Natl. Acad. Sci. U.S. 58 (1967) 45.
[3] L.F. ten Eyck and W. Kauzmann, Proc. Natl. Acad. Sci. U.S. 58 (1967) 888.
[4] R. Josephs and W.F. Harrington, Proc. Natl. Acad. Sci. U.S. 58 (1967) 1587.
[5] J.L. Bethune and G. Kegeles, J. Phys. Chem. 65 (1961) 433.
[6] J.L. Bethune and G. Kegeles, J. Phys. Chem. 65 (1961) 1755.
[7] J.L. Bethune and G. Kegeles, J. Phys. Chem. 65 (1961) 1761.
[8] J.L. Bethune, Ph.D. Thesis, Clark University, 1961.
[9] A.S. Spirin, N.V. Belitsina and E.B. Lishnevskaya, FEBS Lett. 24 (1971) 219.
[10] M. Noll, B. Hapke, M.H. Schreier and H. Noll, J. Mol. Biol. 75 (1973) 281.
[11] O.P. van Diggelen and L. Bosch, Eur. J. Bochem. 39 (1973) 499.
[12] O.P. van Diggelen, H. Oostrom and L. Bosch, Eur. J. Biochem. 39 (1973) 511.
[13] L.A. Ball, P.M. Johnson and I.O. Walker, Eur. J. Biochem. 37 (1973) 12.
[14] A.S. Spirin, B. Sabo and V.A. Kovalenko, FEBS Lett. 15 (1971) 197.
[15] R.S. Zitomer and J.G. Flaks, J. Mol. Biol. 71 (1972) 263.
[16] A. Wishnia, A. Boussert, M. Graffe P. Dessen and M. Grunberg-Manago, J. Mol. Biol. 93 (1975) 499.
[17] H. Walters, W. Smits, J. van Kessel and G. van Os, Eur. J. Biochem. 58 (1975) 269.
[18] G.A. Gilbert and R.C. Ll. Jenkins, Proc. Roy. Soc. London A253 (1959) 420.
[19] J.B. Chaires, M. Tai, C. Huang, G. Kegeles, A.A. Infante and A.J. Wahba, Biophys. Chem. 7 (1977) 179.
[20] H. Görisch, D.J. Goss and L.J. Parkhurst, Biochemistry 15 (1976) 5743.
[21] J.R. Cann and G. Kegeles, Biochemistry 13 (1974) 1868.
[22] B. Demple and A.A. Infante private communication.
[23] B. Hapke and H. Noll, J. Mol. Biol. 105 (1976) 97.
[24] A.A. Infante and M. Krauss, Biochim. Biophys. Acta 246 (1971) 81.
[25] J.B. Chaires and A.A. Infante, private communication.