ON SOME ARTIFACTS OF SUCROSE GRADIENT SEDIMENTATION OF RIBOSOMES

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1. Introduction

Sedimentation analysis of ribosomes is fraught with a number of artifacts. At the same time both analytical moving boundary sedimentation and moving zone sedimentation in sucrose gradients are widely utilized in biochemical practice without taking these hazards into account. It was only recently shown that the increasing hydrostatic pressure along the centrifuge tube may induce dissociation of ribosomes into subparticles during sedimentation [1, 2]. Since then the possibility of this artifact was accepted by some researchers as an explanation of a number of abnormal sedimentation patterns. However, this must be sooner regarded just as one of the artifacts resulting from *induction of dissociation in the course of sedimentation*.

One more artifact of this type can be the sucrose-induced dissociation of ribosomes in the course of sedimentation: it was recently shown by analytical ultracentrifugation that increasing sucrose concentrations (from 5% to 20%) have a progressive dissociating effect on *E. coli* ribosomes [3].

At the same time it was shown that dynamic equilibrium can exist between non-translating 70 S monoribosomes and 50 S and 30 S subparticles, i.e., the mixture of ribosomal particles can be a reversibly-interacting 50 S · 30 S ⇌ 50 S + 30 S system [4]. The sedimentation analysis of such systems requires a special complicated interpretation [5, 6]; in any case apparent sedimentation patterns may not reflect the number and amount of real components in the analyzed mixture and the observed velocities may not correspond to real sedimentation coefficients of particles. In other words, artifacts during centrifugation may originate independently of the dissociating effect of pressure [1, 2] or sucrose [3], and simply be the result of an existing *reversible interaction (dynamic equilibrium) of particles* in the system studied [4].

The following artifacts as a result of an existing reversible interaction (association–dissociation) of ribosomal particles can be expected in the case of moving zone sedimentation in sucrose gradient:

(1) The complete or partial disappearance of the coupled (70 S) component as a result of continuous washing off of the products of its dissociation (50 S and 30 S) during the moving of its zone through sucrose. Although the couple is present in the initial equilibrium mixture it is not revealed by this method. The artifact is typical of cases when the rate of separation of components during centrifugation is greater than the rate of the association reaction.

(2) The revealing of a zone which does not represent individual particles but a sedimenting reversibly-interacting mixture (e.g., 30 S + 50 S ⇌ 50 S · 30 S) with a corresponding weight-average sedimentation coefficient value. It looks like a "slow" ("loose") couple. This artifact is analogous to that in the case of boundary sedimentation during analytical ultracentrifugation [4]. It occurs when the rate of component separation during centrifugation is essentially slower than the rates of the forward and reverse reactions.

(3) The revealing of the zones, which, by the end

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of the centrifugation run, represent pure individual subparticles, located not at the 50 S and 30 S positions but further from the meniscus (the so called "fast 50 S and 30 S subparticles"). This artifact is also the result of the reversibly-interacting 50 S · 30 S ⇌ 50 S + 30 S system: the mixture zone during its movement gradually loses one subparticle, enriching itself with the other until it becomes a pure subparticle zone. It must arise when the rate of component separation is comparable with the rates of the forward and reverse reactions.

Variations of the speed and type of rotor, of ionic conditions and of ribosome concentration will change the ratio between the component separation rate and the rates of the reactions, thus giving one or another type of artifacts.

All three enumerated artifacts following from the existence of a dynamic equilibrium between the ribosomal subparticles are demonstrated experimentally in this paper.

2. Materials and methods

Preparations of uncharged *E. coli* (strain MRE-600) ribosomal particles were obtained as described previously [4, 7]. In the preparations a portion of the ribosomal subparticles was incapable of associating into the couples and thus did not participate in the equilibrium; in sucrose gradient centrifugation experiments they could be used as 50 S and 30 S markers. In some experiments, for "freezing" the equilibrium (to stop both the reaction of subparticle association and the dissociation reaction of couples) a one-tenth volume of 40% formaldehyde was added before centrifugation to the suspension of ribosomal particles [8]. Centrifugation was done in the SW-25.1 or SW-41 rotors of the Spinco L50 or L2 centrifuge. One of the three or six parallel (simultaneously centrifuged) tubes was, as a rule, used for sedimenting pure 50 S subparticles as a marker for calculating the sedimentation coefficients in the other tubes. The sedimentation coefficients of components were estimated proceeding from the assumption that the velocity of particles in the sucrose gradient is constant (the distance travelled from the meniscus is directly proportional to the sedimentation coefficient, at least within the limits from 30 S to 70 S). Accuracy of

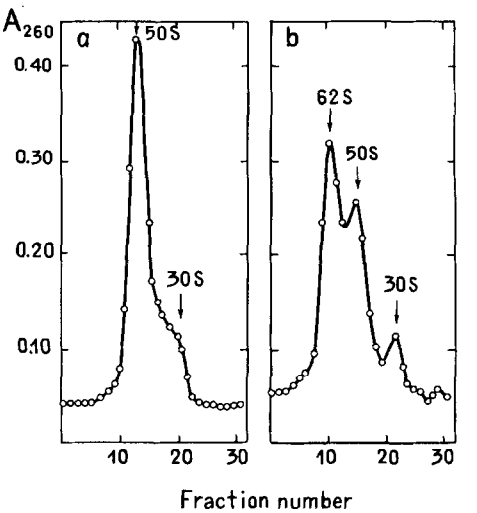

Fig. 1. Sucrose gradient sedimentation distribution of uncharged ribosomal particle mixture at two different Mg^{2+} concentrations. Gradient from 5 to 20% sucrose. Buffer in sucrose: 5 or 10 mM $MgCl_2$ – 50 mM KCl – 1 mM K_2HPO_4, pH 7.5. Ribosome suspensions (0.45 ml) with a concentration of about 1 mg/ml were layered onto the gradients. Centrifuged at 25,000 rpm (SW-25) for 5 hr at 4°.
a) 5 mM $MgCl_2$; b) 10 mM $MgCl_2$.

measurement made with the 50 S marker was within the limits of ±1 S. The ultraviolet absorption of the fractions obtained as a result of centrifugation was measured at 260 nm after dilution. When necessary, the fractions after sucrose gradient centrifugation were analyzed in the Spinco E ultracentrifuge with ultraviolet absorption optics.

3. Results

3.1. *Disappearance of couples*

The first artifact of centrifugation is demonstrated in fig. 1a: sucrose gradient centrifugation of the mixture of uncharged ribosomal particles 50 S · 30 S ⇌ 50 S + 30 S at 5 mM Mg^{2+} reveals no 50 S · 30 S couples but only 50 S and 30 S subparticle peaks. Together with this, 50 S · 30 S couples were present in the equilibrium mixture at the same 5 mM Mg^{2+} prior to centrifugation: if a study is made of the mixture where the equilibrium has been "frozen" by

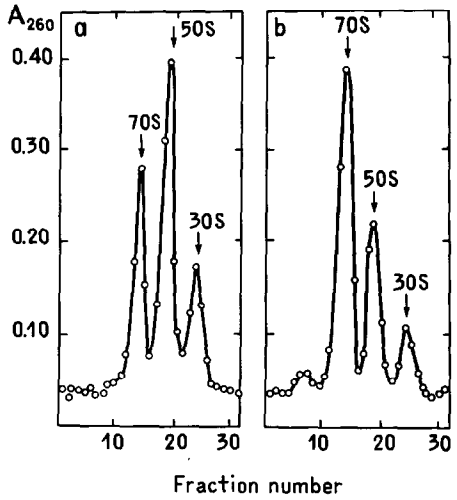

Fig. 2. Sucrose gradient sedimentation distribution of uncharged ribosomal particle mixture fixed with formaldehyde at two different Mg^{2+} concentrations. Centrifuging conditions same as in fig. 1 with the exception of time which was 3 hr 40 min.
a) Ribosomal subparticle mixture was fixed with formaldehyde at 5 mM $MgCl_2$ and then layered onto the gradient with the same Mg^{2+} concentration;
b) ribosomal subparticle mixture was fixed with formaldehyde at 10 mM $MgCl_2$ and then layered onto the gradient with the same Mg^{2+} concentration.

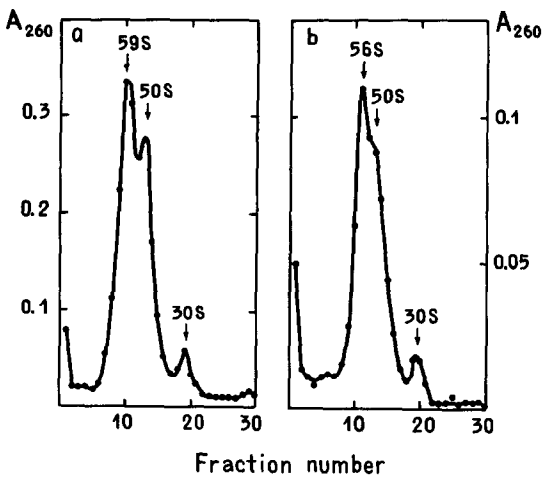

Fig. 3. Sucrose gradient sedimentation distribution of uncharged ribosomal particle mixture at two different ribosome concentrations. Gradient from 5 to 20% sucrose. Buffer in sucrose: 14 mM $MgCl_2$ – 100 mM NH_4Cl – 10 mM Tris-HCl, pH 7.2. Centrifuged at 40,000 rpm (SW-41) for 3 hr at 4°.
a) 0.37 mg of the 50 S and 30 S subparticle equimolar mixture was layered in a 0.3 ml volume onto the gradient;
b) 0.12 mg of the 50 S and 30 S subparticle equimolar mixture was layered in a 0.3 ml volume onto the gradient.
Five drop fractions were collected and diluted with 2 ml of the buffer for UV absorption measurements.

adding formaldehyde [8], a distinct 70 S component is observed (fig. 2a). (In special experiments on the kinetics of formaldehyde action, it was shown that it practically instantaneously stops the reaction of ribosomal subparticle association and blocks the dissociation reaction a little more slowly [9]; it follows that formaldehyde in the conditions used cannot increase the proportion of couples in the mixture.)

3.2. *Appearance of "slow couples"*

Fig. 1b shows that at 10 mM Mg^{2+} the more rapidly sedimenting zone occupies a position near 60 S. At the same time, after formaldehyde fixation of the initial mixture in the same conditions (10 mM Mg^{2+}) a more distinct separation of the sedimenting zones is observed and the zone of 50 S · 30 S couples occupies the 70 S position (fig. 2b). This indicates that in the first case (fig. 1b) the 60 S component may represent the sedimenting zone of reversibly-interacting (comparatively rapidly associating and dissociating) particles moving with a weight-average velocity (70 S $\rightleftharpoons$ 50 S + 30 S) and not real "slow" couple particles.

Experiments on diluting the initial suspensions of ribosomal particles before their layering onto the sucrose gradient corroborate the assumption of the 60 S component as an equilibrium mixture zone: as would be expected its velocity (apparent sedimentation coefficient) decreases with the decrease of the ribosomal particle concentration (fig. 3a and b).

The fact that the 60 S zone does not simply represent "slow" 50 S · 30 S couples but is a sedimenting mixture of 50 S · 30 S $\rightleftharpoons$ 50 S + 30 S can also be proved by the following. If a fraction corresponding to the 60 S component (fig. 4) is collected separately, fixed with formaldehyde and re-centrifuged, it is seen that the 60 S component is transformed into three distinct components, the 70 S, 50 S and 30 S (fig. 5). (In conditions of 10–20 mM Mg^{2+}, the dissociation reaction rate seems to be essentially slower than the rate of formaldehyde fixation of the 70 S ribosomal particles [9], so that formaldehyde can hardly induce

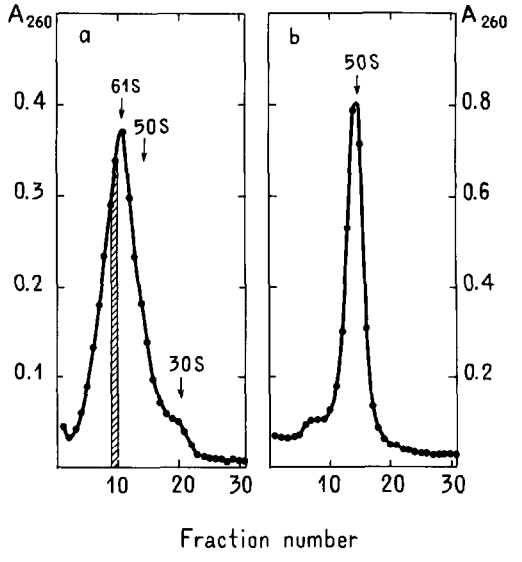

Fig. 4. Sucrose gradient sedimentation of ribosomal particles for following the isolation of the 60 S component. Gradient from 5 to 20% sucrose. Buffer in sucrose: 10 mM $MgCl_2$ – 100 mM KCl – 10 mM triethanolamine-HCl, pH 7.3. Centrifuged at 20,000 rpm (SW-25.1) for 10 hr at 4°.

a) 4.3 mg of the 50 S and 30 S subparticle equimolar mixture was layered in a 0.5 ml volume onto the gradient. 10 drop fractions were collected after centrifugation. Of these the first drop was diluted with 2 ml of the buffer for UV absorption measurement and the remaining 9 drops were fixed with formaldehyde and used in recentrifugation experiments (see fig. 5);

b) (marker): 0.47 mg of 50 S subparticles in a 0.5 ml volume was layered on the gradient; after centrifugation 10 drop fractions were collected and diluted with 2 ml of the buffer for UV absorption measurements.

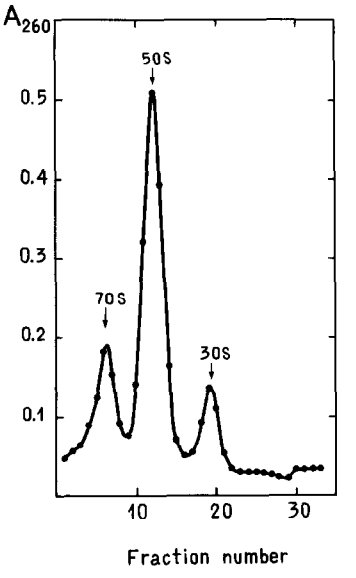

Fig. 5. Sucrose gradient sedimentation distribution of the formaldehyde fixed 60 S component. Gradient from 15 to 30% sucrose. Buffer in sucrose: 10 mM $MgCl_2$ – 100 mM KCl – 10 mM triethanolamine-HCl, pH 7.2. Centrifuged at 22,000 rpm (SW-25.1) for 16 hr at 4°. The formaldehyde fixed fraction corresponding to the hatched region in fig. 4 (fraction No. 9) was layered on the gradient. After centrifugation 10 drop fractions were collected and diluted with 1.4 ml of water for UV absorption measurements.

dissociation of part of the couples.)

3.3. *Appearance of "fast" subparticles*

To illustrate the third type of artifacts the results of centrifugation of identical portions of the same mixture of uncharged ribosomal particles at different Mg^{2+} concentrations in the sucrose gradient can be demonstrated (fig. 6). In the given experiment components were observed at positions near 30 S, 50 S and 60 S–55 S at Mg^{2+} concentrations from 20 mM to 14 mM; at 10–12 mM Mg^{2+} apparent components occupied 30 S, 42–44 S and 52–54 S positions; at a Mg^{2+} concentration between 10 mM and 7 mM, the components after centrifugation were located at 30 S, 40 S and 50 S; and at Mg^{2+} from 6 mM and lower two subparticle components were seen at 30 S and 50 S positions. In order to interpret this strange picture of a gradual shift of the 60 S component to the 50 S component, the appearance of the 40 S component, and then the shift of the 40 S component to the 30 S component, the contents of real 30 S and 50 S subparticles were analyzed in fractions of the sucrose gradient. For this purpose, the fractions were dialyzed to lower the Mg^{2+} concentration to 1–3 mM, necessary for the complete dissociation of couples into 30 S and 50 S subparticles, and then studied in the analytical ultracentrifuge. The distribution of 30 S and 50 S subparticles over the fractions of the sucrose gradient with 20 mM Mg^{2+} and 10 mM Mg^{2+} is shown in fig. 7a and b. It appeared that there were four components after centrifugation in the sucrose gradient: the first is at the 60 S–55 S position mainly representing 50 S subparticles; the second is at the 50 S position also representing 50 S subparticles; the third is at a

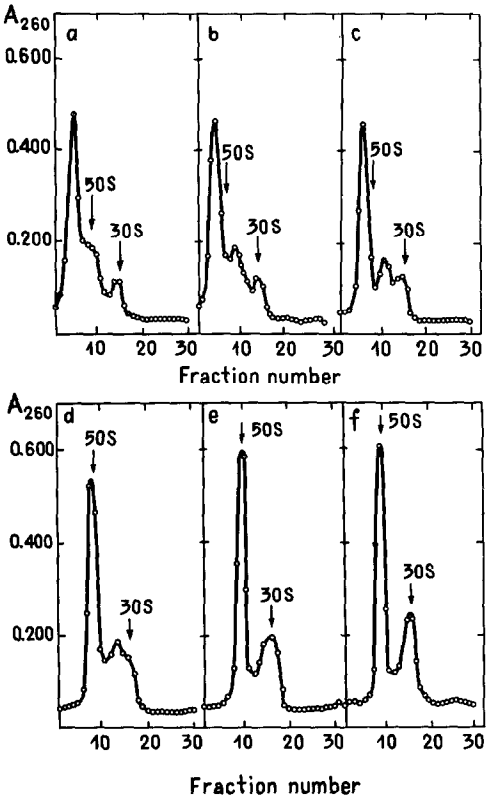

Fig. 6. Sucrose gradient sedimentation distribution of uncharged ribosomal particle mixture depending on the Mg^{2+} concentration. Gradient from 5 to 20% sucrose. Buffer in sucrose: from 5 to 20 mM $MgCl_2$ – 100 mM NH_4Cl – 10 mM Tris-HCl, pH 7.3. Identical (0.13 mg 30 S plus 0.26 mg 50 S) portions of the same ribosome suspension in a volume of 0.2 ml at 20 mM $MgCl_2$ were layered on the gradient. Centrifuged at 40,000 rpm (SW-41) for 3 hr 30 min at 4°.
a) 14 mM $MgCl_2$;
b) 12 mM $MgCl_2$;
c) 10 mM $MgCl_2$;
d) 8 mM $MgCl_2$;
e) 7 mM $MgCl_2$;
f) 6 mM $MgCl_2$.

position near 40 S–45 S chiefly representing 30 S subparticles; and the fourth is at a position near 30 S representing 30 S subparticles. The 40 S–45 S component is not a 30 S particle dimer, and the 60 S–55 S component is not a dimer of the 50 S particles: even with 20 mM Mg^{2+} in the sucrose gradient a preparation of pure 50 S particles does not display dimers (fig. 7d), while a preparation of pure 30 S

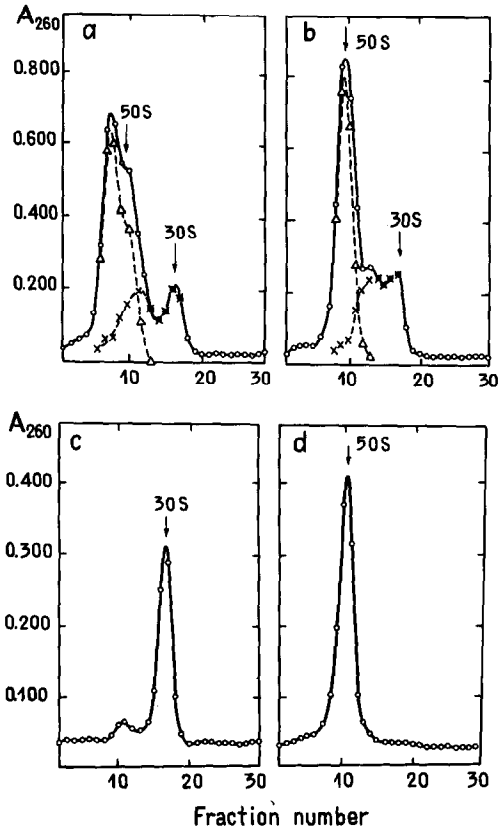

Fig. 7. Tracing of distribution of real 30 S and 50 S subparticles through the sedimentation distribution profile of uncharged ribosomal particle mixture. Gradient from 5 to 20% sucrose. Buffer in the gradient: 20 mM or 10 mM $MgCl_2$ – 100 mM NH_4Cl – 10 mM Tris-HCl, pH 7.3. Centrifuged at 40,000 rpm (SW-41) for 3 hr 30 min at 4°. After centrifugation each fraction was analyzed in the analytical ultracentrifuge under conditions of complete dissociation into 50 S and 30 S subparticles and the amount of 50 S and 30 S subparticles in each fraction of the gradient was thus measured.
(o—o—o): Distribution of total UV absorption at 260 nm;
(X - - - X - - - X): distribution of 30 S subparticles;
(Δ - - - Δ - - - Δ): distribution of 50 S subparticles.
a) Equimolar mixture of 30 S and 50 S subparticles (0.5 mg in a 0.3 ml volume) centrifuged in the sucrose gradient at 20 mM Mg^{2+};
b) equimolar mixture of 30 S and 50 S subparticles (0.5 mg in a 0.3 ml volume) centrifuged in the sucrose gradient at 10 mM Mg^{2+};
c) (control): only 30 S subparticles (0.13 mg in a 0.2 ml volume) centrifuged in the sucrose gradient at 20 mM Mg^{2+} (the same preparation as in a and b, but other centrifugation run);
d) (Control): only 50 S subparticles (0.2 mg in a 0.2 ml volume) centrifuged in the sucrose gradient at 20 mM Mg^{2+} (the same preparation as in a and b, but other centrifugation run).

particles, though it does show some dimers, displays them in an incomparably lesser amount than the apparent 40 S component (cf. fig. 7a or b, dotted line, and fig. 7c). Consequently, the faster movement of part of the 50 S subparticles and of part of the 30 S subparticles can be explained by the fact that they participated in the reaction 50 S · 30 S ⇌ 50 S + 30 S and for this reason at first moved faster, but little by little lost one of its partners during centrifugation and became pure subparticles. The decrease of the Mg^{2+} concentration, shifting the equilibrium towards dissociation, leads to an earlier washing off of the partners from the equilibrium mixtures during centrifugation; that is why, with the lowering of Mg^{2+} concentrations, the initial 60 S–55 S component progressively approaches the 50 S position and the initial 45 S–40 S component approaches the 30 S position.

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