SUPPRESSION OF RNA PRECIPITATION DURING Cs_2SO_4 DENSITY GRADIENT CENTRIFUGATION

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Equilibrium sedimentation in CsCl density gradients, as introduced by Meselson, Stahl and Vinograd (1957), provides a most useful tool for fractionation of DNA and for determination of its many properties. On the other hand, this ingenious technique has not been directly applicable to all kinds of RNA, since under the usual conditions CsCl is not sufficiently soluble to provide gradients applicable for RNA. This problem has been circumvented by centrifugation at 40 to 50° C, temperatures at which the buoyant density of saturated CsCl solutions is high enough to band RNA (Bruner and Vinograd, 1965; Kelly et al., 1965). Technical difficulties, however, including fogging of the lenses and the inherent thermal lability of RNA, limit the usefulness of this procedure. The use of cesium formate (Davern and Meselson, 1960) and cesium acetate solutions (Stanley, 1963) is hampered by their high viscosity. Cs_2SO_4, which has been utilized for the density gradient analysis of DNA (Wake and Baldwin, 1962; Erikson and Szybalski, 1964), would be readily applicable to the banding of RNA, were it not for the phenomenon of aggregation and precipitation observed with many RNA's. The precipitated RNA forms ultra-sharp bands at a buoyant density usually somewhat higher than that characteristic for the dissolved RNA, with a large part of the material sedimenting toward the bottom of the cell. Furthermore, with two or more kinds of RNA present, their co-precipitation makes evaluation of the analytical banding patterns hardly possible.

It occurred to us that the precipitation of RNA might be closely related to the hydrogen-bonded aggregations observed between RNA and denatured DNA

(Opara-Kubinska, Kubinski and Szybalski, 1964) or between RNA molecules (Grunberg-Manago and Gros, 1964) at high salt concentrations, a phenomenon which is averted in the presence of formaldehyde or other agents interfering with the formation of ordered structures between complementary base sequences. Thus, addition of formaldehyde to Cs_2SO_4 solutions should prevent the aggregation and precipitation of RNA. Experiments have confirmed this expectation.

MATERIALS and METHODS

Bacillus subtilis RNA was isolated by the classical phenol procedure and fractionated into 5s (sRNA), 18s, and 23s RNA (rRNA) by sucrose gradient centrifugation or on the methyl-esterified albumin-kieselguhr column, as outlined by Opara-Kubinska et al., (1964). R17 coliphage RNA and its replicative, RNase-treated, double-stranded form were kindly supplied by Dr. R. L. Erikson. Tobacco mosaic virus (TMV) RNA, normal or labeled with 5-fluorouracil (FU·RNA; 50% uracil replacement), was prepared as described previously (Lozeron and Gordon, 1964).

Samples for routine Cs_2SO_4 density-gradient centrifugation were prepared by mixing 0.1 ml of SPC buffer (0.15 M-NaCl + 0.10 M-Na_2HPO_4 + 0.02 M-Na_3-citrate, pH 7.1) containing 1 to 1.5 μg RNA (per band) with 0.1 ml water (control) or 0.1 ml formaldehyde (usually 5% HCHO), and adding (very slowly with stirring) ten minutes later 0.3 ml of Cs_2SO_4 solution (saturated at 25°C), followed by final refractometric density determination and adjustment, usually to 1.61 to 1.64 g/cm^3. Samples for mixed CsCl-Cs_2SO_4 gradients were prepared as described above by adding saturated solutions of CsCl (1.9 g/cm^3) and Cs_2SO_4 (2.0 g/cm^3) (added in this order or as a mixture) to RNA-containing buffer, with or without formaldehyde. Samples transferred to 2°, 12mm Kel-F lined cells were centrifuged at 25°C, 44,770 r.p.m. for 22 or 44 hrs under conditions customary in this laboratory (Erikson and Szybalski, 1964).

The Cs_2SO_4 and CsCl were the selected technical grades of American Potash & Chemical Corp., New York 10016, N. Y., and were purified by boiling with a large excess of acid-washed Norit A, filtration, and recrystallization from

hot water. Optical grades of Cs_2SO_4 and CsCl manufactured by Rare Metals Derivatives, Inc., Ambler, Penna., Harshaw Chemical Co., Cleveland 44106, Ohio, and S. H. Cohen Assoc., Yonkers 10710, N. Y. (99.99%) were also evaluated.

RESULTS

In formaldehyde-free Cs_2SO_4 gradients, coliphage R17 RNA, in its single-stranded (M.W. 1.2×10^6) or double-stranded configuration, and 5s (M.W. 2.3×10^4) or 18s (M.W. 0.6×10^6) B. subtilis RNA form symmetrical, Gaussian-like bands at the buoyant densities of 1.620, 1.607, 1.643, and 1.649 g/cm^3, respectively. Under the same conditions, however, B. subtilis 23s RNA (M.W. 1.2×10^6), TMV RNA (M.W. 2×10^6) and TMV FU·RNA form precipitates or co-precipitates, which appear as hyper-sharp, often faint bands (Figs. 1A,2A,3A). When 1% formaldehyde is employed, under the conditions described in Materials and Methods, all RNA's tested form symmetrical, Gaussian-like bands at the densities 1.606 (RF·RNA), 1.636 (5s RNA), 1.634 (18s RNA), 1.636 (23s RNA (Fig. 3B)), 1.627 (TMV RNA (Figs. 1B, 2B)), and 1.652 (TMV FU·RNA (Fig. 2B)). Thus, the presence of formaldehyde prevents the precipitation of high molecular weight RNA and often decreases its buoyant density. Intermolecular cross-

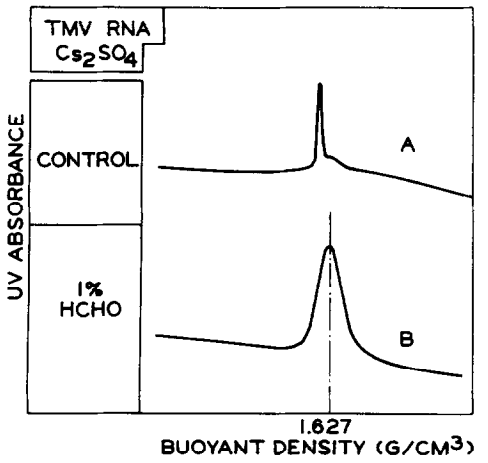

Fig. 1. Tracings of photographs taken after 44 hrs of Cs_2SO_4 density-gradient centrifugation (44,770 r.p.m., 25°C) of TMV-RNA in the absence (A), and in the presence of 1% formaldehyde (B).

linking of RNA by formaldehyde (Freifelder and Davidson, 1963) was neither expected nor observed under the present experimental conditions, as evidenced by complete separation of the TMV RNA and FU·RNA bands (Fig. 2B). Although sRNA does not form a hyper-sharp band in the absence of formaldehyde, the profile of this relatively broad band becomes more symmetrical and more easily discernible when 1% formaldehyde is employed.

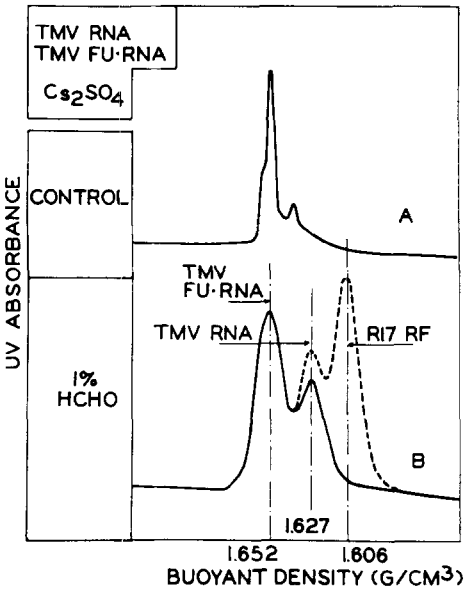

Fig. 2. Tracings of photographs taken after 44 hrs. of Cs_2SO_4 density-gradient centrifugation (44,770 r.p.m., 25°C) of TMV-RNA and TMV FU·RNA in the absence (A), and in the presence (B) of 1% formaldehyde (solid line). The replicative form (RF) of coliphage R 17 RNA (1.606 g/cm^3) was added as the density reference (dotted line) in experiment B.

In the experiments described above, 1% formaldehyde suppresses the precipitation of RNA, provided the range of initial density of Cs_2SO_4 is 1.55 - 1.64 g/cm^3. Within this density range TMV RNA, TMV FU·RNA, and 23s RNA band in the region between the bottom and the middle of the analytical cell. When attempts were made to band TMV RNA in the upper half of the cell by employing initial densities greater than 1.65 g/cm^3, partial or complete disappearance of the RNA bands was noted, in the absence or even in the presence of formaldehyde. This difficulty, which will be designated here as 'loss' of RNA, can be

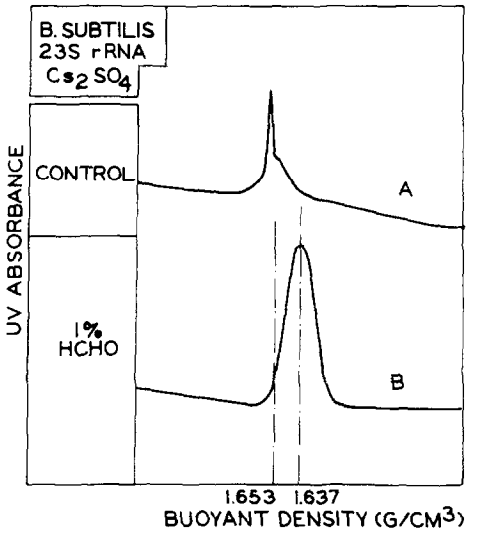

Fig. 3. Tracings of photographs taken after 44 hrs of Cs_2SO_4 density-gradient centrifugation (44,770 r.p.m., 25°C) of B. subtilis 23S ribosomal RNA in the absence (A), and in the presence of 1% formaldehyde (B).

remedied by using mixtures of CsCl and Cs_2SO_4. If a CsCl-Cs_2SO_4 mix (1.25:1) is used (0.05 ml buffer 0.25 ml CsCl, 0.20 ml Cs_2SO_4; see Materials and Methods), TMV RNA and TMV FU·RNA form separate bands close to the meniscus. A low degree of co-precipitation between the two RNA's is still apparent in the absence of formaldehyde but the bands become Gaussian when 1% formaldehyde is present. On the other hand, if a mixed gradient with a high ratio of CsCl:Cs_2SO_4 (8:1) is employed (0.05 ml buffer, 0.40 ml CsCl, 0.05 ml Cs_2SO_4), conditions in which TMV RNA and TMV FU·RNA band near the cell bottom, precipitation, co-precipitation, and 'loss' of the RNA's are eliminated even in the absence of formaldehyde, as evidenced by the complete resolution of both RNA's and the formation of symmetrical, Gaussian-like bands. A more complete description of RNA behavior in various mixed gradients will be presented elsewhere.

Extensive purification of Cs_2SO_4 has been reported to suppress, at least in part, the RNA precipitation phenomenon (Riley et al., in press). For TMV RNA, however, the formation of precipitate bands and the loss of RNA at high Cs_2SO_4 concentrations could not be eliminated using the purest optical grades of

Cs_2SO_4 employed by Riley et al. (in press) and described in Materials and Methods.

CONCLUSIONS and SUMMARY

Two phenomena observed during density-gradient centrifugation of high molecular weight RNA's in the Cs_2SO_4 gradient are precipitation with the formation of hyper-sharp bands, and 'loss' of RNA bands when high initial Cs_2SO_4 concentrations are employed. The first phenomenon can be suppressed by the addition of 0.5 to 2% formaldehyde; dimethylformamide is less effective at these concentrations. The 'loss' of RNA bands can be eliminated by the use of mixed CsCl-Cs_2SO_4 (1.25:1) gradients in the presence of formaldehyde. With extreme CsCl:Cs_2SO_4 ratios (8:1) the precipitation of RNA can be almost totally suppressed even in the absence of formaldehyde. Also, the resolution of TMV RNA and TMV FU·RNA bands increases by approximately 50% compared to the resolution obtained in Cs_2SO_4.

These techniques permit the fractionation of various RNA's, including viral RNA in the presence of host ribosomal RNA, free of the confusing artifacts caused by the co-precipitation of RNA fractions. Whenever formaldehyde does not interfere with assays of RNA, the Cs_2SO_4 gradient of initial density 1.6 g/cm^3 containing 1% formaldehyde is quite satisfactory. With very high molecular weight RNA, mixtures of 0.05 ml buffer, 0.35 ml CsCl, 0.10 ml Cs_2SO_4 (TMV RNA bands approximately at the cell center) or 0.10 ml buffer, 0.20 ml CsCl, 0.20 ml Cs_2SO_4, supplemented with 1.0% formaldehyde, would be recommended. When formaldehyde cannot be tolerated, a gradient containing 0.05 ml buffer, 0.40 ml CsCl, and 0.05 ml Cs_2SO_4 permits RNA fractionation with a minimum of precipitation or other interfering phenomena, but only the lower one-third of the cell can be utilized with this solvent.

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