THE POSSIBILITY OF AGGREGATION OF RIBOSOMAL RNA DURING HOT PHENOL-SDS DEPROTEINIZATION

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Under certain conditions, the widely used hot phenol-SDS method of deproteinizing ribosomal RNA (r-RNA) can give rise to non-covalently bonded aggregates of the two species of r-RNA. This effect is dependent upon the concentration of RNA in solution and on the temperature of extraction. Since aggregation would be a serious artifact, the phenomenon was studied in some detail. The results indicate that such artifacts can best be avoided by extracting relatively low concentrations of RNA at a lower temperature than previously recommended.

MATERIALS AND METHODS

HeLa cells (type 3) were grown in suspension culture, labeled and harvested according to the methods of Eagle (1959) and Penman, Scherrer, Becker & Darnell (1963). Hot phenol-sodium dodecyl sulfate (SDS) deproteinization of the cell cytoplasm was carried out according to the method of Scherrer & Darnell (1963) as modified by Penman (1966) except that the temperature of the extraction was varied as indicated. This procedure, and the rationale for the various steps (S. Penman, unpublished observations) is as follows. The cell extract to be deproteinized is prepared in a small volume of buffer, which contains 0.5% SDS and 10^{-2}M EDTA. The nuclei from 100 ml of cells is usually extracted in a 2 ml volume, while 100 ml of entire HeLa cells are usually concentrated into 4 ml for extraction. The SDS will denature the proteins present, and the addition of an equal volume

of phenol completes the process. Heating the phenol-aqueous mixture partially releases RNA which has become trapped in the SDS-protein interface which forms between the phenol and aqueous phases. This heating is particularly important for quantitative recovery from preparations containing nuclei. EDTA is used to remove divalent ions, so that this SDS-protein-RNA complex can be disrupted. After shaking against phenol, chloroform containing 1% (v/v) isoamyl alcohol (as an anti-foaming agent) equal in volume to the phenol is added, and the mixture again shaken vigorously with heating. The chloroform-phenol phase is much denser than the aqueous phase, giving a well defined stable interface after centrifugation. The chloroform also removes SDS from the interface complex, releasing the trapped RNA. The chloroform-phenol phase is then removed, and the remaining aqueous phase and the interface are shaken against several changes of an equal volume of chloroform, to complete the release of the RNA from the interface and also to remove the last traces of phenol. The aqueous layer, which contains the RNA is then removed, leaving the protein behind in the interface. After extraction, the RNA is precipitated by addition of two to three volumes of cold ethanol and collected by centrifugation. The precipitated RNA is in each case dissolved in SDS buffer (0.1M NaCl, 0.01M TRIS-Cl^-, 0.001M EDTA, 0.5% SDS; pH 7.4) and analyzed by sucrose density gradient centrifugation.

Zonal centrifugation of the RNA was carried out at 25°C using 15-30% SDS-sucrose gradients as described by Gilbert (1963). The sedimentation profiles of the RNA in the gradients were analyzed with a Gilford recording spectrophotometer and fractions were collected when necessary. Dissociation of the aggregated RNA by use of dimethylsulfoxide (DMSO) was carried out by the method of Katz & Penman (1966, 1967) using polyadenylic acid as a co-precipitant.

RESULTS AND DISCUSSION

The effect of the temperature of extraction of HeLa cell cytoplasmic

RNA was investigated as follows: approximately 6 x 10^7 cells (150 ml of cell culture) were separated into nuclear and cytoplasmic fractions (Penman, et al., 1963). The cytoplasmic fraction was divided into two parts; one part was extracted with phenol-SDS at 55° and the other at 65°. These samples were then analyzed on sucrose density gradients. The results of the experiment, shown in Figure 1, indicate that extraction at 65° results in the formation of a considerable amount of RNA that sediments faster than 28S.

The aggregation of RNA is also a function of the concentration of RNA during extraction. Extraction of high concentrations of RNA even at 55° leads to the formation of a considerable amount of aggregated RNA, as was seen as follows. The ribosomes from 1.5 x 10^8 cells were obtained by centrifugation of cytoplasmic extract for one hour at 150,000 x g.

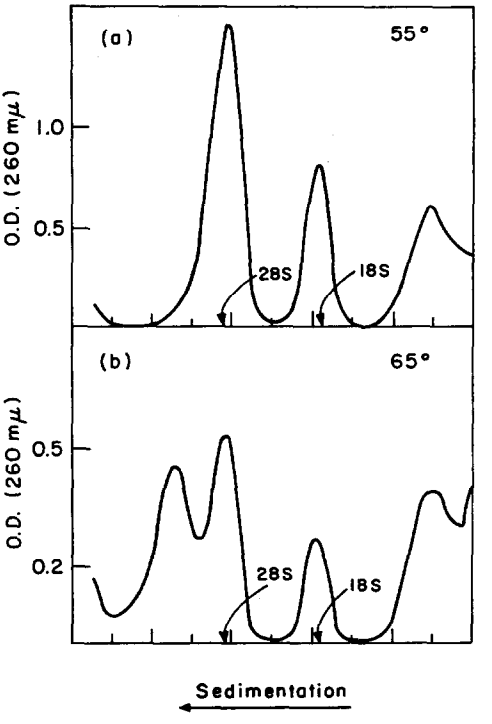

Figure 1. The effect of temperature on the sedimentation profile of hot phenol extracted HeLa cell cytoplasm. (a) Extracted at 55°. (b) Extracted at 65°.

The ribosomes were resuspended in 1 ml of SDS buffer to form a very concentrated solution and extracted with phenol at 55°. The resulting concentration of RNA was approximately 4 mg/ml. Ribosomal RNA was also extracted at 1/3 and 1/9 this concentration. The results of sucrose density gradient analysis are shown in Figure 2. It is evident that the amount of material sedimenting faster than 28S r-RNA depends upon the concentration of RNA subjected to hot phenol-SDS extraction.

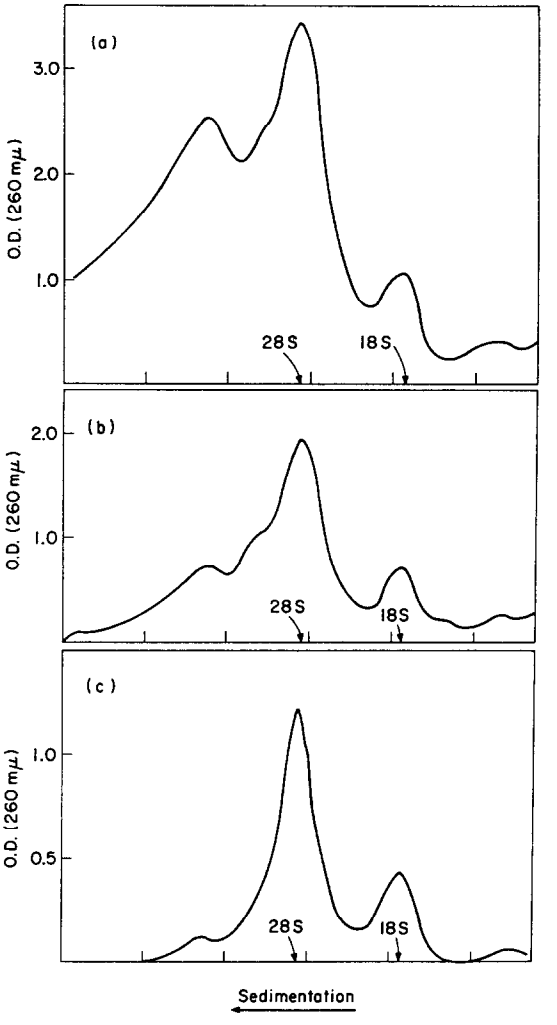

Figure 2. The effect of concentration on the sedimentation profile of hot phenol extracted HeLa cell ribosomes. (a) The cytoplasmic ribosomes from 10^8 HeLa cells were extracted in 1 ml of SDS buffer at 55°. (b) The cytoplasmic ribosomes of 3 x 10^7 HeLa cells were extracted as above. (c) The cytoplasmic ribosomes from 10^7 HeLa cells were extracted as above.

In addition to the formation of an aggregated species of RNA containing both 28S and 18S r-RNA (see below), aggregation of 28S r-RNA alone and 18S r-RNA alone occurs. To show this more clearly, the r-RNA from 1.6 x 10^8 cells was centrifuged on a sucrose density gradient. The 28S and 18S r-RNA species were collected separately, precipitated with ethanol, and dissolved in SDS buffer. Aliquots containing 28S r-RNA and 18S r-RNA alone were separately heated to 65° in the presence of phenol for five minutes. The phenol was removed by three chloroform extractions and the RNA then analyzed by sucrose density gradient centrifugation. The results of this experiment are shown in Figures 3a and 3b. 28S RNA alone forms an aggregate of higher S value than 28; 18S RNA alone forms an aggregate which has a sedimentation coefficient slightly less than 28.

It can be demonstrated that the aggregates found are not covalently cross-linked. A small amount of very high specific activity H^3-18S ribosomal RNA was mixed with a large excess of unlabeled 28S RNA. This mixture was heated in the presence of phenol at 65° for 15 minutes and then

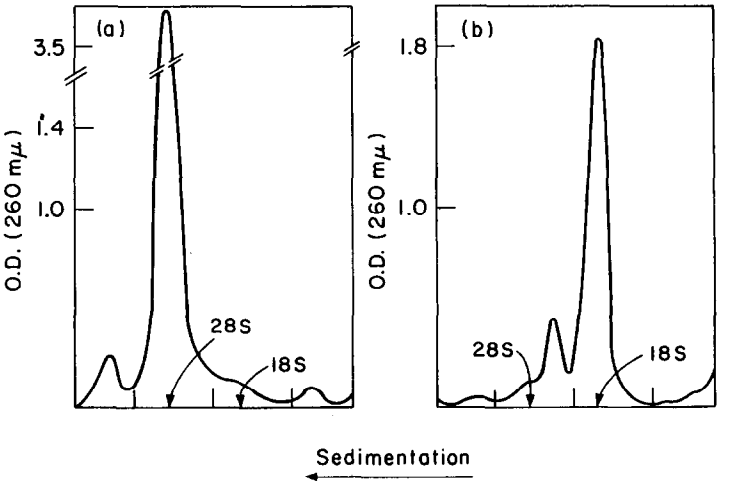

Figure 3. The aggregation of HeLa cell r-RNA in the presence of phenol. HeLa cell r-RNA was isolated as described in the text. (a) Approximately 0.5 mg of 28S r-RNA was heated to 65° for five minutes in the presence of phenol and subjected to sucrose density gradient centrifugation. (b) Approximately 0.3 mg of 18S r-RNA were treated as above.

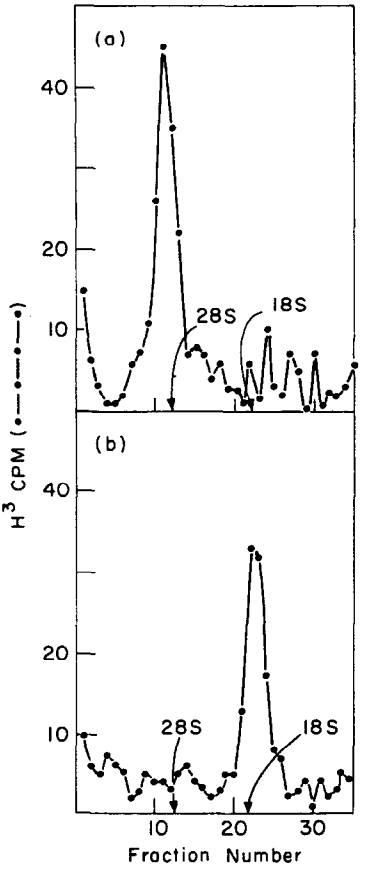

Figure 4. The effect of DMSO upon the sedimentation behavior of 28S : 18S r-RNA aggregate. (a) Sedimentation profile of the radioactivity isolated from the material sedimenting faster than 28S, as described in the text. (b) Sedimentation profile of the same material after DMSO treatment. The arrows indicate the positions of untreated ribosomal RNA components sedimented in a parallel gradient.

centrifuged on a sucrose gradient. A small peak of radioactivity was found sedimenting faster than 28S which was absent in a control gradient of unheated RNA. This material was isolated from the gradient, precipitated with ethanol and resuspended in SDS buffer. The RNA was then subjected to the solvent denaturation process described by Katz and Penman (1966, 1967). The result of this treatment is shown in Figure 4. The radioactivity in the untreated material retains its sedimentation properties, while the material treated in dimethylsulfoxide has been disaggregated,

and the radioactivity now sediments at the 18S position. The concentration of the 18S material used in the formation of the aggregate was extremely low, about 0.025 mg/ml, thus precluding the self-aggregation of the 18S species. The lability to DMSO treatment indicates that the aggregate is not a covalently cross-linked one. In order to make the above demonstration, it was necessary to use polyadenylic acid as a carrier species during the denaturation and subsequent precipitation steps to insure the separation of the aggregate strands and to prevent the formation of new aggregates from the input material (Katz & Penman, in preparation).

We have thus demonstrated that use of too high a temperature during the hot phenol extraction procedure can lead to an aggregation of 18S and/or 28S ribosomal RNA into faster sedimenting species of RNA. These experiments demonstrate that HeLa cell RNA aggregates in a manner similar to that described for E. coli r-RNA by Marcot-Queiroz & Monier (1965) and that areas of complementarity between the two species of RNA of ribosomes may be a general feature. It should be emphasized that these results do not in any way cast doubt upon the previous finding of a heavy precursor to ribosomal RNA (Scherrer & Darnell, 1963). That the 45S RNA precursor is not an artifact produced by the hot phenol extraction is clear from the following observations. The 45S RNA first extracted by Scherrer and Darnell was preferentially labeled during short pulses. It chases completely and rapidly in actinomycin (Darnell, 1962) into 32S and 18S RNA. The concentration of cellular RNA used in the experiments of Scherrer and Darnell were below that shown to result in aggregation. Furthermore, the 45S and 32S precursors of ribosomal RNA are found when purified nuclei (Penman, 1966) and nucleoli (Penman, Smith & Holtzman, 1966) are extracted in hot phenol-SDS or with SDS alone (J. Warner, personal communication). Furthermore, these species do not change their sedimentation behavior after DMSO treatment (Katz & Penman, in preparation).

The aggregation phenomenon described here is not often a serious problem when working with extracts obtained from tissue culture since the concentration of RNA is usually quite low. Extraction from an animal tissue such as rat liver is often performed at higher material concentration and considerable caution should be exercised. The use of the lower temperature is recommended, as extraction at 55° appears to be as efficient as at 60°, and results in considerably less aggregation.

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