A SIMPLIFIED PROCEDURE FOR THE SIMULTANEOUS ISOLATION OF 4S AND 5S RNA

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In recent years it has become apparent that most of the commonly used methods used for the preparation of tRNA, such as phenol extraction of whole cells (Monier, Stephenson & Zamecnik, 1960), centrifugation of total phenol-extracted RNA on a sucrose density gradient (e.g. Gilbert, 1963), or differential precipitation with salt (e.g. Smith, 1960), yield preparations contaminated with other species of low molecular weight RNA (Rosset & Monier, 1963; Reynier, Aubert & Monier, 1967). In order to prevent contamination of tRNA with 5S ribosomal RNA it is necessary to completely free the extract of ribosomes and ribosomal subunits, but even so, the low molecular weight RNA is still heterogeneous (Schleich & Goldstein, 1966; Reynier et al, 1967). On the other hand, it is not possible to obtain 5S RNA free of 4S by any of the above procedures, because even highly purified ribosomes still contain some tRNA.

Of the methods available for separating the various classes of low molecular weight RNA from each other, such as chromatography on methylated albumin-Kieselguhr (MAK) columns (e.g. Brown & Littna, 1966), electrophoresis on polyacrylamide gels (e.g. Gould, 1966), and chromatography on long columns of Sephadex G-100 or G-200 (e.g. Schleich & Goldstein, 1966; Reynier et al, 1967), only the latter is suitable as a preparative procedure.

It is common practice to deproteinize the RNA prior to the application of separation techniques. Despite the great usefulness of phenol extraction, however, its use on a large scale is cumbersome and time-consuming. In the case of crude extracts, containing a large excess of protein, multiple extractions are usually required, and emulsion problems or loss of RNA in the preci-

pitate forming at the interphase are not uncommon. We were further struck by the usual apparent absence of peptidyl-RNA even in samples prepared from actively growing cells, which should contain an appreciable amount of this form. The partition characteristics of peptidyl-RNAs in the phenol-water system have not been studied, and it is possible that RNAs with larger peptides attached to them are extracted by phenol. Bresler et al (1966) isolated peptidyl-RNA by direct chromatography on G-200 Sephadex of ribosomes in sodium dodecyl sulfate (SDS), but their columns were too short to resolve different classes of low molecular weight RNA.

We sought to develop a method for the separation of different classes of RNA which would not require prior deproteinization of the RNA, and which would allow the recovery of all the RNA in an extract, including that covalently bound to nascent protein. We found that direct chromatography of ribosomes or of crude extracts on G-75 Sephadex, in the presence of small amounts of SDS, was fast and efficient and yielded separate peaks of 5S and 4S RNA. These peaks are contaminated by some low molecular weight proteins and lipids, but these contaminants can easily be removed by a single phenol extraction and/or precipitation with ethanol.

EXPERIMENTAL

An arg^-, met^-, RC^{rel} mutant (687) of E. coli K_{12} was used for the preparation of crude extracts. (The mutant was kindly supplied by Dr. B. D. Davis' laboratory). The bacteria were grown in minimal medium with various amounts of the two essential amino acids. SDS (99% pure) was obtained from Pfaltz and Bauer, New York. The use of pure SDS is essential, as the usual commercial product (lauryl sulfate, 93% pure) leads to degradation of RNA (see also Maaløe & Kjeldgaard, 1966; Crestfield, Smith & Allen, 1955).

The bacteria were disrupted in a French Press at high pressure (15,000-17,000 psi) so as to disrupt polysomes mechanically (McQuillen, Roberts & Britten, 1959). The extract was then centrifuged to yield an S-30 fraction (Matthaei & Nirenberg, 1961). Ribosomes were prepared from frozen E. coli B according to the method of Kurland (1966).

Both ribosomes and crude extracts were adjusted to a given SDS concentration just prior to chromatography on Sephadex columns which had been preswollen in SDS for several days and prewashed with a given buffer. The concentration of SDS is not critical, but 0.25% usually was sufficient to clear turbid extracts, although with some ribosome suspensions the sample had to be made 0.5% in SDS in order to achieve clearing. Concentrations as low as 0.05% SDS are sufficient to cause disruption of ribosomes. All runs were made at room temperature to prevent precipitation of the SDS.

RESULTS

Preliminary runs on G-100 of crude extracts in the presence of SDS had shown that the RNA pattern obtained was exactly like that obtained with phenol-isolated total RNA (see Reynier et al, 1967). It was also found that most of the protein in the extract is in the excluded portion. However, the flow rate of columns of the required length (180 cm) is extremely slow, several days being required for complete elution.

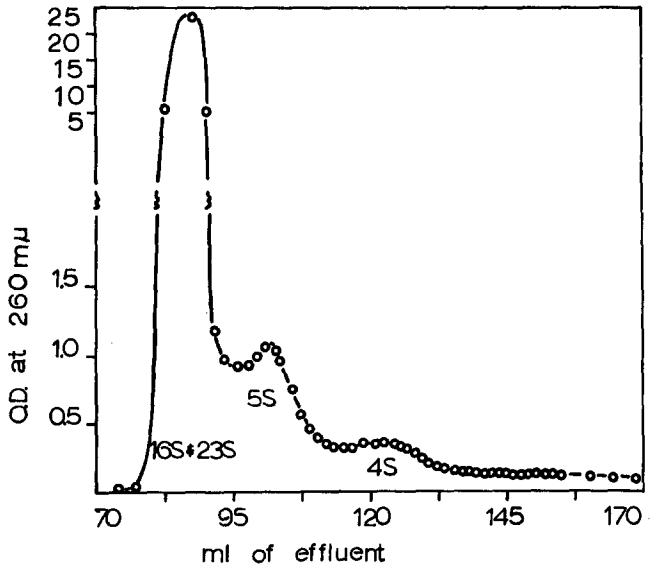

Fig. 1. Chromatography of Ribosomes on G-75 Sephadex in the Presence of SDS.

Purified ribosomes corresponding to 11.2 mg of RNA were chromatographed on a column measuring 60 x 2.5 cm in a buffer containing 0.01M Tris, 0.1M NaCl and 0.25% SDS, at pH 7.3. Recovery of UV-absorbing material in the three peaks was 92%. The balance of the optical density appeared in the region of small molecules.

G-75 Sephadex, which has not yet been used in the literature for the isolation of 5S RNA, probably because of fear of exclusion of this RNA by the gel, was tested with purified ribosomes, which yielded the pattern shown in Fig. 1. From the number and distribution of the peaks it is apparent that 5S RNA is not excluded by G-75; in addition it was found to give better separation between the 5S and 4S regions than G-100, and allowed the use of a shorter column (60-100 cm versus 180 cm). G-75 also has a better flow rate. Therefore, G-75 was used in all subsequent experiments.

It is well-known that E. coli 5S RNA is devoid of methylated bases (Brownlee, Sanger & Barrell, 1967), so that this is a suitable property for checking on the identity and purity of the putative 5S RNA peak.

A crude extract prepared from bacteria methylated with $^{14}CH_3$-methionine

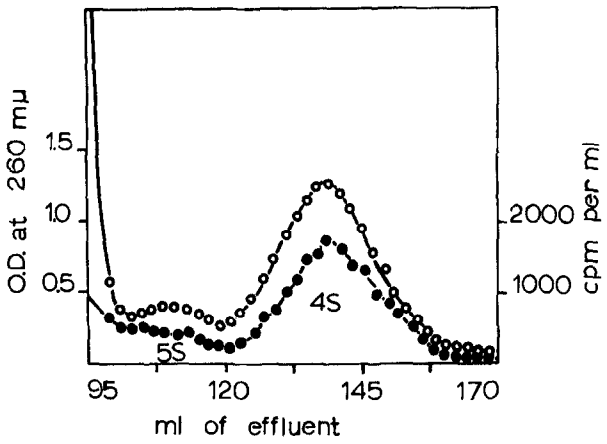

Fig. 2. Chromatography on G-75 Sephadex of a SDS-Treated Crude Extract of E. coli Labeled with $^{14}CH_3$-Methionine in the Absence of Protein Synthesis.

Bacteria were grown in minimal medium in the presence of methionine (5 μg/ml) and arginine (25 μg/ml) to an optical density at 650 mμ of 0.4. They were then washed in minimal medium and resuspended. After 10 min at 37°, chloramphenicol was added to a concentration of 100 μg/ml and the incubation was continued for one additional hour. At this point C^{14}-Met was added (0.01 mc, or 0.014 mg/liter of culture). After all the labeled methionine had been taken up, the cells were harvested and washed in minimal medium, then resuspended in complete medium and grown to an optical density of 0.7. A crude extract was prepared and an aliquot containing 7.35 mg of RNA was made 0.1% in SDS and chromatographed on a column measuring 100 x 1.8 cm in 0.1M NH_4Ac, pH 5.2, and 0.1% SDS (Galibert, Larsen, Lelong & Boiron, 1966). The recovery of O.D. in the macromolecular components was 92%. Another 9% was recovered in smaller material. Open circles represent optical density; closed circles represent radioactivity.

in the absence of protein synthesis was chromatographed on G-75 in the presence of SDS. The results are shown in Fig. 2. It is seen that contrary to the results obtained with preisolated RNA, there are some counts in the 5S region. The fractions were therefore pooled, and the RNA precipitated with 67% ethanol at -20°. In the case of the 5S peak no counts would be recovered in the precipitate, whereas the 4S region, as expected, contained C^{14}-methyl groups. Whatever counts there were in the 5S region can therefore be attributed to contamination by methylated lipid--and would thus not be seen if, for example, the discs had been dried by ethanol-ether.

In order to test for the degree of protein contamination in the 5S region, bacteria were labeled with a limited amount of C^{14}-methionine in the presence

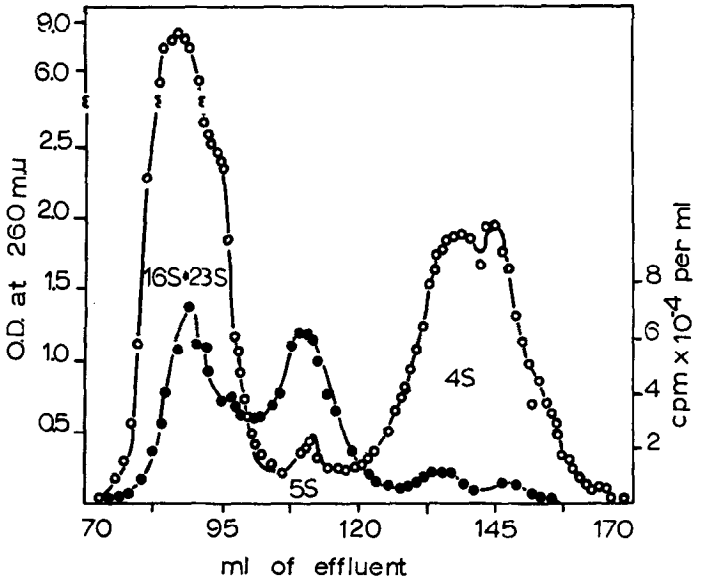

Fig. 3. Chromatography on G-75 of a SDS-Treated Crude Extract of E. coli Pulse-Labeled with $^{14}CH_3$-Methionine in Complete Medium.

Bacteria were grown in minimal medium containing 2.5 mg/liter of Met and 100 mg/liter of arginine. Growth leveled off at an O.D. of 0.45, at which point 0.175 mg C^{14}-Met, containing 1.7 x 10^7 cpm, were added to the 1 liter culture. When all the labeled methionine had been taken up, 5 mg C^{12}-Met were added and growth allowed to continue to an O.D. of 0.6.

Crude extract corresponding to 8 mg of RNA was chromatographed under the conditions described in Fig. 1. The recovery was 97%. Open circles represent optical density; closed circles represent radioactivity.

of arginine, i.e., under conditions where radioactivity from the methionine would be expected to be used for methylation as well as for protein synthesis. When such an extract was chromatographed on G-75, the results shown in Fig. 3 were obtained. It is seen that now the 5S peak is substantially labeled. Each peak was pooled, and submitted to a single extraction with 90% phenol. In the case of the 5S peak, 100% of the counts were extracted into the phenol, whereas in the case of the 4S, 74,000 cpm per mg RNA remained in the aqueous phase.

Preliminary experiments with extracts containing peptidyl-RNA labeled both *in vivo* and *in vitro* have shown that complete recovery of label is possible.

In summary, then, our results show that deproteinization is unnecessary for the chromatographic separation of different RNA fractions on Sephadex columns in the presence of SDS. The method can be applied either preparatively, or analytically for labeled RNAs and peptidyl-RNAs. Besides representing a very substantial saving in time and effort, the method has the advantage of allowing essentially complete recovery of both counts and UV absorbing material even from turbid or partially precipitated extracts. Furthermore, the SDS inhibits ribonuclease action (Crestfield *et al*, 1955).

All RNA fractions were contaminated with protein and with lipid, but these contaminants could be removed preparatively by a single phenol extraction of the pooled fractions, after chromatography, and analytically by proper washing procedures of the discs used for the determination of radioactivity, according to Mans and Novelli (1961).

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