The Preparation and Characterization of Ribonucleic Acid Obtained by Direct Phenol Extraction of Intact Cells of Escherichia coli from Low Ionic Environment

Michael Artman, Michael Fry and Hanna Engelberg
Department of Bacteriology, Hebrew University-Hadassah Medical School,
Jerusalem, Israel

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The various methods used for the isolation of soluble and ribosomal ribonucleic acids from bacterial cells have recently been reviewed by Kirby (1964). The procedures for the extraction of ribosomal ribonucleic acid involve the use of spheroplasts or disrupted cells with subsequent extraction of nucleic acids with phenol or/and sodium dodecyl sulfate (SDS). Soluble ribonucleic acid (s-RNA) a mixture of species of molecular weight 25,000 to 30,000 with a sedimentation coefficient of about 4S can be isolated from intact cells by direct phenol extraction in the cold from media containing 10^{-2} M Mg^{2+} (Zubay, 1962), or by extraction with pyrophosphate containing isoamyl alcohol (Rammler et al., 1965). Inasmuch as ribosomal RNA is not extractable under these conditions it has been assumed that bacterial cell wall acts as a molecular sieve allowing RNA of low molecular weight to escape into the extracting medium while retaining high-polymer ribosomal RNA.

In this communication we wish to report on a method for the isolation of ribosomal RNA from intact cells of E.coli which involves direct phenol extraction of bacteria from low ionic environment.

E.coli strain B was grown overnight on a shaker at 37° in Bacto - tryptone broth containing 0.5% NaCl. The overnight cultures were diluted with the same fresh medium to contain 10^8 cells/ml and reincubated until a titer of 10^9 cells/ml was attained. The exponentially growing cells with a generation time of about 70 minutes were poured on to crushed frozen Tris-Mg buffer (0.01M Tris, pH 7.4, containing 0.01M Mg^{2+}), collected by centrifugation and once washed with the same buffer. The cells (about 70 mg wet weight of cells) were suspended in 3 ml of 0.01M Tris buffer, pH 7.4, containing bentonite (1 mg/ml) to which one of the following additions was made: a) Mg^{2+} in varying concentrations, b) Na^+ in varying concentrations, c) 0.01M EDTA and d) 0.01M EDTA containing varying concentrations of Na^+.

The cells in the various suspending media were gently agitated on a vortex

(Super-Mixer,Lab.Line Instruments Inc.,) with an equal volume of freshly distilled 90% phenol for 2 to 5 minutes in the cold.

The phenol mixture was centrifuged at 10,000xg for 10 minutes in the Servall RC-2 refrigerated centrifuge.The top aqueous layer was carefully collected,traces of phenol removed by ether extraction and the dissolved ether removed by air blowing.To the aqueous phase 0.1 volume of a 10% solution of NaCl was added followed by two volumes of ice-cold 95% ethanol.The precipitated nucleic acids sedimented out and were collected by low-speed centrifugation. The precipitate was dissolved in 0.01M Tris buffer,pH 7.4. After complete dissolution,the concentration of Mg^{2+} was raised to 0.01M and DNase (10 ug/ml) added.After the end of a 20 min.incubation at 4° the RNA was recovered by alcoholic precipitation.The final RNA precipitate was dissolved in 0.005M Tris buffer,pH 7.4,containing 0.1M NaCl and clarified by low-speed centrifugation,when necessary.

As a control for the total RNA content of the bacterial cells used for the experiments,the following method which allows quantitative recovery of E.coli RNA was used.The cells grown and harvested as described above were suspended in the Tris-Mg buffer containing 1.5% of SDS and disintegrated in the 20KC MSE ultrasonic disintegrator.The sonicated cells were extracted with phenol and RNA isolated as described above.

To determine the composition and recovery of RNA obtained by the direct phenol extraction of intact cells from solutions of various ionic strength and composition the RNA preparations were assayed for their RNA content by absorbancy measurements at 260 mu,the size distribution determined by the sedimentation pattern of RNA preparations in sucrose density-gradients and compared to those of the control which consisted of RNA obtained by phenol extraction of a similar weight of cells treated with SDS and disintegrated by sonication.Summation of the ultraviolet absorbing material in the various RNA fractions obtained by the different extraction methods yielded the desired data.

The composition of the various RNA preparations was determined by sucrose density-gradient centrifugation. 3 to 5_{A260} units of RNA solutions in 0.005M Tris,pH 7.4,containing 0.1M NaCl were carefully layered on top of a 4.4 ml of a linear 5% to 20% sucrose gradient prepared in 0.005M Tris,0.1M NaCl (pH 7.4). This was centrifuged at 37,000 rev/min for 4 hours in a SW-39 swinging bucket head of the model L Spinco Ultracentrifuge.Thirty fractions from each centrifuge tube were collected,the volume made up to 1 ml with 0.1M NaCl and assayed for absorbancy at 260 mu.

In Table 1 and Fig.1 the effects of the composition of the suspending media

Table 1

Effect of ionic strength of the medium on the extractability of ribosomal RNA from intact cells of E.coli

Extracting medium composition	ionic strength	RNA extracted relative amounts of s-RNA	r-RNA	percent of total r-RNA
Tris-Mg^{2+}	0.01	100.0	0.0	0.0
Tris-Mg^{2+}	0.0001	100.0	0.0	0.0
Tris-Mg^{2+}	0.00001	42.0	58.0	74.0
Tris-Na^{+} ^^	0.01	100.0	0.0	0.0
Tris-Na^{+} ^^	0.0001	100.0	0.0	0.0
Tris-Na^{+} ^^	0.00001	31.0	69.0	88.0
Control(RNA from sonicated cells)	-	21.0	79.0	100.0

^^ Identical results were obtained when the extracting medium contained 0.01M EDTA, in addition to sodium ions .

on the extractability of ribosomal RNA from intact cells of E.coli are shown.The results of the experiments showed that when ionic strength of the suspending solutions was 0.01 and down to 0.0001,quantitative extraction of s-RNA was achieved. At the same time no ribosomal RNA escaped into the extracting medium which contained esclusively s-RNA.Addition of EDTA to the various suspending media containing sodium ions (ionic strength 0.01 to 0.0001) had no effect on the extractability of nucleic acids.

When ionic strength of the suspending solutions was lowered to 0.00001 or when extractions were carried out from 0.01M Tris-0.01M EDTA (pH 7.4), 74 to 88 percent of total ribosomal RNA became extractable.

It is noteworthy that when ribosomal RNA was extracted from intact cells the ratio of 16S : 23S RNA was higher than for RNA extracted from sonicated cells.This may indicate that there still is some retention of 23S RNA under the conditions employed.

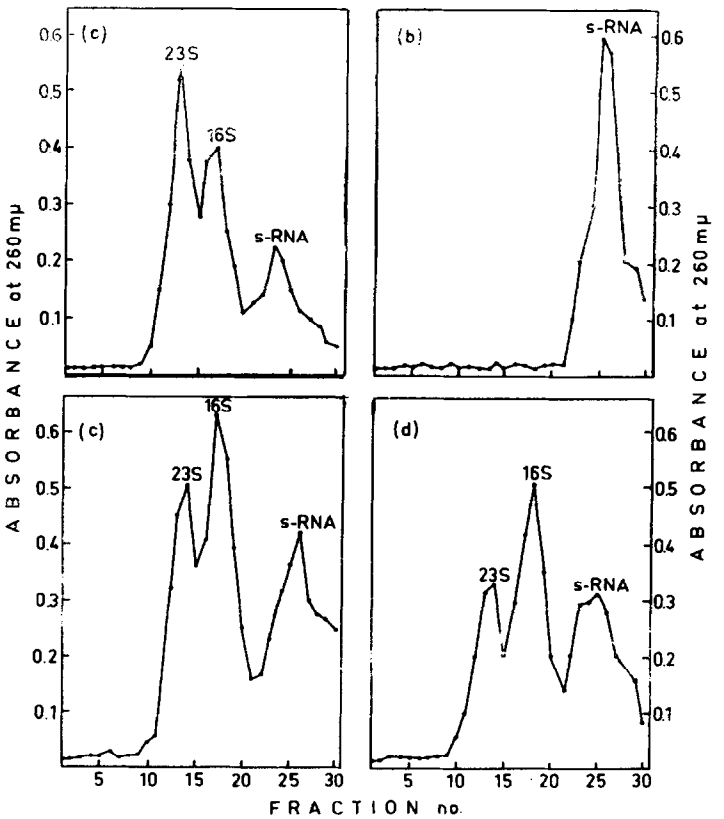

Fig.1. Sedimentation distribution of RNA obtained by direct phenol extraction of intact cells of E.coli.Sucrose gradient (5% to 20%) contained 0.005M Tris buffer, pH 7.4, and 0.1M NaCl.Centrifugation was performed in a Spinco SW-39 rotor at 37,000 rev./min for 4 hours with refrigeration.RNA extracted from: a) sonicated cells(control); b)intact cells in 0.01M Tris,0.01M EDTA and 0.01M Na^+; c)intact cells in 0.01M Tris,0.01M EDTA and 0.00001M Na^+; d) intact cells in 0.01M Tris and 0.01M EDTA.

It is known from the studies of Weidel et al.,(1960) that treatment of E.coli cell wall with phenol dissolves the outermost soft lipoprotein layer,removes the in-between soft lipopolysaccharide layer,leaving intact the innermost rigid layer, the so called R layer,which is believed to be composed of a great number of rigid spheres linked together by lysozyme-sensitive structures.

It would appear,therefore,that in our experiments on the extractability of RNA from intact bacterial cells by phenol we are dealing with the permeability of the R layer of E.coli cell wall to high-polymer compounds.Thus,it can be assumed that it is the rigid innermost layer of E.coli cell wall which acts as a molecular

sieve which at moderate ion concentrations allows the escape of s-RNA and retains the high-polymer ribosomal RNA.

As to the mechanism underlying the permeability of the R layer of E.coli cell wall to high-polymer RNA in solutions of low ionic strength, the latter may affect the RNA or the R layer of the cell wall itself.

Thus, two explanations for the effect of ionic strength of the medium on the extractability of ribosomal RNA may be offered. One is that the apertures between the rigid spheres of the R layer of E.coli cell wall act as a sieve retaining the compact high-polymer RNA with secondary or/and tertiary structure while allowing RNA of low molecular weight or the extended unfolded chains of high-polymer RNA to escape into the extracting medium. This explanation is based on that there is a remarkable correlation between the ionic strength at which ribosomal RNA is converted into unfolded state (Spirin, 1963) and that at which ribosomal RNA begins to pass through the phenol-resistant R layer.

Another possibility to be considered is that mono- or divalent cations are required for the preservation of the structural integrity of the R layer. In low ionic environment the organization of the only cell wall layer which remains intact after phenol treatment is destroyed and all high-polymer species become extractable regardless of their size or structure. This would be the first indication that monovalent cations play a role in the organization and structure of a portion of bacterial cell wall. Hitherto, only multivalent cations were shown to be essential for the integrity of bacterial cell walls, specifically for that of the lipopolysaccharide fraction of cell walls (Asbell and Eagon, 1966).

Studies aimed at distinguishing between the two alternative mechanisms are under way.

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