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ISOLATION OF THE CYTOPLASMATIC 5S RIBOSOMAL RNA FROM COTTON SEEDS BY PREPARATIVE ELECTROPHORESIS

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The cytoplasmatic ribosomes of the seeds of the cotton plant of variety 108-F were isolated by a method described previously [1]. The total ribosomal RNA was obtained from the ribosome by sodium dodecyl sulfate (SDS)—phenol treatment. Analysis of the total rRNA by electrophoresis in 10% polyacrylamide gel (PAG) showed that the preparation contained 95-96% of high-molecular-weight ribosomal RNAs (28S + 18S), 1.2-1.5% of 5S rRNA, and 2.4-3% of tRNA, which agrees with literature information [2].

For the fractionation of RNA and the separation of the 5S rRNA from other components, some authors have used the method of gel filtration on Sephadex G-1000 [3] or G-75 [4], or on Sephadex G-100 followed by rechromatography on G-75 [5], chromatography on MAK (methylated albumin on kieselguhr) [6], and fractionation by electrophoresis in PAG [7, 8]. Electrophoresis in PAG possesses a higher resolving capacity than the other methods of fractionation and permits the 5S rRNA fraction to be separated in homogeneous form even when products of the degradation of high-molecular-weight rRNA are present in the preparation of total RNA.

Various instruments are used for preparative electrophoresis in polyacrylamide gel [9, 10]. Following the design of Maizel's instrument [11], with some modifications, we have put together an instrument for preparative gel electrophoresis with continuous elution of the substances being separated which permits the fractionation and separation of homogeneous fractions of 5S rRNA and of the total tRNA from the total rRNA.

To enrich the total rRNA with the 5S rRNA fraction and also to avoid the wrinkling of the surface of the gel and its detachment from the walls of the chamber caused by an excess of high-molecular-weight rRNAs, the latter were separated from the preparation by salting out with sodium chloride [12]. We used NaCl solutions of various concentrations (from 1 M to saturation) at concentrations of the sample of total rRNAs of 150-200 o.u./ml and at pH values of the solution of 5.6, 7, and 8.

At a concentration of total rRNA in the solution of 150 o.u./ml, the percentage of high-molecular-weight rRNAs in the preparation after salting out with 1 M NaCl solution was 56%, with 2 M solution 46%, with 3 M 39%, and with 4 M 38%. However, an increase in the concentration of total rRNA to 200 o.u./ml and of the concentration of NaCl to saturation (26 g per 100 g of solution) led to a decrease in the amount of the high-molecular-weight rRNA fraction in the preparation of total rRNA. Thus, after salting out with 4 M NaCl solution the amount

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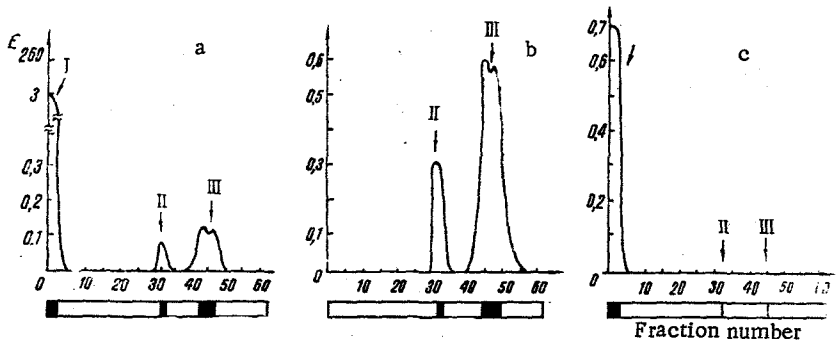

Fig. 1. Electrophoresis of the total rRNA (a, b) and of the high-molecular-weight rRNA (c) in 10% PAG at 5 mA per tube: I) high-molecular-weight rRNA; II) 5S rRNA; III) tRNA; a) total rRNA before purification, b) total rRNA after purification; c) high-molecular-weight rRNA after salting out.

of high-molecular-weight rRNAs in the preparation fell to 13.5%, and after salting out with saturated NaCl solution it fell to 10%.

A study of the influence of the pH of the solution on the salting out of the high-molecular-weight rRNAs from the total rRNA showed that in the case of a concentration of total rRNA in the solution of 200 o.u./ml, a change in pH within the range from 5.6 to 8 gave an insignificant advantage in the salting out of the high-molecular-weight rRNAs: at pH 5.6, 10%; at 7, 12%; and at 8, 13%.

The further purification of the preparation of total rRNA from residues of high-molecular-weight rRNAs and other impurities was carried out by chromatography on a column of DEAE-cellulose [13] and achieved the practically complete elimination of the high-molecular-weight rRNA fraction. In the preparation obtained, the amount of the 5S rRNA fraction was 22% and of tRNA 78%.

The completeness of the elimination of the high-molecular-weight rRNAs from the preparation was checked by electrophoresis in 10% PAG.

Simultaneously with the elimination of the high-molecular-weight rRNAs an almost 20-fold enrichment of the preparation of total rRNA with the 5S rRNA fraction was achieved.

In the analysis of the precipitate of high-molecular-weight rRNAs after salting out with saturated NaCl solution by electrophoresis in PAG, trace amounts of low-molecular-weight 5S rRNA and tRNA were detected visually, although they were not recorded spectrophotometrically after the hydrolysis with 0.3 N KOH of a piece of the gel with the corresponding RNA fraction. This means that practically no coprecipitation takes place (Fig. 1c). After such a considerable enrichment, fractionation and the isolation of the homogeneous 5S rRNA was carried out by electrophoresis.

Instrument for Preparative Electrophoresis. The instrument, made from Plexiglas [Lucite] consists of an upper electrode vessel with a rectangular chamber (10 × 2 × 10 cm) for the separating gel fitted with a collecting chamber and a lower electrode vessel.

The thickness of the internal walls of the chamber for the separating gel with a water jacket is 1.5 mm (for better heat exchange, since on the passage of the current the gel heats up) and the thickness of the outer jacket is 3 mm.

The main part of the instrument — the collecting chamber where the continuous elution of the RNA fractions migrating from the gel takes place — consists of a Plexiglas frame having lateral apertures with a diameter of 1 mm for the passage of the eluting solution (the external dimensions of the chamber are 13 × 5 × 0.3 cm and the internal dimensions 10 × 2 × 0.3 cm), and of a frame of the same size without apertures. The chamber is hermetically sealed by two thin rubber gaskets. All the elements of the collecting chamber are fastened together by 10 plastic bolts.

The upper and lower electrode vessels (25 × 15 × 15 cm) each have a capacity of 5 liters. In the upper vessel a rectangle 10 × 2 cm has been cut out in the center and the chamber for

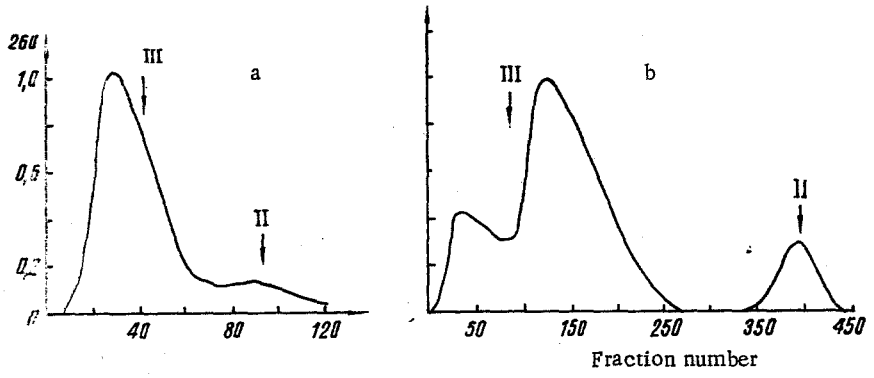

Fig. 2. Elution profile of the components of the total rRNA during preparative electrophoresis (I = 150 mA, 1 ml/min, 4-ml fractions): II) 5S rRNA; III) tRNA; a) electrophoresis in 13% PAG, height of the gel 5 cm; b) electrophoresis in 15% PAG, height of the gel 7 cm.

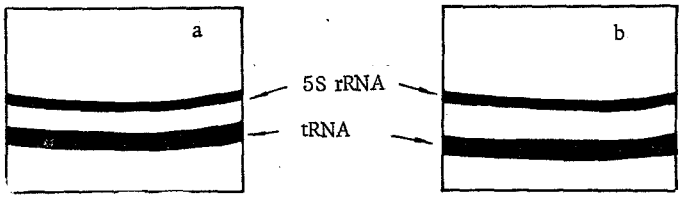

Fig. 3

the separating gel has been glued on. The upper vessel with the chamber for the separating gel is fixed to the lower vessel. The buffers in the upper and lower vessels were cooled by coils arranged on the perimeter through which mains water was passed.

For the successful fractionation of the total rRNA and the separation of the low-molecular-weight 5S rRNA from the other components such as tRNA and the degradation products of the high-molecular-weight rRNAs, the optimum conditions for preparative electrophoresis in PAG were selected (the current strength, the voltage, the concentration of monomer in the separating gel, the ratio of monomer and comonomer in the polymerization of the gel, the method of polymerizing the gel, the ionic strength and pH of the gel solutions, the electrode buffers, the eluting buffer, the rates of elution of the substances being separated, the amount and volume of the preparation deposited, etc.).

As a result of the experiments performed, it was found that with an increase in the current strength from 150 to 200-250 mA the migration front of the dye Bromophenol Blue became highly distorted with appreciable lagging at both edges.

Effective resolution of the 5S rRNA fraction from tRNA was achieved at an acrylamide concentration of 15% and a height of the gel of 7 cm (Fig. 2). Under these conditions it was possible to successfully separate up to 20 mg (500 o.u.) of a preparation of the total rRNA freed from the high-molecular-weight rRNA fraction. The time of electrophoresis was 42 h from the moment of depositing the sample on the gel.

Preparative electrophoresis was performed in a uniform buffer system C: 0.04 M Tris, 0.02 M CH_3COONa, 0.002 M disodium EDTA, pH 7.2 (CH_3COOH was added at room temperature). To maintain the pH and the ionic strength of the electrode buffers, the buffers were circulated with a Peripump, a peristaltic pump of the Hungarian firm MTA Kutesz.

As a result of the polymerization of the polyacrylamide gel by forming a layer of the gel solution under the buffer [11], a gel was obtained with larger pores. This was observed on comparing the gel produced by a polymerization process in which a layer of the gel solution was formed under the buffer (Fig. 3a) with a gel formed by a polymerization process in which a layer of buffer solution was formed above the gel solution (Fig. 3b). In the second case, the distance between the bands of the 5S rRNA on preparative electrophoresis was far greater (Fig. 3). From this the conclusion may be drawn that in the polymerization of the gel by the first method some dilution of the gel solution took place which led to the forma-

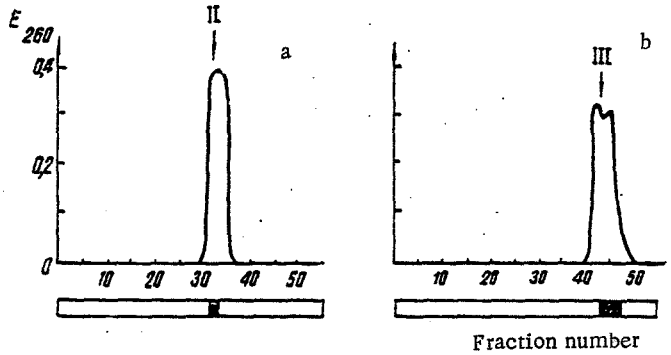

Fig. 4. Electrophoresis of the components of the total rRNA isolated by preparative electrophoresis: II) 5S rRNA; III) tRNA; a) fraction of the 5S rRNA/ b) fraction of the total tRNA.

tion of a gel with larger pores and therefore it is necessary to polymerize the gel by the second method.

To obtain narrow bands in the migration of the substance in the gel, the volume of the preparation of total rRNA must not exceed 1.5-2 ml, and the solution must be deposited in a thin layer on the surface of the gel.

No unimportant role is played by the rate of elution of the substances being separated. The optimum rate proved to be 1 ml/min, with the collection of 4-ml fractions. A pump was attached to the collecting chamber to pump out the substances migrating into it. If the pump is connected the other way round, then, because of the pressure created by it, the gel and the membrane will be highly bent, which leads to an increase in the volume of the collecting chamber, to a poor resolution of the substances being separated, and, possibly, to leakage of the chamber.

An increase in the ionic strength of the eluting solution (buffer B) by the addition of NaCl to a final concentration of 0.2 M did not lead to a decrease in the adsorption of the nucleic acids in the membrane. The losses in the membrane were not more than 1% of the total rRNA. The yield of the fractions of the preparation being separated amounted to 95% of the initial amount.

The homogeneity of the fractions of 5S rRNA and of total tRNA obtained were subjected to analytical electrophoresis in 10% PAG (Fig. 4).

EXPERIMENTAL

Isolation and Purification of the Total rRNA. The proteins were eliminated from the isolated ribosomes by SDS-phenol treatment and the total rRNA was precipitated from the aqueous phase by the addition of three volumes of ethanol (96%) cooled to −20°C [1]. The resulting precipitate was collected by centrifuging at 3000g for 15 min.

Part of the total rRNA was dissolved in 0.2 M NaCl in buffer B (0.25 M CH_3COONa, pH 5.6) to a concentration of the material of 150 o.u./ml (four test-tubes with 5 ml each), and NaCl was added to final concentrations of 1, 2, 3, and 4 M. The solution was left in the refrigerator at −20°C for 12 h.

A solution of the total rRNA with a concentration of the material of 200 o.u./ml (four test-tubes with 5 ml in each) was prepared in the same way, and NaCl was added to a final concentration of 4 M and saturation (tubes I and II); in tubes III and IV the pH values of buffer B were 7 and 8, respectively, and NaCl was added to saturation and they were left in the refrigerator.

After thawing out, the precipitates of high-molecular-weight rRNAs that had deposited were separated by centrifuging at 16,000 rpm in a K-24 centrifuge for 1 h. The supernatant was treated with 2.5 volumes of ethanol (96%) that had been cooled to −20°C and was left in the refrigerator for the formation of a precipitate (3-4 h). The precipitates of total rRNA that deposited were separated by centrifuging at 3000g for 15 min, were washed three times

with 80% ethanol to eliminate traces of NaCl, and with ethanol and ether, and were dried in vacuum. In all the preparations of total rRNA the percentage compositions of the components — 28S + 18S rRNA, 5S rRNA, and tRNA — were determined.

The total rRNA after the elimination of the high-molecular-weight rRNAs by salting out with NaCl at saturation (pH 5.6) was chromatographed on a column of DEAE-cellulose [13] to free it from traces of high-molecular-weight rRNA and other impurities.

Separation of the Low-Molecular-Weight 5S rRNA and tRNA by Preparative Electrophoresis. A stock was prepared of 20 liters of buffer C: 0.04 M Tris, 0.02 M CH_3COONa, 0.002 M EDTA disodium salt, pH 7.2 (CH_3COOH was added at room temperature). In 140 ml of buffer C was dissolved 21 g of acrylamide (A) that had been recrystallized from chloroform (15% gel, height 7 cm) and then N,N'-methylenebisacrylamide (0.3%) was added and the solution was degassed.

The solution was cooled to 0°C and TEMED (0.033 ml per 1 g of A) and a solution of ammonium persulfate (0.33 ml per 1 g of A) were added. The gel solution was rapidly poured into the chamber for the separating gel where the collecting chamber had been replaced by a plate with a solid rubber gasket and was carefully formed into a layer above buffer B. After the end of the polymerization process (1 h), the plate with the rubber gasket was removed and the collecting chamber was fixed in the following sequence: to the chamber with the separating gel was applied a rubber gasket and then the frame with the apertures for elution, a semipermeable membrane (regenerated cellulose), a rubber gasket, and the Plexiglas frame (13 × 5 × 0.3 cm), and they were fixed with plastic bolts.

Buffer B containing SDS (0.3%) was pumped through the collecting chamber to check its liquid-tightness and to eliminate possible nuclease contaminants.

The upper electrode vessel with the glued-on chamber with the separating gel was placed above the lower electrode vessel and was filled with buffer B, and water was passed through the coils of the outer and inner vessels and the water jacket of the separating gel until the temperature had fallen to 6-8° (15-20 min). Then stirrers were switched on to stir the buffers.

On the gel a layer of 1.5-2 ml of a 0.001% solution of Bromophenol Blue with 20% of sucrose was formed, and the source of constant current (UIP-2) and then the pump for circulating the buffer solutions were switched on. Electrophoresis was carried out at I = 150 mA (V = 100 volts) for 1 h, by which time the dye had migrated 1-1.5 cm in the gel.

A solution of total rRNA (1.5-2 ml) at a concentration of 200-250 o.u./ml, purified on a column of DEAE cellulose, was prepared with 20% sucrose and was carefully layered onto the gel. Electrophoresis was continued for 42 h. As soon as the dye began to issue into the collecting chamber, the pump was switched on and the migrating fractions of total rRNA were collected at the rate of 1 ml/min in 4-ml fractions. The UV absorption at λ_{260} was measured on an SF-4 instrument and graphs were plotted of the elution profile of the separated fractions of total rRNA (see Fig. 2).

The fractions corresponding to the 5S rRNA were combined and so were those corresponding to the tRNA and they were deposited on two small columns of DEAE-cellulose (1 × 1 cm) that had been washed with 1 M NaOH, distilled water, 1 M HCl, and distilled water again. The columns were then washed with 0.2 M NaCl in buffer B and eluted with 1 M NaCl solution in the same buffer. To each of the eluates was added three volumes of ethanol cooled to —20°C and they were kept in the refrigerator at —20°C. The precipitates of 5S rRNA and tRNA that deposited were collected separately by centrifuging, washed with ethanol and ether, and dried in vacuum.

Electrophoretic analysis for homogeneity was carried out as described previously [1].

SUMMARY

1. The complete separation of low-molecular-weight 5S rRNA and tRNA from fractions of high-molecular-weight rRNAs (28S + 18S) in a preparation of the total rRNA from cotton seeds has been achieved.

2. The optimum conditions have been selected for the preparative electrophoresis and isolation of homogeneous preparations of 5S rRNA and total tRNA.

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SUPPORTS FOR THE SOLID-PHASE SYNTHESIS OF OLIGONUCLEOTIDES WITH A 5'-TERMINAL PHOSPHATE GROUP*

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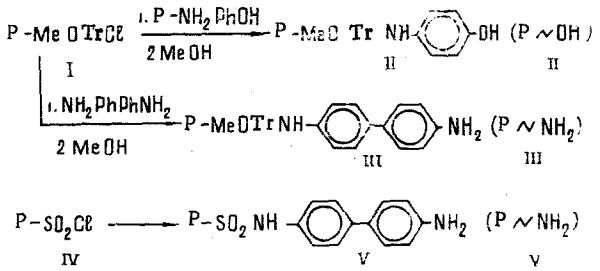
The advances achieved in the synthesis of five- to seven-membered oligodeoxyribonucleotides by the solid-phase method [1-3] are, at the present time, permitting this approach to be regarded as promising for the rapid preparation of oligonucleotides of a given composition. However, practically none of the oligodeoxyribonucleotides synthesized have contained a 5'-terminal phosphate group, since the supports used for the synthesis were modified by the introduction of trityl [2, 3] or carboxy [4, 5] anchor groups. The methods of modifying polymers suitable for the production of 5'-nucleotides described in the literature are, as a rule, extremely laborious.

In the present paper we consider the synthesis of supports containing aromatic hydroxy and amino groups as the centers for the addition of a 5'-nucleotide. The introduction of such anchor groups in the polymers was carried out by the following schemes:



In the first case, a methoxytritylated polymeric support (I) that is commonly used in the solid-phase synthesis of oligonucleotides [3] was subjected to modification and, therefore, the modification of the polymer amounted to the addition of p-aminophenol or benzidine, which enabled the synthesis of oligonucleotides with a phosphate group at either end of the chain to be carried out on the same initial support (I).

*The following abbreviations are adopted in this paper: P, polymeric support; TPS, 1,3,5-triisopropylbenzenesulfonyl chloride, C.A., condensing agent; pN(N), deoxynucleoside 5'-phosphate (deoxynucleoside protected at an exocyclic amino group).

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