Fractionation of Soluble Ribonucleic Acid on Diethylaminoethyl Sephadex with a pH and Salt Gradient for Elution

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SUMMARY

A new method for the chromatographic fractionation of soluble ribonucleic acid (sRNA) is described. A gradient of increasing salt and pH is employed to elute sRNA from diethylaminoethyl Sephadex. The resolution of sRNA species for 10 different amino acids obtained with this method is presented. There is evidence that resolution is dependent upon a change in sRNA from a partially denatured state at the beginning of elution to the native state at the end. The method, which has a large input capacity, provides good resolution of certain sRNA species. It is convenient to use and can be adapted for either preparative or analytical work.

Numerous methods for fractionating soluble ribonucleic acid into its component species are now available; however, each presents problems. These include poor recovery of amino acid acceptor activity, the need for expensive special equipment, poor flow rates which prolong chromatographic techniques, difficulty in recovering sRNA from eluting solutions, and low capacity for input sRNA. In addition, few methods provide good resolution of more than a few of the sRNA species in a single step. Particularly needed are additional convenient methods comparable to chromatography on methylated albumin and Kieselguhr (1), which can be used by investigators desiring to perform analyses of changes in sRNA patterns. The present communication offers a new method which, in some situations, provides better resolution of sRNA species than the methylated albumin-Kieselguhr columns. This method consists of column chromatography on diethylaminoethyl Sephadex with elution by a combined gradient of increasing salt and increasing pH. The recovery of amino acid acceptor activity is good, the fractions can be assayed without special treatment, and the column has a large input capacity so that the method can be adapted to either preparative or analytical work.

The abbreviation used is: sRNA, soluble RNA or transfer RNA.

EXPERIMENTAL PROCEDURE

Materials—sRNA was prepared from Salmonella typhimurium, strain LT-2, by the method of Holley et al. (2). The cells which were used had been grown aerobically in a minimal medium containing glucose; they were harvested in late log phase. They were stored by freezing before they were used. The sRNA preparation was stripped of amino acids at pH 9.0 for 1 hour at 37°.

DEAE-Sephadex, A-50, coarse (Pharmacia), was washed according to the instructions of the manufacturer, and the fines were decanted several times. It was then equilibrated with the first eluting solution of the gradient which is described below and stored at 4°. The columns were washed with an amount of the first eluting solution equal to several bed volumes before the sRNA was applied.

The following 14C-L-amino acids were obtained from Nuclear-Chicago: lysine, 0.256 mM, 200 mC per mmole; isoleucine, 0.281 mM, 174 mC per mmole; leucine, 0.294 mM, 170 mC per mmole; serine, 0.418 mM, 120 mC per mmole; proline, 0.541 mM, 185 mC per mmole; methionine, 0.572 mM, 29.1 mC per mmole; aspartic acid, 0.481 mM, 106 mC per mmole; valine, 0.50 mM, 200 mC per mmole; and glycine, 0.888 mM, 67 mC per mmole. L-Histidine-14C, 0.45 mM, 223 mC per mmole, was purchased from New England Nuclear.

Methods—sRNA fractions were assayed for amino acid acceptance with fresh extracts from sonified S. typhimurium. The extracts, which contained approximately 10 mg per ml of protein, were passed through Sephadex G-50 in lieu of dialysis. The assay system contained 40 μmoles of triethanolamine, pH 7.5; 3 μmoles of MgCl₂; 0.30 μmole of ATP (Sigma); 0.025 mg of crystalline bovine serum albumin; and approximately 0.8 μmole of the 14C-amino acid being tested. sRNA, 0.050 to 0.750 absorbance unit (260 μm, 1-cm light path), was assayed in each preparation, and 0.001 ml of bacterial extract was used. The total volume of each assay was 0.461 ml.

The tubes containing the assays were incubated for 15 min at 37°, and the contents were precipitated by the addition of 2 ml of cold 5% trichloroacetic acid. The albumin, which functioned as a carrier, was found to be necessary when small amounts of sRNA were present. The tubes were chilled for at least 5 min, and the precipitates were collected on 25-mm Millipore filters (0.45-μ
pore size) and washed three times. The filters were then glued to
planchets, and the amount of ¹⁴C-amino acid incorporated into a
precipitable form was determined in a Nuclear-Chicago low
background counter.

The presence of LiCl and other salts at concentrations over
approximately 0.20 M prevents the formation of aminoseryl-
sRNA. For this reason, aliquots of no more than 0.10 ml of
column eluates were assayed in the volume described above.
Standardized solutions of sRNA were assayed along with column
fractions so that the proportionality of counts incorporated to
the amount of sRNA present was established throughout the
range of acceptance activities found in the fractions. Recovery

\[
\text{AMINO ACID ACCEPTOR ACTIVITY (cpm/0.1 ml of eluate)}
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\text{FRACTION NUMBER}
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\text{FRACTION NUMBER}
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Fig. 1. The resolution on DEAE-Sephadex of the sRNA species
for 10 amino acids. sRNA (50 mg) was applied to a DEAE-Sepha-
dex column measuring 1.5 × 15 cm. Elution was performed with
the 2-liter linear gradient described in the text. The flow rate was
20 ml per hour, and fractions were collected every 30 min. The
pattern of absorbance is shown at the top of the figure, and patterns
of acceptor activity for the different amino acids are shown below it.
In general, every fifth tube was assayed for amino acid acceptance.
A scale of counts incorporated into the fractions tested is shown at
the right of each pattern. The concentration of the linear LiCl
gradient is superimposed on the pattern of absorbance.

of total acceptance activity was determined by assaying a known
amount of column input. Blank values to be subtracted from
the total count obtained for each fraction were determined by
measuring the amount of amino acid incorporated into an assay
mixture containing bacterial extract but no sRNA.

RESULTS

Early experiments in which sRNA was eluted from DEAE-
Sephadex by a gradient of LiCl revealed that sRNA is eluted by
lower salt concentrations at neutral pH (6.0) than at an acidic pH
(3.0). This result suggested that a pH gradient could be applied
along with a salt gradient with the hope of improving sRNA
resolution. A variety of gradients were tried with both increasing
pH and decreasing pH. An acetic acid-lithium acetate (0.02
M) buffer was used to control the pH. sRNA is not soluble below
pH 2.8 in 0.5 M LiCl. Based on patterns of optical density, it
appeared that gradients from pH 3.0 to 4.0 or from pH 3.5 to 4.0
gave good resolution of material eluted early but poor resolution
toward the end of the gradient. A gradient from 4.0 to 4.5 gave
poor resolution at the beginning of elution. Optimal resolution
was obtained by using a gradient from pH 3.5 to 4.5. Because
the gradient results from the titration of acetate, the rate of pH
change is not quite linear. It decreases slightly as the pK of
acetate is approached (i.e., toward the end of the gradient).

A relatively flat linear gradient of increasing LiCl concentra-
tion from 0.50 to 0.70 M was used. The linearity was checked by
measuring the conductivity of the fractions. The distribution of
ultraviolet-absorbing material throughout the gradient indicated
that it could not be made flatter, because when further flattening
was attempted, material eluted early in the gradient was not
resolved, or material normally eluted late failed to come off the
column. LiCl was used because its solubility in ethanol allowed
eluted sRNA fractions to be desalted readily. In preparative
work, sRNA in solutions of LiCl was precipitated with 3 volumes
of cold ethanol, or the solutions were lyophilized and the LiCl was
extracted from the dried material with ethanol.

Columns of various sizes were tried, and resolution remained
essentially unchanged until the capacity of the diethylaminoethyl
groups present was reduced to about four times that required
(according to the manufacturer's specifications) to absorb the in-
put, assuming one sRNA phosphate group per diethylaminoethyl
group. In practice a column 13 cm long and 1.5 cm in diameter
was used for inputs from 30 mg to 100 mg. A 2-liter linear
gradient as described above gave optimal resolution. Larger
eluting volumes resulted in unnecessary dilution of the sRNA and
wider peaks for the sRNA species. Flow rates of no more than 1
liter per day provided the best resolution.

Columns packed with DEAE-cellulose (Schleicher and Schuell)
were also tried. Elution of sRNA occurred at a lower salt con-
centration (0.40 to 0.60 M LiCl), but there was very little resolu-
tion of sRNA species.

A small column was developed for input samples of 0.5 to 2.0
mg. The small column had a bed volume of about 2.5 ml and
dimensions of 0.3 cm (diameter) by 25 cm. The same LiCl and
pH gradient was used, and a total volume of 70 ml was used for
elution. As in the case of the larger column, the best resolution
was obtained when elution occurred over a period of 48 hours or
more.

Columns have been run both at room temperature and at 4°,
and there is little difference in the quality or pattern of resolu-
tion. Because of the possibility of instability of sRNA at acidic
pH and of microbial growth in the fractions, all results presented are from experiments performed in the cold.

Fig. 1 shows the resolution of several species of sRNA which is obtained by use of the larger column. Two major peaks of ultraviolet-absorbing material are seen, and most of the sRNA species studied are eluted in the larger one with only modest separation from each other. Some, however, like the first peak of methionine and the peaks of valine, are associated with the absorbing material that is eluted early. A few clearly multiple peaks of sRNA species which accept the same amino acid are seen, but the shoulders and wide and asymmetrical peaks which are more commonly seen also suggest multiple species of sRNA which accept the same amino acid.

Fig. 2 shows the resolution possible with the smaller column. The pattern of ultraviolet absorbance is similar to that shown for the larger column, and the peaks of acceptor activity for the three different amino acids tested are comparable to those of the larger column. These patterns of ultraviolet absorbance and acceptor activity have been reproduced many times.

There is little if any loss of ultraviolet-absorbing material or amino acid accepting activity while the sRNA is on the column. Some recovery data for the column illustrated in Fig. 1 are as follows: absorbance (260 mr), 85%; histidine acceptor activity, 107%; lysine, 89%; proline, 76%; methionine, 80%; and aspartic acid, 112%.

A single passage through the column results in an enrichment of acceptor activity of 2- to 5-fold depending on the sharpness of the peak and its location in the absorbance pattern. Little additional enrichment is obtained by rerunning peaks on a similar column, even if the gradient is flattened appropriately. sRNA species which were rechromatographed were eluted at the same salt concentration both times.

The column can be a useful adjunct to other purification procedures because it can resolve certain mixtures of species not resolved by other methods. For example, as shown in Fig. 3, a mixture of a leucine sRNA species and a tyrosine sRNA species (Tyr II), which had not been entirely resolved by three counter current passages (3), was readily resolved by one of the small DEAE-Sephadex columns.2

The column can also be used to separate species of aminooacylsRNA. The low pH of the gradient permits good recovery of the esterified amino acid, and the pattern of elution, in the few cases which have been examined, appears to be the same as for non-acylated sRNA.

**DISCUSSION**

Attempts to fractionate sRNA by chromatography on DEAE-cellulose, -Sephadex, and -paper have been more successful when conditions which might be expected to alter the secondary structure of sRNA have been utilized during elution (4, 5). Cherayil and Bock (6) have achieved good resolution by DEAE-Sephadex and -cellulose chromatography in the presence of urea concentrations up to 7.0 M. They have used various gradients of urea concentration, and they have also studied the effect of constant low pH (4.5) in the presence of urea and have found that patterns of resolution were altered from those obtained at neutral pH. Although we have not used urea in eluting solutions, we have found that a combined pH and salt gradient results in better resolution than a salt gradient at constant pH. Bergquist and his co-workers (7) have shown that sRNA has a greater affinity for DEAE-cellulose at high temperatures. Their resolution is

Fig. 3. Resolution of two species of sRNA from a previously purified input. Approximately 0.5 mg of sRNA previously purified by countercurrent distribution, which consisted of a mixture of tyrosine sRNA II and one of the leucine sRNA species, was placed on a column like the one described in Fig. 2. Elution was accomplished with a linear gradient from pH 3.5 to 4.5 with 0.55 to 0.70 M LiCl. The separation of these two species by the DEAE-Sephadex column is nearly complete.

2 The partially purified sRNA used in this experiment was obtained from Dr. B. P. Doctor.
better at high temperatures, and they have published chromato-
graphic patterns of Escherichia coli sRNA (8) obtained at 65°
which have some similarity to those described in this report.
They also described a pattern of resolution which can be obtained
with DEAE-Sephadex columns and elution with a solution of
constant salt concentration in the presence of decreasing tem-
perature (9).

The present study has shown that sRNA has a greater affinity
for DEAE-Sephadex in acidic solutions than near neutrality. It
is of interest that this occurs despite the assumption of positive
charges by the protonated bases and the absence of any anionic
charge contribution by the secondary phosphate groups in acidic
solution. A possible explanation of this observation is that the
denaturation could result from the disappearance
of hydrogen bonds between base pairs as the bases are protonated.

The method described here is convenient and reasonably rapid.
It provides different patterns of sRNA resolution than other
methods and therefore offers possibilities for separating some
species which are less successfully resolved by other methods.
It has a large input capacity, and amino acid acceptor activity is
stable under conditions of fractionation. When used along with
other methods, it should be useful in the preparation of purified
sRNA species and in the analysis of patterns of sRNA from
cells in various physiological states.

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