A Column Chromatographic Procedure for the Fractionation of s-RNA*

Joseph D. Cherayil and Robert M. Bock

ABSTRACT: Examination of several key variables which influence s-RNA chromatography on DEAE-cellulose and DEAE-Sephadex has shown that the order of displacement of specific amino acid acceptor s-RNA's is a function of the *p*H, exchanger type, and urea concentration. The order of elution by salt gradients is altered by partial protonation of the cytosine and adenine bases. In addition, strong nonelectrostatic interactions which occur between s-RNA and the cellulose matrix can be overcome by urea gradients which further alter the order of elution. These interactions are less pronounced when the matrix is cross-linked dextran. The ion exchange, base protonation, and urea displacement provide three or more complementary variables which alter the order of elution of specific acceptor activities. When yeast s-RNA is eluted from DEAE-cellulose at *p*H 7.6 by a gradient increasing in urea concentration, arginine, valine, and proline acceptor RNA's emerge with the front of ultraviolet absorbancy and, while separated from several other amino acid acceptors, are superimposed. Rechromatography of the fraction containing these three acceptors on a column of DEAE-Sephadex at *p*H 4.5 using a salt gradient displaces valine well before arginine and proline. Subsequent chromatography of the arginine and proline acceptor peak on DEAE-cellulose at *p*H 4.5 using a urea gradient results in their separation from each other. Tyrosine and leucine acceptor RNA's elute together from DEAE-cellulose at *p*H 7.6 but are resolved into separate peaks at *p*H 4.5 on the DEAE-Sephadex column.

These combined procedures are useful in isolation and enrichment of most specific acceptor activities and for separation of multiple acceptor species for a single amino acid. The principles underlying the separation processes are discussed. A method is also described for simple micro assay of amino acid acceptor activity by conducting the entire measurement on filter paper disks.

The amino acid transfer ribonucleic acids (s-RNA) are an important class of compounds which play a major role in the synthesis of proteins. There is an s-RNA (in some cases more than one) which specifically combines with each of the amino acids. Each amino acid–bearing s-RNA then participates in a complex in which it interacts with a ribosome and a specific base triplet in the messenger RNA bound to that ribosome. The polypeptide attached to the s-RNA which occupies the neighboring message triplet is then enzymically transferred to the α-amino group on the newly bound aminoacyl s-RNA. This process of polypeptide chain growth is well documented, but whether special s-RNA molecules or codons correspond to peptide chain initiation and termination is unknown. A knowledge of the primary structure and conformation of s-RNA is essential to understand its specificity and to get an insight into the mechanism of protein synthesis (see Berg, 1961, review). For most studies of sequence and conformation, highly purified specific s-RNA is required. Pure s-RNA's, or at least partially purified s-RNA's, are useful in other studies such as the determination of the nucleotide sequence in codon triplets (Leder and Nirenberg, 1964). The metabolic variability of s-RNA in response to nutritional and environmental changes is largely unknown, but several theories (Stent, 1964; Tissières and Gros, 1963; Neidhardt, 1964) which implicate s-RNA, or s-RNA-like molecules as key participants in induction and repression of specific enzyme synthesis have been proposed. Convenient high-resolution methods for separation of specific s-RNA's will facilitate the study of these metabolic mechanisms.

Methods which have been reported for the purification of specific s-RNA's include countercurrent distribution (Apgar *et al.*, 1962; Zachau *et al.*, 1961), partition chromatography (Tanaka *et al.*, 1962; Muench and Berg, 1964), column chromatography on methylated albumin (Sueoka and Kano-Sueoka, 1964), DEAE-cellulose (Kawade *et al.*, 1963), and hydroxyapatite (Hartman and Coy, 1961), as well as chemical methods involving oxidation of amino acid–free s-RNA with periodate and subsequent separation of the dialdehyde RNA from aminoacyl RNA by various methods (Sapcnara and Bcck, 1961; Zubay, 1962; Stephenson and Zamecnik, 1962). Treatment of aminoacylated s-RNA's with amino acid *N*-carboxyanhydride (Mehler and Bank, 1963; Simon *et al.*, 1964) or with coupling reagents specific for the amino acid on the RNA (Brown, 1960) has also been used for specific RNA purification.

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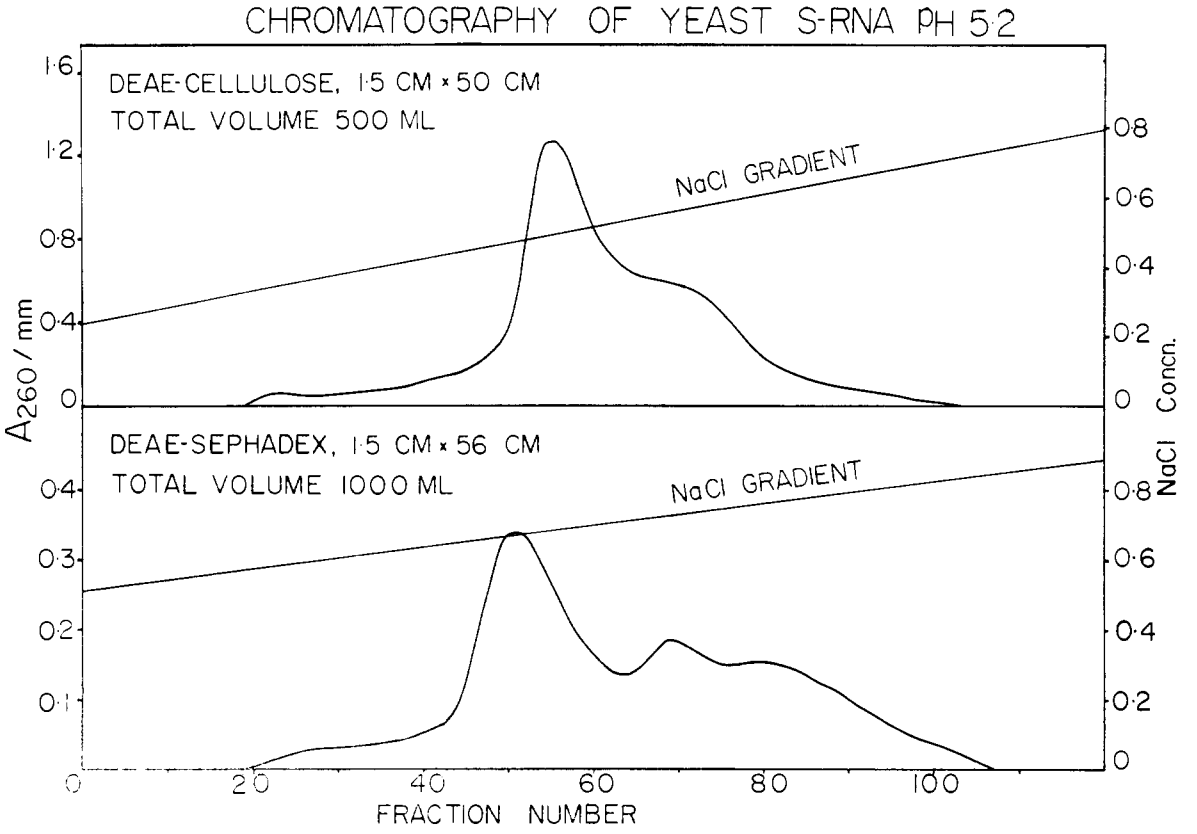

FIGURE 1: Chromatography of yeast s-RNA at *p*H 5.2. The DEAE-cellulose column was equilibrated with 0.3 M NaCl in 0.02 M acetate buffer. s-RNA (90 mg) was applied to the column and the chromatogram was developed with a linear gradient of NaCl. The DEAE-Sephadex column was equilibrated with 0.5 M NaCl in 0.02 M acetate buffer and 56 mg of s-RNA was applied to it. Elution was performed by a linear gradient of NaCl.

We have studied the fractionation of yeast s-RNA on DEAE-cellulose and DEAE-Sephadex columns under a variety of conditions. This is a report concerning our observations on several parameters which influence the behavior of various specific s-RNA's on these columns. A simple micro method for the assay of s-RNA chromatographic fractions for amino acid acceptor activities is also reported.

Experimental

DEAE-Cellulose. DEAE-cellulose of low capacity, 0.4 meq/g (Calbiochem, Los Angeles) was used throughout this work. The ion exchanger was washed with water and 1.0 M NaCl, suspended in a solution approximately 0.4 M in NaCl and 0.02 M in the buffer to be used, and packed into the column under gravity. The column was then equilibrated with a solution containing NaCl, urea, and buffer of appropriate concentrations by pumping this solution through the column at a flow rate of approximately 10 ml/hr per cm^2 column cross section.

DEAE-Sephadex. DEAE-Sephadex A-50 (Pharmacia, Uppsala) medium grade of capacity 3.3 meq/g was repeatedly washed with 0.1 M HCl and NaOH and 1.0 M NaCl in order to remove the colored solutes, and then packed into the column as described in the case of DEAE-cellulose.

Preparation of s-RNA. Yeast (*Saccharomyces lactis*), grown in the University of Wisconsin pilot plant 500-gallon fermenter, was harvested while in logarithmic phase, washed with cold 0.25 M NH_4Cl, and stored in liquid nitrogen. RNA from 1-kg lots of pulverized frozen yeast cells was extracted with boiling sodium dodecyl sulfate solution in phosphate buffer (Crestfield *et al.* 1955) and treated with phenol to remove the protein. s-RNA was separated from polysaccharide and ribosomal RNA by adsorption on DEAE-cellulose and subsequent elution (Brunngraber, 1962). After repeated treatments with phenol and precipitation in 75% ethanol, the s-RNA was dissolved in 0.1 M NaCl and stored frozen. The yield varied from 1 to 1.4 g/kg of yeast. The various amino acid acceptor activities varied from 0.6 to 1.5 mμmoles/mg of RNA. These are minimum values obtained under assay conditions known not to be saturating and without correction for isotope dilution.

Preparation of Activating Enzyme. Frozen yeast cells (50 g) were crushed and mixed with 60 ml of glass beads (superbrite 120 μ) and 30 ml of a buffer consisting

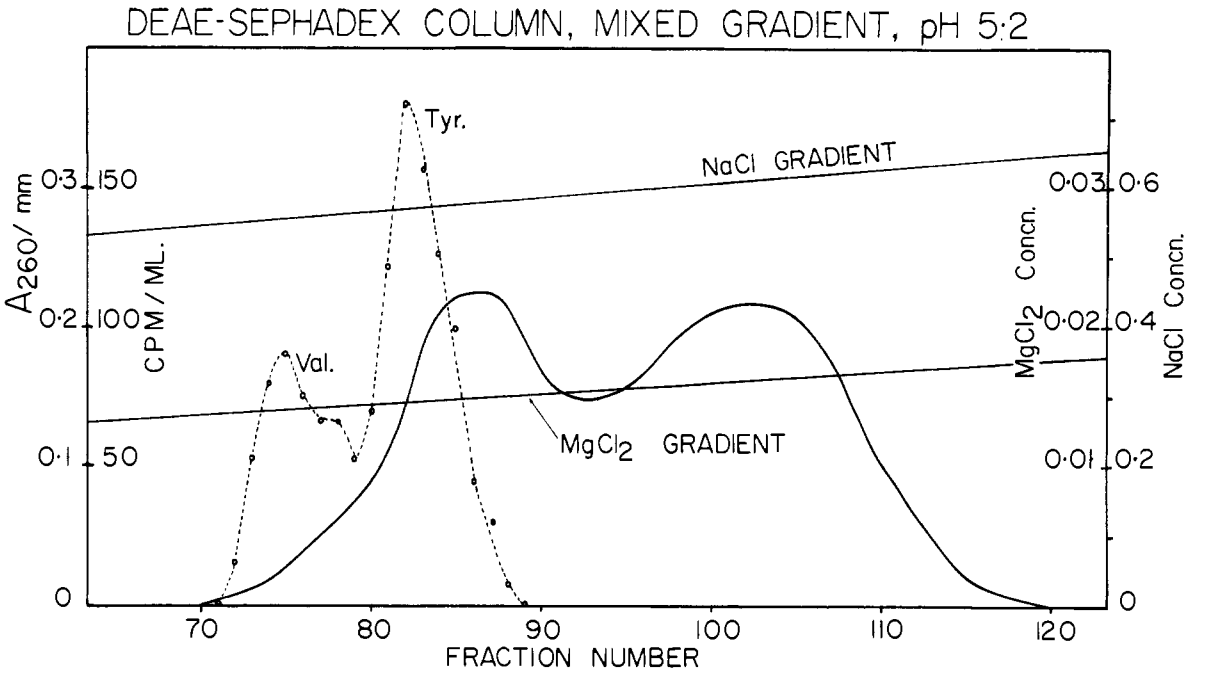

FIGURE 2: Elution of s-RNA with NaCl and $MgCl_2$ gradient at *p*H 5.2. s-RNA (14 mg) prelabeled with [^{14}C]valine and [^{14}C]tyrosine was applied to a DEAE-Sephadex column (1.5 × 100 cm) equilibrated with 0.5 M NaCl and 0.01 M $MgCl_2$ in 0.02 M acetate buffer. Elution was performed by a gradient linear in NaCl and $MgCl_2$. Fraction size was 6 ml, collected every 30 minutes. Aliquots of the fractions were dried in planchets and radioactivity was determined in a gas-flow counter.

of 0.01 M Tris-HCl, *p*H 7.5, 0.01 M magnesium acetate, 0.03 M ammonium chloride, and 0.005 M β-mercaptoethanol. The mixture was ground in a Minimill (Eppenback Model QV-Q) for 15–20 minutes and centrifuged at 2000*g* for 30 minutes to remove the glass beads and the cell debris. The supernatant solution was decanted and further centrifuged for 3 hours at 40,000 rpm in a Spinco Model L ultracentrifuge. The clear supernatant solution was dialyzed against the above-mentioned buffer for 16–20 hours with three changes of buffer. All operations were done at close to 0°. The dialyzed solution, in small aliquots, was quickly frozen in ethanol–dry ice and stored in liquid nitrogen.

Operation of Chromatographic Columns. The sample was added to the column at the highest salt and urea concentration at which the R_F is close to zero and was eluted by slowly increasing the concentration of salt or urea. The two principal types of gradients employed were linear in sodium chloride in the presence of constant urea (7.0 M) or exponential in urea in the presence of constant sodium chloride (0.34 M). The total volume of the gradient varied from 5 to 10 column volumes and the flow rate was approximately 10 ml/hr per cm^2 of the column bed. The salt and urea concentrations presented in the figures are calculated from the appropriate gradient theory (Bock and Ling, 1954), taking into account the void volume of the column and the volume of the eluent delivered. The eluting solutions were metered by a peristaltic pump (Sigmamotor, Inc.) and the effluent optical density at 260 or 280 mμ was monitored with a recording spectrophotometer (Beckman DB) equipped with a flow cell of 0.1 cm optical path (Quaracell, Inc.). Column temperature was 22 ± 2° unless otherwise specified. The *p*H values listed are for the final solutions including urea unless otherwise noted (Levy and Magoulas, 1962).

Amino Acid Acceptor Assay. The amino acid acceptor assays were performed on filter paper disks by a micro modification of the usual assay procedure (Cherayil and Bock, 1964). Aliquots of 0.05–0.15 ml of the column fractions were applied directly to filter paper disks (Whatman 3MM, 2.3 cm in diameter) numbered and supported in rows by means of pins in a Styrofoam block covered with a Teflon sheet. The paper disks were partially dried in a current of air (hair dryer) and transferred to 75% ethanol containing 0.03 M KCl kept cooled in ice. They were then washed free of urea and excess salt with the cold ethanol-KCl mixture in a large Buchner funnel, with the filter paper disks sandwiched between two large coarse filter papers, and air dried. The RNA on the paper disk was incubated at room temperature with radioactive amino acid, activating enzymes, and other cofactors. The block supporting the paper disks was covered with a glass tray which was humidified inside in order to reduce evaporation. The incubation mixture consisted of 40 μmoles of Tris buffer,[1] *p*H 7.5, 4 μmoles of $MgCl_2$, 0.5 μmole of EDTA, 1 μmole of ATP, 5 μmoles of mercaptoethanol,

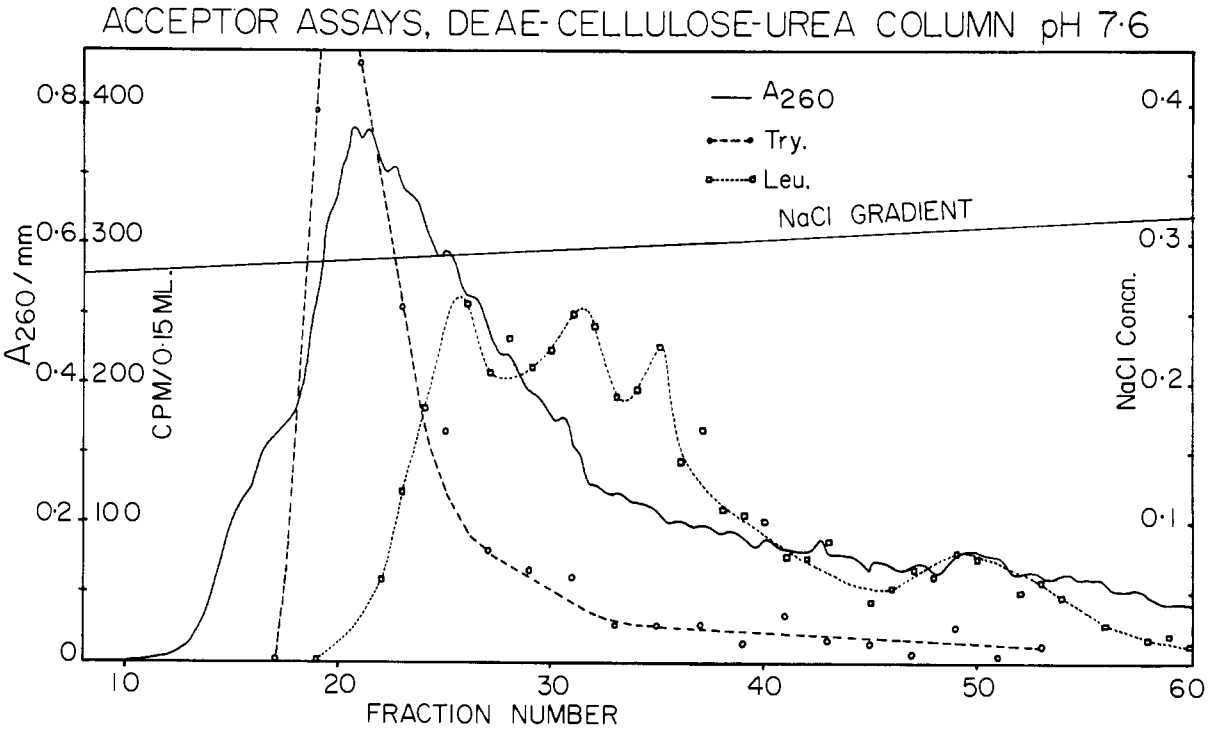

FIGURE 3: Elution of s-RNA in the presence of urea. RNA (20 mg) was applied to a 1- × 50-cm DEAE-cellulose column equilibrated with 0.25 M NaCl, 7.0 M urea, and 0.02 M potassium cacodylate buffer of *p*H 7.0 (*p*H of mixture was 7.6). Elution was performed by a gradient of NaCl, 0.28 M–0.4 M, in a total volume of 500 ml, in 7.0 M urea. Fraction size was 3 ml collected every 30 minutes.

40 μmoles of KCl, 0.3 μc of [^{14}C]amino acid of specific activity 10–25 μc/μmole, 0.01 μmole each of nineteen other cold amino acids, and 0.1 ml of yeast supernatant enzyme (approximately 1.5 mg protein) in a total volume of 1.0 ml; 0.1 ml of this mixture was added to each paper disk.

After incubation (20–30 minutes) at room temperature the paper disks were dried in a current of air until the surface no longer had a wet sheen, and then were put into 10% trichloroacetic acid containing cold amino acid. They were then washed in the Buchner funnel successively with 66% cold ethanol containing 0.5 M NaCl, 10% trichloroacetic acid and 5% trichloroacetic acid containing cold amino acid in each, and finally ethanol-ether (3:1). The paper disks were dried under a heat lamp and radioactivity was determined in a scintillation counter. Linear counts were noted up to 50 μg of s-RNA per paper disk.

Results

Elution of s-RNA in the Absence of Urea. The optical density patterns of elution of s-RNA from a DEAE-cellulose and DEAE-Sephadex columns are shown in Figure 1. s-RNA is eluted from a DEAE-cellulose column between 0.4 M and 0.65 M NaCl and from a DEAE-Sephadex column between 0.7 M and 0.85 M NaCl. Partial separation of some of the specific RNA's has been indicated by the few acceptor assays (not the micro method) performed with the fractions from these columns. Valine acceptor RNA is eluted in the early fractions and tyrosine acceptor in the late fractions. The second optical density peak from the DEAE-Sephadex column (Figure 1) contains serine acceptor RNA more than 10-fold purified relative to the material added to the column.

A small amount of magnesium chloride decreases the concentration of sodium chloride required for elution of the various specific s-RNA's. Figure 2 represents the elution pattern of s-RNA with a magnesium chloride gradient superimposed on a sodium chloride gradient. Very good separation of valine and tyrosine acceptor RNA is indicated, and the latter appears in the early fractions rather than in the late fractions. In this case s-RNA prelabeled with [^{14}C]valine and [^{14}C]tyrosine was used, and the *p*H was kept low (5.2) to stabilize the amino acid s-RNA. The effect of $MgCl_2$ on resolution of other specific s-RNA's has not been explored.

Effect of Urea. When s-RNA was chromatographed on DEAE-cellulose in the presence of 7 M urea at *p*H 7.5, several optical density peaks were observed (Figure 3). Acceptor assays performed on the fractions revealed

[1] Tris has been found to catalyze the removal of amino acid from some s-RNA's (C. Heredia, personal communication). Potassium maleate buffer of *p*H 6.9 with dithiothreitol replacing the mercaptoethanol has been found to give greater and more reproducible attachment of amino acid than the procedure in the studies reported here.

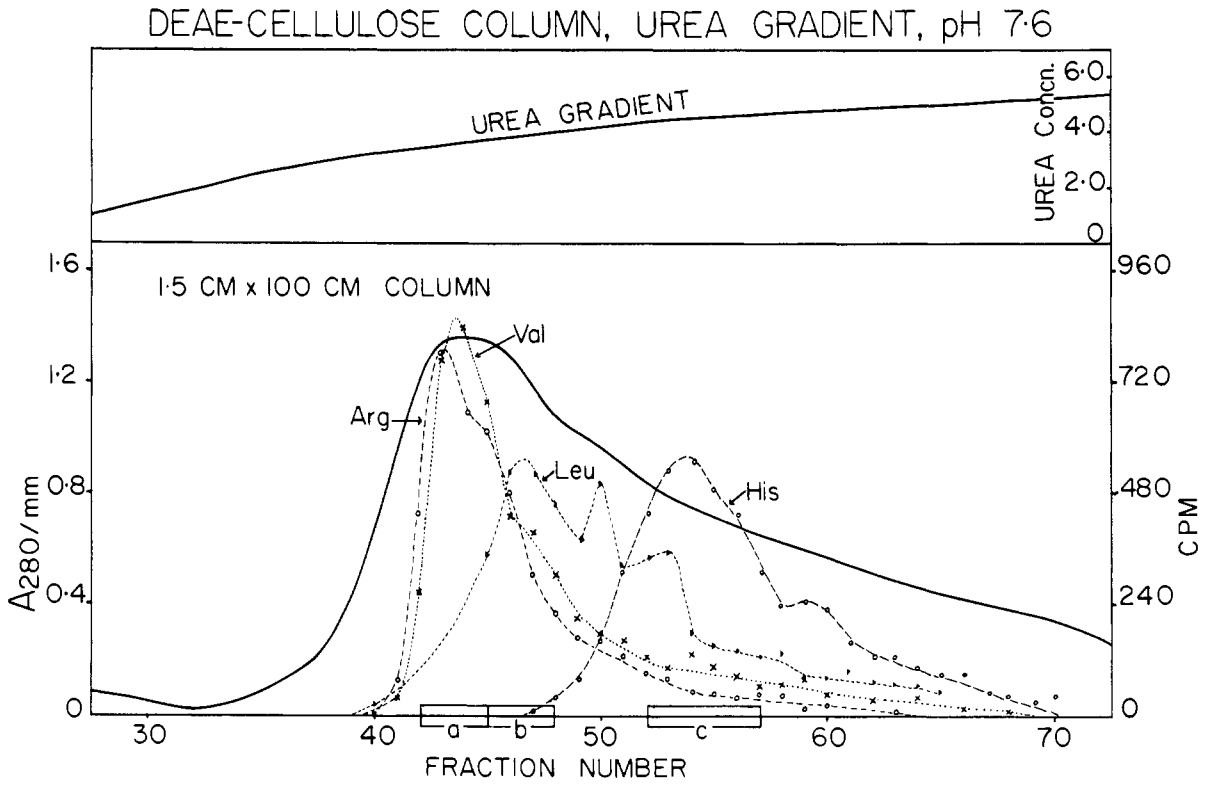

FIGURE 4: Elution of s-RNA with a urea gradient. s-RNA (200 mg) was applied to a DEAE-cellulose column equilibrated with 1.5 M urea in 0.34 M NaCl, 0.02 M Tris-HCl, *p*H 7.5, and was eluted by an exponential gradient of urea; 7.0 M urea in 0.38 M NaCl and buffer was added to a constant-volume mixer containing 300 ml of the mixture used for equilibration. Fraction size was 7.5 ml, collected every 30 minutes. Fractions indicated by a, b, and c were rechromatographed on a DEAE-Sephadex column (see Figure 5).

partial separation of the various acceptors. Tryptophan s-RNA was eluted first from the column, followed in quick succession by glycine, valine, tyrosine, lysine, phenylalanine, and leucine acceptor RNA's. Leucine acceptor RNA was resolved into multiple peaks. The elution pattern is very sensitive to small changes in the salt concentration; a slightly sharper gradient elutes all the s-RNA in one peak, while a very shallow gradient spreads the fractions over inconveniently large volumes; it may be noted that most of the s-RNA is eluted from the column between 0.28 and 0.31 M NaCl. We find optimum practical resolution when this salt range is covered in a gradient of 5–10 column volumes.

Urea Gradient. In the presence of 7.0 M urea the salt concentration required to elute s-RNA is lower than that required in the absence of urea; at a salt concentration of 0.34 M NaCl all the s-RNA is eluted from the column (see Figure 3). However, in the absence of urea all the RNA remains bound to DEAE-cellulose at this salt concentration (see Figure 1). Therefore it is possible to elute s-RNA with a urea gradient, keeping the salt concentration constant. Figure 4 shows the elution pattern of s-RNA with a urea gradient. In this column some of the acceptor activities are well separated from others. For example, histidine s-RNA is almost completely separated from valine, arginine, and proline s-RNA's. Leucine acceptor RNA shows three distinct peaks. Repetition of the assay of the various fractions in duplicate assays has shown that the peaks noted are real. Multiple acceptor activities were observed for serine, valine, and isoleucine s-RNA's. An advantage of this system is that the elution pattern is not sensitive to small changes of urea concentration so that a steep urea gradient can be applied to the column without much loss in resolution. The early fractions and the late fractions from the column representing approximately one-third of the added optical density units do not have amino acid acceptor activities. These can be discarded.

Rechromatography (at Lower pH.) Protonation of cytidylic and adenylic residues can markedly alter the interaction of s-RNA with the ion exchanger and is therefore useful in rechromatography of acceptor activities which failed to resolve on the previously described columns. Figure 5 shows the elution patterns upon rechromatography on DEAE-Sephadex at *p*H 4.5 of fractions from the DEAE-cellulose-urea column. In this column several distinct optical density peaks are observed. Arginine and proline s-RNA's are well separated from valine s-RNA (Figure 5a); in the previous column all three acceptors were eluted together. Proline s-RNA shows signs of splitting into two peaks. The purity of arginine s-RNA in the peak activity fractions

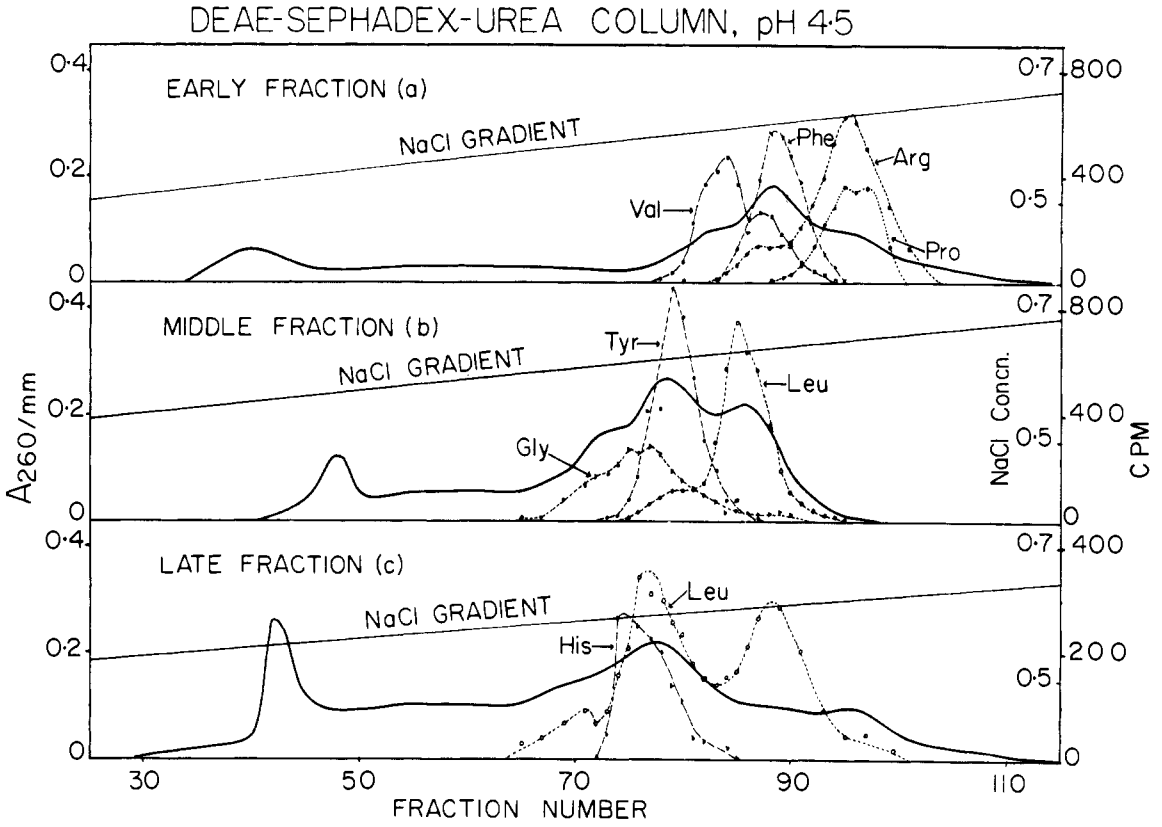

FIGURE 5: Rechromatography on DEAE-Sephadex at *p*H 4.5. A 1.2- × 100-cm column was equilibrated with 0.4 M NaCl in 7.0 M urea and 0.03 M acetate buffer, *p*H 4.5. Fractions a, b, and c from the previous column (Figure 4) were adjusted to approximately 7.0 M urea with solid urea and to *p*H 4.5 with acetic acid. The samples were applied to the column and eluted by a linear gradient of NaCl, 0.52–0.70 M in a total volume of 500 ml, in the presence of 7.0 M urea and buffer. Fraction size was approximately 4 ml, collected every 30 minutes.

has been determined to be approximately 50%. Attempts to detect acceptor activity in the first optical density peaks were not successful.

At this *p*H, three or more leucine acceptor activities are well resolved from each other (Figure 5b,c). Distinct separation of leucine s-RNA from tyrosine s-RNA is indicated in Figure 5b. Repetition of this experiment using various amounts of s-RNA under slightly modified conditions has given the same pattern of results. However, slight variations of the salt concentrations at which different specific s-RNA's are eluted are observed, depending on the column size, the flow rate, the salt concentration at which the sample is added, the amount of RNA, and the gradient. The resolution and purification of the various specific acceptor RNA's are improved by raising from 0.4 M to 0.45 M the NaCl concentration at which the sample is applied to the column and eluting by a shallower gradient (Figure 6). The resolution of glycine acceptor RNA into two peaks is clearly seen in this case.

Separation of Arginine and Proline s-RNA's. As has been shown in Figure 5, arginine and proline s-RNA's may be separated completely from valine s-RNA by rechromatography on the DEAE-Sephadex column. On this column, the separation between arginine and proline s-RNA's is only partial (see Figure 5a). The two may be separated from each other by rechromatography on DEAE-cellulose at *p*H 4.5 with a urea gradient (Figure 7).

When the experiment was repeated using a longer column (1 × 48 cm), arginine and proline acceptor RNA's were separated well from each other and the latter gave two acceptor activity peaks. Leucine and tyrosine acceptor activities were noted between the arginine and proline acceptor activity peaks.

Discussion

The parameters *p*H, temperature, salt type, DEAE column matrix type, and urea concentration have been tested as variables which influence the resolution of specific amino acid acceptor RNA's. Within these variables are found three complementary principles for resolution of the s-RNA mixture: chain length of RNA, fraction of protonatable bases, and degree of interaction (probably mainly by purines) with cellulose (Tomlinson and Tener, 1963). The purine base interaction is strong if cellulose is the DEAE matrix, weak if dex-

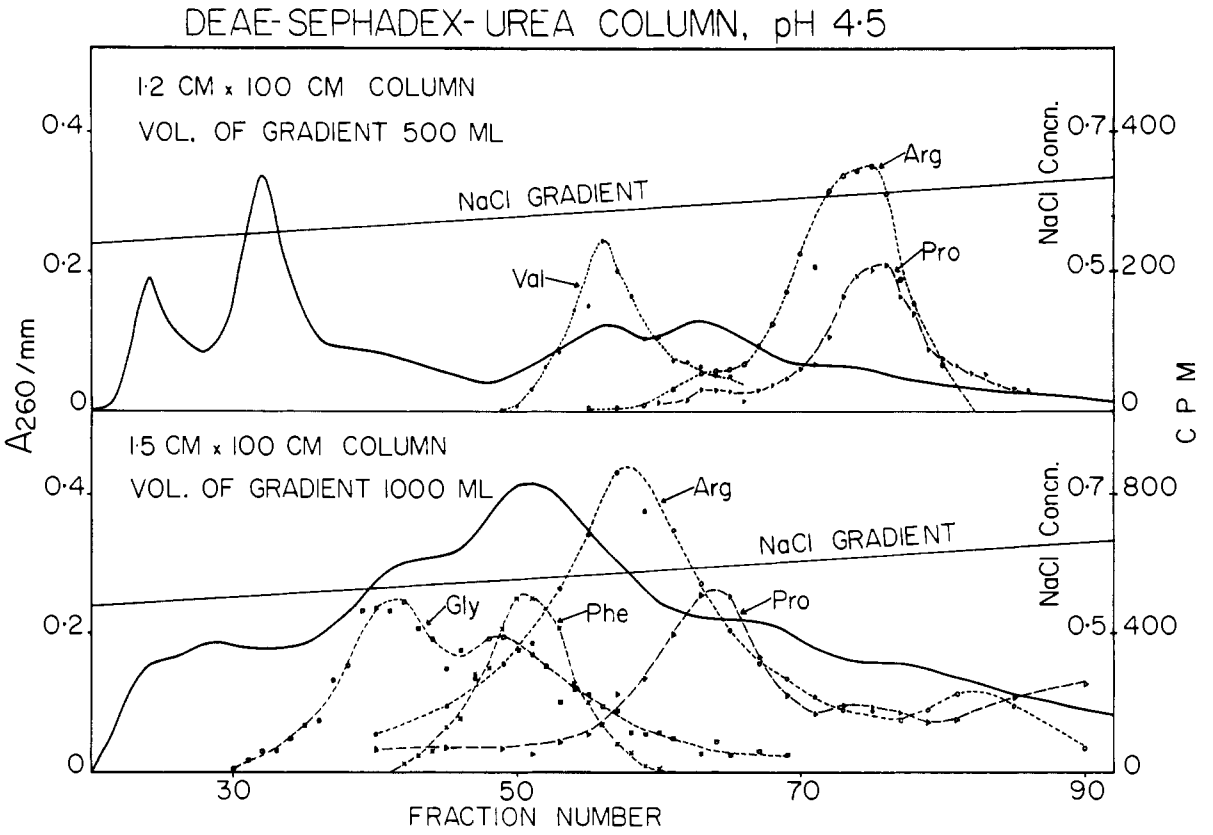

FIGURE 6: Rechromatography on DEAE-Sephadex at *p*H 4.5 under slightly modified conditions. Both columns were equilibrated with 0.45 M NaCl in 7.0 M urea and buffer. The gradient was linear, 0.54–0.70 M, in NaCl in both cases. To the smaller column (upper) was added in 25-ml volume 12 mg of partially purified s-RNA from an early fraction from a previous column (see Figure 4). Fraction size was 4.6 ml, collected every 30 minutes. To the larger column (lower) 80 mg of s-RNA from an early fraction of a previous separation was applied in 7.0 ml. Fractions of 8.5 ml were collected every 30 minutes.

tran (Sephadex) is the matrix (Rushizky, *et al.*, 1964), and is decreased upon the addition of urea.

When the combination of high urea concentration and dextran-supporting matrix is used to remove nonelectrostatic effects and to weaken the conformational rigidity of s-RNA molecules, the separation process is highly selective for chain length of the polyanion (Stewart and Stahmann, 1962). The observed strong dependence of R_F on salt concentration is characteristic of long polyanions of similar chain length.

The net charge of these polyanions can be decreased by lowering the *p*H and subsequent partial protonation of cytosine and adenine. The lowest practical *p*H is limited by the acid-catalyzed hydrolysis of phosphodiester linkages. At *p*H 2.0 and 23° in 7 M urea and 0.6 M NaCl, the half-life of acceptor activity is about 16 hours. At *p*H 4.5, less than one-fourth of the cytosine residues have been protonated as judged by hyperchromicity at 280 mμ, corrected for denaturation hypochromicity as observed at 247 mμ. This slight protonation markedly altered the order of elution of acceptor activity. At *p*H 3.5, the cytosine bases are mostly protonated and yet another elution sequence is observed. The spectral studies of protonation were performed on crude fractions; so it is uncertain if the acceptor activities which were shifted most had some residues unusually available for protonation. The influence of temperature has not yet been found useful. At 50° markedly higher salt concentrations are needed for elution, and the resolution and allowable flow rate do not significantly improve relative to experiments at 25°.

The three complementary principles of chromatographic separation can be conveniently applied in sequence because effluent fractions from a DEAE-cellulose column eluted with a urea gradient containing 0.34 M NaCl at *p*H 7.6 can be adjusted to 7 M urea with solid urea and added directly to a DEAE-Sephadex column, and then developed with a salt gradient in constant urea at *p*H 7.6 (elution range 0.35–0.6 M NaCl). Effluent fractions from this second column may be adjusted to *p*H 4.5 or 3.3 and added directly (or after slight dilution) to a DEAE-Sephadex column which is then developed with a salt gradient in 7 M urea (0.54–0.7 M NaCl at *p*H 4.5 or 0.3–0.6 M NaCl at *p*H 3.3). The solutions added to the columns are such that the effective R_F of the RNA is less than 0.05, so that even if the sample volume approaches a full column volume, it is adsorbed in a narrow zone at the top of the column

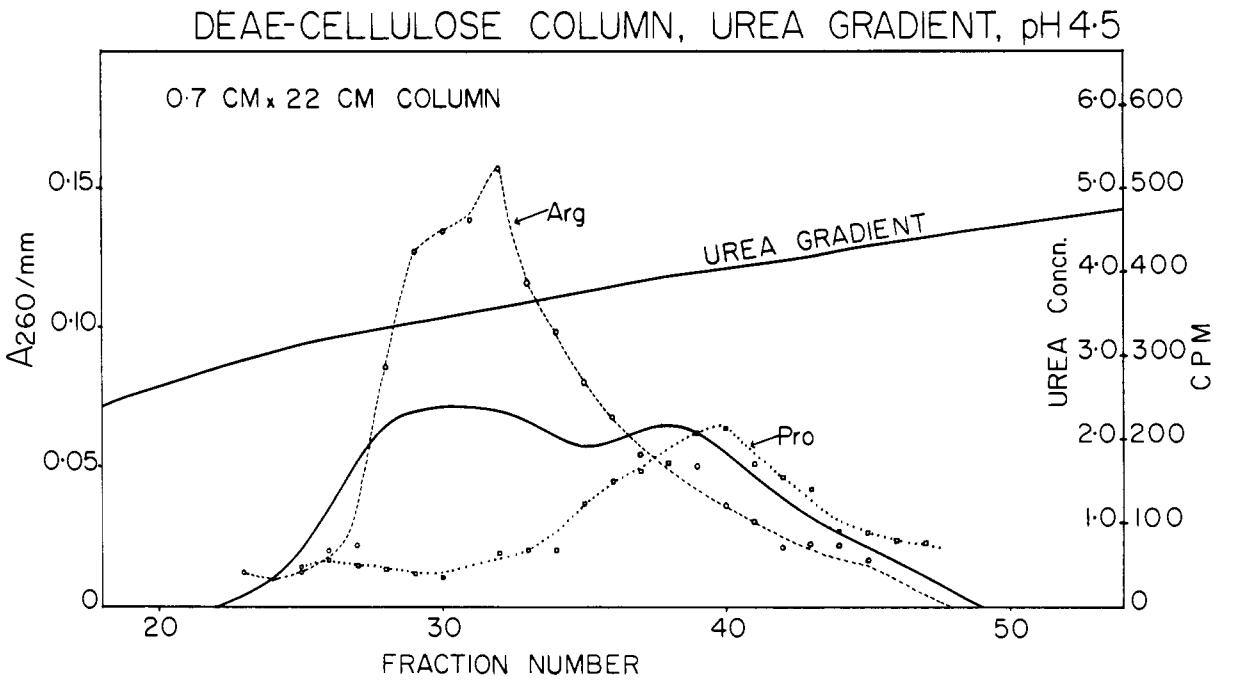

FIGURE 7: Separation of arginine and proline acceptor RNA's. The column (DEAE-cellulose) was equilibrated with 1.0 M urea in 0.34 M NaCl and 0.03 M acetate buffer of *p*H 3.7. The fractions containing arginine and proline acceptor RNA's (see Figure 5a) were pooled, adjusted to the urea and NaCl concentrations of the column, and slowly added to the column. Elution was performed by an exponential gradient of urea; 7.0 M urea in 0.4 M NaCl and 0.03 M buffer was added to a constant-volume mixer containing 100 ml of the mixture used for equilibration. Fraction size was 2 ml, collected every 30 minutes.

and gives high resolution upon the ensuing chromatography.

It has been found empirically that development of the chromatogram in less than 30 hours decreases resolution detectably, while periods longer than 60 hours do not markedly improve the resolution. Small gradients (5–10 column volumes) and slow flows permit operation at high loading and elution of the RNA at concentrations of up to 3 mg/ml with no apparent loss of resolution from overloading. A column 1 meter long gives resolution comparable to that shown here with initial loading of at least 50 mg RNA/cm^2 of the column bed. The techniques described here have been applied to samples ranging from a few mg to 1 g. The procedures appear well suited for scale-up to about 10 g/column.

In some instances it is desirable to preload the s-RNA with radioactive amino acids and chromatograph under conditions such that the alkali-labile amino acid ester remains attached to the s-RNA. The DEAE-Sephadex-urea column with a salt gradient at *p*H 4.5 and a complementary column, DEAE-cellulose at *p*H 4.5 in constant salt with a urea gradient, are suitable for separation of aminoacyl s-RNA species.

The recovery of RNA from these columns is almost quantitative (95–100%). The recovery of acceptor activities is believed to be not less than 70–80%. No definite estimate of the latter could be made, as the enzyme used in these studies was a crude yeast extract containing nuclease activity; the effect of nuclease becomes more pronounced as purity increases and less RNA is used for assay. Fractions from DEAE-Sephadex-urea column (*p*H 4.5) were stored in the refrigerator for several months without loss of acceptor activity.

It is known that in some of the acceptor s-RNA's one or more of the 3′-terminal nucleotides is missing; a stimulation in acceptor activity is noted by the addition of CTP.[2] With proline s-RNA a 2-fold stimulation is noted by the addition of CTP equivalent to one-fourth that of ATP, while some other acceptor activities show no CTP requirement.

The practical value of manipulation of the independent complementary variables is readily apparent. Figure 4 shows a column separation in which arginine, proline, and valine acceptors were found superimposed. When the *p*H was lowered, valine acceptor emerged well before proline and arginine. The urea displacement of the nonelectrostatic interactions with the cellulose matrix permitted separation of arginine from proline acceptor. Similar studies on other sets which were poorly separated by one of the DEAE chromatographic conditions have, in every case, led to a procedure by which the overlapping acceptors could be separated. The pro-

[2]Abbreviations used in this work: CTP, cytidine-5′-triphosphate; ATP, adenosine-5′-triphosphate.

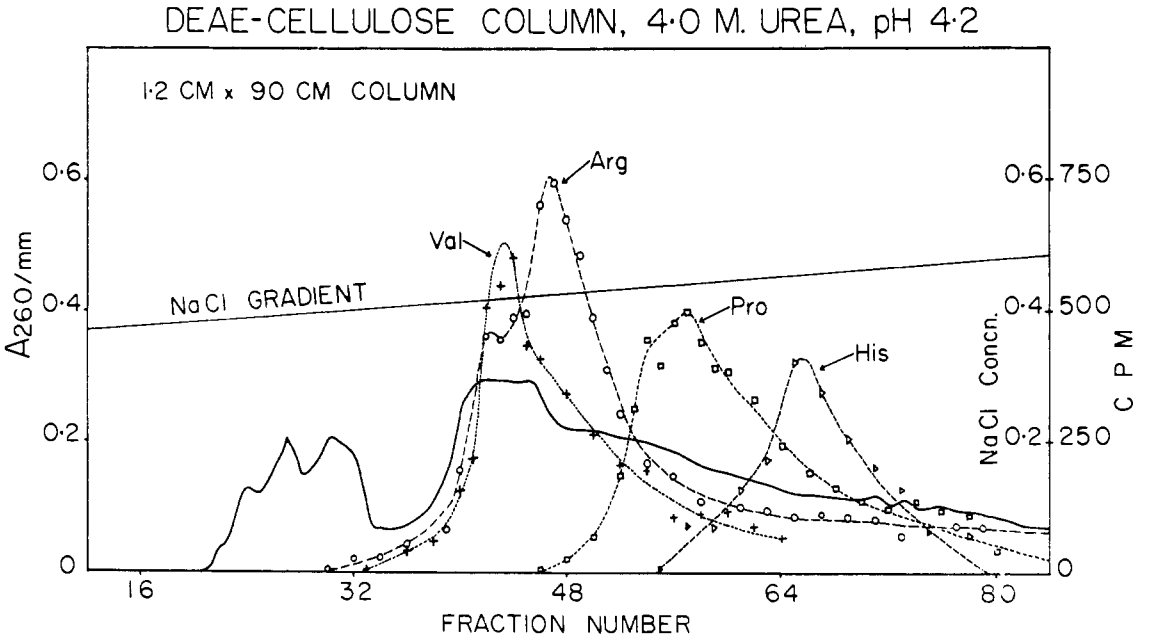

FIGURE 8: Chromatography of s-RNA on DEAE-cellulose at *p*H 4.2 in the presence of 4.0 M urea. Crude yeast s-RNA (20 mg) was applied to a column equilibrated with 0.33 M NaCl in 4.0 M urea and 0.02 M sodium acetate buffer. Elution was accomplished with a gradient linear in NaCl, 0.35–0.6 M, in a total volume of 500 ml containing 4.0 M urea and buffer. Fraction size was 4.5 ml, collected every 30 minutes.

cedure has been found applicable without modification to *Escherichia coli* s-RNA and *Micrococcus pyogenes* s-RNA (B. Weisblum, and J. Strominger, personal communications). If the investigator is not attempting isolation of a certain pure s-RNA but needs a single high-resolution display of the pattern of acceptor activities (Sueoka and Kano-Sueoka, 1964), a DEAE-cellulose column, at *p*H 4.2 in 4 M urea developed with a sodium chloride gradient from 0.3 to 0.6 M, exploits a maximum number of the chromatographic differences found among amino acid acceptor RNA's. An example of the resolution obtained on such a column is shown in Figure 8. This system also gives convenient flow properties and can be reused without repacking the column.

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Sulfur-containing Metabolites Secreated by an Ethionine-Resistant Mutant of *Neurospora**

Sara B. Galsworthy and R. L. Metzenberg

ABSTRACT: Several sulfur compounds are secreted by a mutant of *Neurospora crassa* which has lost control over methionine biosynthesis. The present paper describes the identification of these compounds as *S*-methyl-α-keto-γ-mercaptobutyric acid, *S*-methyl-α-hydroxy-γ-mercaptobutyric acid, the corresponding sulfone, *S*-methyl-β-mercaptopropionic acid, and the corresponding sulfone.

During the course of studies of the biochemical lesions involved in temperature-sensitive, irreparable mutations of *Neurospora crassa*, Metzenberg *et al.* (1964) isolated a temperature-sensitive, ethionine-resistant mutant of *Neurospora*, *r-eth*-1, which had lost control over methionine biosynthesis. In order to measure total production of methionine by *r-eth*-1, it was necessary to measure any methionine metabolites secreted into the medium as well as methionine incorporated into protein. Minimal medium in which *r-eth*-1 had grown was capable of supporting growth of a methionine-requiring strain of *Escherichia coli*. The latter mutant, which was isolated by the method of Davis (1949), was able to grow on methionine, but not on cystathionine, homocysteine, or other intermediates in the methionine biosynthetic pathway. Methionine per se, however, was shown to be absent from *r-eth*-1 culture filtrates. This paper describes the identification of the compounds derived from methionine which appeared in culture filtrates of *r-eth*-1.

Materials and Methods

Materials. The calcium salt of DL-*S*-methyl-α-hydroxy-γ-mercaptobutyric acid[1] was purchased from Mann Research Laboratories. L-[^{3}H]Methionine, specific activity 160 mc/mole, was purchased from Nuclear Chicago Corp. Snake venom (*Crotalus adamanteus*) was purchased from the Ross Allen Reptile Institute. Catalase was purchased from Sigma Chemical Co.

Fractionation of Radioactive Materials. The sample was adjusted to *p*H 8 and applied to a 1- × 20-cm column of Dowex 1 (formate). Distilled water (50 ml) was run through the column. Substances were eluted from the column by a closed-system gradient of formic acid consisting of a mixer containing 50 ml of distilled water and a reservoir containing 50 ml of 1 M formic acid. Fractions (3 ml) were collected. When the contents of the reservoir had passed into the mixer, the reservoir was replenished with 50 ml of 4 M formic acid.

Bioassay. The assay was carried out in 19- × 150-mm cuvets. Aliquots of column fractions, considered to be reasonably aseptic, were evaporated *in vacuo* in the cuvets and used without sterilization. Because the assay is quite rapid, it is necessary to avoid only gross contamination. Solutions of MKMB, MHMB, and *N*-acetylmethionine were filter sterilized. The samples were reconstituted in the salts medium of Davis and Mingioli (1950) supplemented with sodium succinate (0.005 M) to give a volume of 5.0 ml. Cells of the *E. coli* methionineless auxotroph were grown in a similar medium supplemented with methionine, harvested during the exponential growth phase, and washed with 0.5 M glucose. The cells were suspended in sufficient 0.5 M glucose so that an inoculum of 0.2 ml in the assay tubes gave an initial absorbancy of 0.010 at 650 mμ when read against distilled water. The assay tubes were read at hourly intervals until a stable terminal reading was obtained (usually 5 hours).

Synthesis of MKMB. MKMB was prepared by incubating L-methionine with snake venom L-amino acid oxidase and catalase as described by Meister (1952).

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[1] Abbreviations and designations used in this work: *S*-methyl-α-keto-γ-mercaptobutyric acid, MKMB; *S*-methyl-α-hydroxy-γ-mercaptobutyric acid, MHMB; *S*-methyl-β-mercaptopropionic acid, MMP.